

**STUDIES ON THE BIOLOGICAL PROPERTIES OF
SELECTED UVARIA SPECIES WITH EMPHASIS ON
ANTICANCER ACTIVITY**

Thesis submitted to



UNIVERSITY OF CALICUT

For the degree of

Doctor of Philosophy in Biochemistry

(Faculty of Science)

By

SMITHA K. R.

under the guidance of

Dr. ACHUTHAN C. R., Ph. D.



**AMALA CANCER RESEARCH CENTRE,
THRISSUR, KERALA, INDIA**

September 2018

DECLARATION

I hereby declare that the thesis entitled “**STUDIES ON THE BIOLOGICAL PROPERTIES OF SELECTED UVARIA SPECIES WITH EMPHASIS ON ANTICANCER ACTIVITY**” is based on the original work carried out by me at Department of Biochemistry, Amala Cancer Research Centre, Thrissur, Kerala, under the guidance of Dr. C.R. Achuthan, Assoc. Professor, Dept. of Biochemistry, Amala Cancer Research Centre, Thrissur, Kerala. The thesis has been subjected to plagiarism check and no part thereof has been presented for the award of any other degree, diploma or other similar titles.

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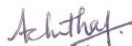


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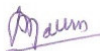
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‘Let us be grateful to people who make us happy; they are the charming gardeners who make our souls blossom.’ - Marcel Proust

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SMITHA K. R.

Abstract

Abstract

Recent global statistics have indicated an increasing trend in the morbidity and mortality related to cancer. The advent of newer technologies has tremendously improved the detection of cancer at early stages and offers successful treatment strategies that increase the survival population. However, in cancers detected at late stages, life expectancy remains low. It is generally agreed that 90-95% cancers can be prevented just by avoiding risk factors, however, a genetic predisposition that accounts for the remaining 5% is hard to prevent. Since all risk factors so far identified contribute to chronic oxidative stress and inflammatory conditions in the body, maintaining innate immunity and oxidative balance or boosting redox status can be adopted as preventive strategies. This can be achieved either by dietary regimen or by exogenous antioxidant supplements, anti-inflammatory or immune stimulatory agents. Many of these antioxidant, anti-inflammatory and immune stimulatory agents can limit cancer recurrence as well as prevent chemotherapy-induced secondary toxicities.

Extensive studies on natural products in drug development and design have identified the importance of pharmacologically active anticancer phytochemicals and many of which are under various stages of clinical trials. Paclitaxel from *Taxus brevifolia* in the trade name Taxol, vincristine and vinblastine from *Vinca rosea*, tamoxifen, etc. are either plant-derived compounds or their synthetic analogs, currently used in chemotherapy. However, these compounds are known to produce secondary toxicity that limits their usage. Search for newer drug candidates with less side effect are still continuing and hence it is important to have more focus on plants species with medicinal value to explore their therapeutic action.

Annonaceous acetogenins are one of the most propitious classes of phytochemicals, isolated and purified from members of Annonaceae family having pronounced antitumor activity. *Annona muricata*, *Annona squamosa*, *Annona vepretorum*, etc. have been identified as the good source. Apart from these, the family also account for pharmacologically active terpenoids (eg. annonaretin A), alkaloids (eg. Arnepavine and annonamine), flavonoids (pinocembrin, pinostrobin, chamanetin, isochamanetin, and dichamanetin) and

phenolic acids (eg. caffeic acid, sinapic acid, and ferulic acid). *Uvaria* species of this family has been extensively studied and identified for their anticancer, antitumor, antioxidant and anti-inflammatory efficacies. Compounds such as uvaretin, diuvaretin, kweichowenol, uvaricin, and isouvaricins are some of the important cytotoxic compounds reported from this species. *Uvaria narum* and *Uvaria macropoda* are locally available species which are less explored. The current study assessed the pharmacological efficacy pertinent to the anticancer potential of these plants.

For preliminary screening of cytotoxic, antioxidant and anti-inflammatory activities, leaf extracts of both *U.narum* (UN) and *U.macropoda* (UM) were prepared using solvents of increasing polarity (Petroleum ether (PE), Chloroform (CHL), acetone (ACT), methanol (MET) and water (AQE)). Phytochemical identification by biochemical and TLC analysis of individual extracts revealed the presence of a more or less similar class of compounds such as terpenoids, sterol, alkaloids, phenols, flavonoids, etc. The cytotoxic potential was evaluated by *in vitro* short-term cytotoxicity assay (Trypan blue exclusion) using DLA (Dalton's Lymphoma Ascites cells), EAC (Ehrlich Ascites Carcinoma cells) and normal spleen cells and long-term antiproliferative assay was performed using HeLa (human cervical cancer cells), HepG2 (human hepatocellular carcinoma cells), HCT-15 (human colon carcinoma cells) and Vero (African monkey normal kidney cells). All the extracts of *U.macropoda* (UMPE, UMCHL, UMACT, UMMET, and UMAQE) were found cytotoxic to the cancer cells as well as normal cells studied in both assays with documented IC₅₀ values above 100 µg/mL. Chloroform (UMCHL) extract was an exception, which showed cytotoxicity towards DLA cells with an IC₅₀ value of 57.60 ± 1.51 µg/mL. On the other hand, individual extracts of *U. narum* such as UNCHL, UNACT, and UNMET were highly cytotoxic (IC₅₀ values below 100 µg/mL) to all cells including normal cells while aqueous extract did not show appreciable toxicity (IC₅₀ value above 100 µg/mL). Interestingly, compounds of UNPE extract showed differential cytotoxicity, being toxic towards cancer cell lines (DLA and EAC) while less toxic to normal spleen cells with IC₅₀ values 19.0 ± 0.57, 38.0 ± 0.74 and >100 µg/mL, respectively in short-term assay and profound antiproliferative activity with IC₅₀ values 20.10 ± 0.97, 5.29 ± 0.49 and 15 ± 0.53

$\mu\text{g/mL}$, respectively towards HeLa, HCT-15 and HepG2. However, in Vero cells, an IC_{50} value obtained was two-fold higher (43.72 ± 1.35) indicating lesser toxicity. Thus from the screening for cytotoxicity among *U.narum* and *U.macropoda* extracts, UNPE extract had been found most active due to its differential cytotoxic/antiproliferative efficacy which is promising. This differential cytotoxicity formed the basis of the selection of UNPE extract for further *in vivo* antitumor studies.

Screening antioxidant efficacy of individual extracts of both plants using *in vitro* antioxidant assays (ferric reducing antioxidant potential (FRAP) 1, 1-diphenyl-2-picrylhydrazyl (DPPH) reducing efficacy, inhibition of lipoxygenase and lipid peroxidation) revealed that all the individual extract possess antioxidant properties in a dose-dependent manner. Supporting these observations, an appreciable amount of polyphenols and flavonoids were found distributed among these extracts. However, *U. narum* plant extracts were comparatively effective than that of *U. macropoda*. Among these extracts, UNMET had the highest antioxidant potential with depicted IC_{50} values 4.13 ± 0.73 , 18.65 ± 0.93 , 50 ± 2.23 and $76 \pm 1.27 \mu\text{g/mL}$, respectively in ferric reducing antioxidant potential (FRAP), 1,1-diphenyl-2-picrylhydrazyl (DPPH) reducing efficacy, inhibition of lipoxygenase and lipid peroxidation assays. In corroboration, UNMET was found to have a higher amount of phenols and flavonoids. Based on these results, UNPE extract which exhibited differential cytotoxicity/antiproliferative efficacy and UNMET extract that showed high antioxidant potential were further analyzed in detail using *in vivo* animal models.

Before going to *in vivo* studies, safety evaluation using acute and sub-acute toxicity models were carried out for the selection of pharmacological doses of the UNPE and UNMET extracts. In the acute oral administration, UNPE extract, above 250 mg/kg b.wt. (2000, 1000, 500 and 300 mg/kg b.wt.) was found highly toxic and led to the death of animals. Hence 250 mg/kg b.wt. was considered as LD_{50} of the extract. On this basis, the doses for UNPE extract for sub-acute toxicity studies were selected as 5, 7.5 and 10 mg/kg b.wt. Daily oral administration of the extract at this specified concentration for 28 days didn't elicit changes in lipid profile, liver functioning, creatinine and urea levels and

hemogram of the mice. Histological examination did not show any toxic alteration in major organs of treated groups animals compared to normal animals. However, UNPE extract in high dose showed toxicity towards the testes and this has to be cautiously evaluated in a systematic manner before concluding its antitumor efficacy. In antitumor studies, the oral administration of UNPE extracts at 5 and 10 mg/kg b. wt. simultaneously with tumor induction showed a significant reduction ($P < 0.01$) in the DLA induced solid tumor volume showing 57.30 and 61.07%, tumor reduction, respectively. At the same dose, the lifespan of ascites tumor-bearing mice was found increased by 47.37 and 56.58%. These doses, however, were well tolerated by the animals without changing the WBC count and total body weight during the entire experimental period (30 days) while maintaining the antitumor efficacy. Simultaneous administration of extract achieved significant tumor reduction compared to the pre-treatment. However, the antitumor efficacy of the UNPE extract was comparatively lesser than cyclophosphamide administered animals. Upon cytotoxicity based fractionation of UNPE extract, the methanolic fraction was found to have better toxic potential with IC_{50} values 27.82 ± 1.02 , 47.27 ± 2.13 and $>100 \mu\text{g/mL}$ towards DLA, EAC, and splenocytes, respectively. The antiproliferative activity of this fraction was found to be dose-dependent exhibiting IC_{50} values of 23.09 ± 2.54 , 98.53 ± 5.47 , 26.05 ± 1.24 and $42.21 \pm 2.08 \mu\text{g/mL}$ in HeLa, Vero, HepG2 and HCT-15 cells, respectively. Further purification of this cytotoxic methanolic fraction resulted in ethyl acetate (EA) and toluene (TOL) sub-fractions. Among this, the sub-fraction EA was found superior to toluene with respect to cytotoxicity and antiproliferative efficacies. When the mechanistic basis of the toxicity was studied using AO/EtBr staining and FACS analysis (Annexin V-FITC combined with propidium iodide staining), both UNPE extract and its EA sub-fraction induced membrane blebbing, chromatin condensation and nuclear fragmentation characteristic of apoptosis in HeLa cells and higher population of Annexin V-FITC stained cells in the Quadrant 2 (Q2) of FACS suggestive of apoptosis. This was further confirmed by cell cycle analysis, using a combination of PI followed by Hoechst 33342 dye, where cell cycle arrest occurred in sub-G1 phase. HPTLC and GC-MS analysis of UNPE extract revealed a number of cytotoxically active sterols and terpenoids such as phytol and squalene, and fatty acid derivatives such as hexadecane and pentadecane. These molecules might be contributing to the

apoptosis-inducing ability of the UNPE extract and its fractions. However, UNPE extract as a whole was found to be most effective than its sub-fractions. Possibly, the separation of cytotoxic compounds into different sub-fractions might have diluted the toxic potential. Another reason could be the modification in the individual molecules of sub-fractions as revealed in the GC-MS analysis. Therefore it is expected that the synergistic effect of phytomolecules in the UNPE extract contributes better cytotoxic potential and needs further exploration.

In the acute toxicity study, the UNMET extract did not produce any mortality up to 14 days at a dose of 2000 mg/kg b.wt. No behavioral changes or weight loss were observed during this period. This suggests that the animals tolerated UNMET extract even up to a dose of 2000 mg/kg b.wt. and therefore the LD₅₀ value may be above this dose. For further sub-acute toxicity studies, doses between 1/10th (200 mg/kg b.wt.) and 1/5th (400 mg/kg b.wt.) of acute dose were selected. The sub-acute oral administration of the doses in this range did not elicit any changes in body weight, the pattern of food and water consumption and organosomatic index. The blood biochemical parameters such as serum electrolytes, lipid profile, liver function marker enzymes, and levels of urea and creatinine remained unchanged following administration of the extracts for 28 days. Histological details of important organs revealed no structural changes between normal and treated animals except for testes. Histological details of testes, however, showed moderate spermatogonial abnormalities. This specific toxicity towards testes was observed in animals treated with 400 mg/kg b.wt. UNMET extract. A careful evaluation of the reproductive toxicity of UNMET extract is thus essential before concluding its pharmacological effect. The presence of phthalic acid mono-2-Ethylhexyl ester, a known reproductive toxicant, was detected in the UNMET extract in LC-MS analysis. Possibly this compound could be responsible for its reproductive organ toxicity. Probably UN extract could be specifically used in reproductive cancers studies which needed to be confirmed using cell culture and animal model. Another possibility is that organ specific toxic compound(s) can be purified from this extract which can be used in pre-clinical researches as a targeting agent.

The detailed investigations of UNMET extract for its antioxidant effect using sodium fluoride (NaF) induced oxidative stress in mice revealed that the extracts, at 200 and 400 mg/Kg b.wt. reversed the NaF induced drop in GSH level, SOD and catalase activities and concomitant hike in TBARS. The histological examination on liver tissues of the treated groups was less affected when compared to the NaF administered control group. This suggested that the protective ability of UNMET extract is due to its ability to maintain redox balance in the animals. Further, UNMET in its two tested doses was found to inhibit PMA-induced superoxide radical generation in murine peritoneal macrophages with 46.37 and 70.88% efficacy. This further suggested the antioxidant efficacy of UNMET extract.

Oral administration of the UNMET extract at 200 and 400 mg/kg b.wt. was also found to reduce carrageenan (by 37.47 and 44.83%) and dextran (19.15 and 25.21%) induced acute paw edema in mice. Studies using Evans blue dye in carrageenan-induced paw edema, it was found that pre-treatment with diclofenac (25 mg/kg b.wt.) as well as UNMET extracts (UNMET-LD: 200mg/kg b.wt. and UNMET-HD: 400mg/kg b.wt.) significantly reduced ($p \leq 0.01$) the extravasation of Evans blue. The standard treated groups exhibited 89.35% inhibition while UNMET-LD-200 mg/kg b.wt. and UNMET-HD- 400 mg/kg b.wt. treated groups were shown 75.93 and 88.92 % inhibition when compared to the control group. Also, the UNMET extract effectively inhibited the formation of formalin-induced chronic paw edema. The histological evaluation of edematous tissue in animals revealed that the treatment with the plant extracts reduced the infiltration of inflammatory cells to the inflamed site. Purification of the UNMET extract resulted in ethyl acetate (EA) and water (AQ) fractions. UNMET-AQ fraction showed better antioxidant potential compared to EA fraction based on DPPH free radical scavenging activity and lipid peroxidation inhibition. Further evaluation of the effect of UNMET-AQ fraction on lipopolysaccharide (LPS)-induced mouse TNF- α secretion in RAW264.7 cells showed that cells exposed to LPS had TNF alpha levels of 342.5 ± 28.93 pg/ mL. However, in UNMET-AQ pretreated (50 and 100 μ g/mL) RAW264.7 cells when exposed to LPS, TNF alpha level was significantly reduced to 226.17 ± 15.14 and 137 ± 15.22 pg/ mL. This was confirmed by real-time PCR analysis of mRNA level expression, in which the

fold change in expression of TNF alpha mRNA with respect to GAPDH (housekeeping gene) in normal untreated cells was found to be 1.03 ± 0.04 and in LPS alone treated cells fold change of 1.91 ± 0.17 was observed. On the other hand, macrophages pre-treated with UNMET-AQ fraction (50 and 100 $\mu\text{g}/\text{mL}$) and challenged with LPS, the fold changes of TNF alpha mRNA in the cells decreased to 1.87 ± 0.10 (ns) and 0.44 ± 0.03 ($P < 0.01$). This strongly suggests the potential of UNMET-AQ fraction to inhibit TNF- alpha-mediated inflammatory signaling; possibly NF- κ B mediated signaling which may be promising in anticancer drug development. Overall, the study concludes that *U. narum* is a good source of anticancer phytochemicals which needs further exploration.

Keywords: *U.narum*, *U.macropoda*, Phytochemicals, antioxidant, anti-inflammatory, antitumor, apoptosis.

Preface

Preface

Plants form the basis for traditional treatment strategies throughout the world for hundreds of years and several plant species have been used in the form of decoctions, crude concentrates and dried powder form to treat various disease conditions. Safety of plant-derived medicine is an important aspect of these medicinal practices. Scientific studies to validate the medicinal use of these plants have identified several important classes of phytochemicals. Last decades witnessed the channelizing phytochemicals for drug development and design. As a result, several plant-derived drugs have been developed and many of them are drug candidates at various stages of clinical trials. It is estimated that approximately 50% of currently used medicine in the modern medical system is either plant-derived or their synthetic analogs. Search for newer drug candidates with less side effect are still continuing and hence it is important to have more focus on plants species with medicinal value to explore their therapeutic action.

Annonaceae has been recorded as one of the most diversified families of tropical forest. For centuries, Annonaceae plants are used in traditional medicines to cure various clinical conditions such as diarrhea, dysentery, arthritis pain, rheumatism, neuralgia, jaundice, and weight loss, etc. Numerous research studies have highlighted the pharmacological activities of the crude extracts as well as isolated compounds from Annonaceae species such as generalized body pain, a potent analgesic, rheumatism, and neuralgia, anti-inflammatory, wound healing, inflammatory bowel diseases, and gastrointestinal ulcers, bronchitis asthma attack. Several indigenous species have been reported to have potent immunomodulatory activity. Phytochemical investigations on plants of Annonaceae family have reported the presence of triterpenes, diterpenes and diterpene flavone glycosides, sterols, lignans, annonaceous acetogenin, alkaloids, and flavonoids, etc. Annonaceous acetogenins are one of the most propitious classes of phytochemicals, having pronounced antitumor activity. For instance, studies on *Annona muricata* have revealed its anticancer, anticonvulsant, anti-arthritic, antiparasitic, antimalarial, hepatoprotective and antidiabetic activities. More than 100 annonaceous acetogenins have been isolated from leaves, barks, seeds, roots, and fruits of *A. muricata*.

The genus *Uvaria* L. under the family Annonaceae contains more than 150 species, known to comprise many bioactive compounds. Traditional medicine has used some *uvaria* plant extract for treating skin allergies, fever, intestinal ulcers, malaria and prostate disorders including Benign prostate hyperplasia (BPH). Recent observations have reported that *uvaria* plants possess antioxidant, antifungal, cytotoxic and antitumor activities (Padmaja et al. 1993). *Uvaria narum* Dunal (Wall.) locally called “narumpanal” has been used for gastrointestinal problems, skin diseases, constipation, low backache, jaundice and fever in traditional medicine and Ayurveda. However, scientific evidence to validate the medicinal use is very less. Various studies have reported that acetogenins and other phytoconstituents in *U. narum* show antifungal, cytotoxic and antitumor activities. Essential oils from different parts of *U. narum* have also been investigated. *Uvaria macropoda* Hook f & Thomson is another *uvaria* plant distributed in South India and Sri Lanka region. At the same time, there is no available scientific data about the pharmacological activities of *Uvaria macropoda* Hook f & Thomson.

This thesis aims to document the pharmacological potential of two locally available *Uvaria plants viz. U.narum and U.macropoda*. The study starts with a comparison of different extracts of these two plants based on cytotoxic and antioxidant properties. The cytotoxic (*U.narum* PE extract) and antioxidant (*U.narum* MET extract) potential fractions are selected for further studies. Antitumor efficacy of cytotoxic principle has been explored and mechanistic basis revealed. Antioxidant and anti-inflammatory components have been identified in the antioxidant active fraction. The present work thus contributes to the understanding of the pharmacological potential of *U. narum*. More detailed studies on the molecular mechanisms and actions are further required to generate candidate drugs.

Table of Contents		
Chapter	Title	Pages
1	Review of Literature	1-46
2	Materials and methods	47-97
3	Preliminary screening for phytochemical constituents, cytotoxicity and antioxidant properties of <i>Uvaria narum</i> and <i>Uvaria macropoda</i>	98-134
4	Safety evaluation of pharmacologically active extracts of <i>U.narum</i> leaves	135-164
5	Cytotoxic and antitumor principles of UNPE extract and their mechanism of action	165-208
6	Exploration of antioxidant and anti-inflammatory potentials of methanolic extract of <i>U. narum</i> (Dunal) Wall. and its partial purification	209-243
7	Summary	244-247
	Bibliography	248-276
	List of publications	277

List of tables

- Table 1.1. Pharmacologically important plants in Annonaceae family
- Table 1.2. Compounds isolated from different plants of Annonaceae family
- Table 1.3. Uvaria plants studied for their biological properties
- Table 1.4. Important phytochemicals isolated from Uvaria species with their activities
- Table 3.1. The percentage yield of different extracts of *U. narum* and *U. macropoda* leaves.
- Table 3.2. Phytochemical screening of different extracts of *U. narum* leaves.
- Table 3.3. Phytochemical screening of different extracts of *U. macropoda* leaves
- Table 3.4. IC₅₀ values of *U. narum* and *U. macropoda* leaf extracts on *in vitro* cytotoxicity study on DLA, EAC and spleen cells by trypan blue method.
- Table 3.5. IC₅₀ values of *U. narum* and *U. macropoda* leaf extracts on *in vitro* antiproliferative study on HeLa, Vero, HCT-15 and HepG2 cells by MTT assay.
- Table 3.6. Total phenols and flavonoids of different extracts of *U. narum* and *U. macropoda* leaves
- Table 4.1. Change in average weight (g) of mice following single dose oral administration of UNMET extract.
- Table 4.2. Relative organ weight of male and female Swiss albino mice following sub-acute administration of UNMET extract of *U. narum* leaves over 28 days.
- Table 4.3. Relative organ weight of male and female Swiss albino mice upon subacute administration of UNPE extract of *U. narum* leaves over 28 days.

- Table 4.4. Hematological parameters of male and female Swiss albino mice following sub-acute oral administration of UNMET extract.
- Table 4.5. Hematological parameters of male and female Swiss albino mice following sub-acute oral administration of UNPE extract.
- Table 4.6. Serum electrolytes levels of male and female Swiss albino mice following sub-acute oral administration of UNMET extract
- Table 4.7. Serum electrolytes levels of male and female Swiss albino mice following sub-acute oral administration of UNPE extract
- Table 4.8. Levels of liver function markers following sub-acute oral administration of UNMET
- Table 4.9. Levels of liver function markers following sub-acute oral administration of UNPE extract
- Table 4.10. Blood lipid profile of Swiss albino mice following oral administration of UNMET extract
- Table 4.11. Blood lipid profile of Swiss albino mice following oral administration of UNPE extract
- Table 4.12. Creatinine and urea levels in the blood following oral administration of UNMET extract
- Table 4.13. Creatinine and urea levels in the blood following oral administration of UNPE extract
- Table 5.1. The inhibitory effect of *U.narum* PE leaf extract on DLA induced solid tumor
- Table 5.2. Mean survival days and % increase in lifespan of ascites tumor-bearing mice treated with UNPE extract.
- Table 5.3. Total compounds identified in the of UNPE extract of *U.narum* leaves by GC-MS analysis

Table 5.4. Compounds in PE extract of *U.narum* leaves identified by GC-MS analysis and their reported biological activity

Table 5.5. Percentage of the cell population in control and ethyl acetate sub-fraction treatment after staining with Annexin V-FITC staining and analyzed using FACS scanner

Table 5.6. Percentage distribution of HeLa cells exposed to ethyl acetate sub-fraction in the cell cycle.

Table 5.7. Compounds reported in the ethyl acetate sub-fraction of UNPE extract through GCMS analysis

Table 6.1. The compounds identified in UNMET by UPLC-Q-TOF-MS analysis

Table 6.2. List of unidentified peaks with RF (min), Mass and m/z ratio obtained after UPLC-Q-TOF-MS analysis of UNMET extract

Table 6.3. List of pharmacologically important compounds identified in UNMET by UPLC-Q-TOF-MS analysis

Table 6.4. Percentage inhibition by UNMET on PMA induced superoxide radical generation in peritoneal macrophages

Table 6.5. Effect of UNMET extract administration on carrageenan-induced acute paw edema

Table 6.6. Effect of UN methanolic extract administration on dextran-induced acute paw edema

Table 6.7. Effect of UNMET extract on chronic formalin-induced paw edema

List of Figures

- Figure 1.1. Global cancer mortality and new cases worldwide in 2018 based on GLOBOCAN 2018 report (Bray et al. 2018)
- Figure 1.2. Estimated age-standardized incidence rates worldwide in 2018, in both sexes of all age group (World cancer fact sheet 2018, WHO)
- Figure 1.3. Role of alcohol intake in cancer development
- Figure 1.4. Effect of ROS on signaling pathways
- Figure 1.5. Inflammation and cancer
- Figure 1.6. Mechanism of action of different phytochemicals on cellular pathways
- Figure 1.7. Mechanism of action of Paclitaxel on apoptosis process
- Figure 1.8. Different cellular targets of Curcumin
- Figure 1.9. The action of kaempferol on different cellular pathways
- Figure 1.10. *Uvaria narum* (Dunal) Wall.
- Figure 1.11. *Uvaria macropoda* Hook. f. & Thomson
- Figure 3.1. TLC of the UNPE extract obtained by developing sample (10 $\mu\text{g/mL}$) using mobile phase, n-hexane: diethyl ether: glacial acetic acid (35:15: 0.75) and observed under (A) visible light and (B) iodine vapour and with spray reagents such as (C) vanillin (D) Liebermann–Burchard reagent, (E) Liebermann–Burchard UV (F) anisaldehyde (G) anisaldehyde UV, (H) 20% sulphuric acid (I) Dragendorff's, (J) ferric ferrocyanide, (K) methanol- potassium hydroxide and (L) ammonia.
- Figure 3.2. Thin layer chromatogram of the UNCHL extract obtained by developing sample (10 $\mu\text{g/mL}$) using mobile phase, benzene: ethyl acetate (5:1), and observed under (A) visible light, (B) iodine vapor, and sprayed with (C) vanillin reagent, (D) Liebermann reagent, (E)

Liebermann–Burchard UV (F) anisaldehyde reagent, (G) anisaldehyde UV, (H) 20% sulphuric acid reagent, (I) methanol potassium hydroxide reagent and (J) ammonia reagent

Figure 3.3. Chromatogram of the UNACT extract obtained by developing sample (10 $\mu\text{g}/\text{mL}$) using mobile phase chloroform: methanol: water (16:5:0.8 V/ V/ V), and observed under (A) visible light and (B) iodine vapour and spraying with (C) vanillin reagent, (D) Liebermann–Burchard reagent, (E) Liebermann–Burchard UV (F) anisaldehyde reagent, (G) anisaldehyde UV, (H) 20% sulphuric acid reagent, (I) methanol potassium hydroxide reagent and (J) ammonia reagent

Figure 3.4. TLC of the UNMET extract obtained by developing sample (10 $\mu\text{g}/\text{mL}$) using mobile phase chloroform: methanol: water (16:5:0.8 V/ V/ V), and observed under (A) visible light and (B) iodine vapour and spraying with (C) vanillin reagent, (D) Liebermann–Burchard reagent, (E) Liebermann–Burchard reagent (UV) (F) anisaldehyde reagent, (G) anisaldehyde (UV), (H) 20% sulphuric acid reagent, (I) dragondorff's reagent, (J) methanol- potassium hydroxide reagent and (K) ammonia reagent

Figure 3.5. TLC of the UNAQE by developing sample (10 $\mu\text{g}/\text{mL}$) using mobile phase methanol: water: acetic acid (18:9:1 V/ V/ V), and observed under (A) visible light and (B) iodine vapor

Figure 3.6. Thin layer chromatogram of the leaf UMPE obtained by developing sample (10 $\mu\text{g}/\text{mL}$) using mobile phase, n-hexane: diethyl ether: glacial acetic acid (35:15: 0.75) and observed under (A) visible light, (B) iodine vapour (C) UV light (254nm), and with spray reagents (D) anisaldehyde reagent, (E) vanillin reagent and (F) Dragondorff's reagent

Figure 3.7. TLC of the UMCHL extract obtained by developing sample (10 $\mu\text{g}/\text{ml}$) using mobile phase, benzene: ethyl acetate (5:1), and observed under (A) visible light, (B) iodine vapor (C) UV light (254nm), and

with spray reagents (D) Anisaldehyde reagent , (E) vanillin reagent and (F) Dragondorff's reagent

Figure 3.8. TLC of the UMACT extract obtained by developing sample ($10\mu\text{g/ml}$) using mobile phase chloroform: methanol: water (16:5:0.8 V/ V/ V), and observed under (A) visible light, (B) iodine vapour (C) UV light (254nm), and with spray reagents (D) Anisaldehyde reagent and (F) Dragondorff's reagent

Figure 3.9. TLC of the UMMET extract obtained by developing sample ($10\mu\text{g/mL}$) using mobile phase chloroform: methanol: water (16:5:0.8 V/ V/ V), and observed under (A) visible light, (B) iodine vapor (C) UV light (254 nm), and with spray reagents (D) anisaldehyde reagent, (E) vanillin reagent and (F) Dragondorff's reagent

Figure 3.10. TLC of the UMAQE extracts obtained by developing sample ($10\mu\text{g/ml}$) using mobile phase butanol: acetic acid: water (60:15:25 V/ V/ V) and observed under (A) visible light, (B) iodine vapor (C) UV light (254nm)

Figure 3.11. Cytotoxicity of *U.narum* leaf extracts towards (A) DLA, (B) EAC and (C) spleen cells

Figure 3.12. *In vitro* cytotoxicity of *U.macropoda* leaf extracts on (A) DLA, (B) EAC and (C) spleen cells

Figure 3.13. Antiproliferative activities of *U.narum*. A) UNPE, B) UNCHL, C) UNACT, D) UNMET and E) UNAQE extracts against HeLa, Vero, HCT-15 and HepG2 cell growth detected by MTT assay after 48 hr treatment.

Figure 3.14. Morphological changes observed under phase contrast microscope 200X in HeLa, HepG2, HCT-15, and Vero cells, after 48 hr treatment with different extracts of *U.narum* leaves. a) Control b) UNPE c) UNCHL d) UNACT e) UNMET and f) UNAQE

Figure 3.15. Antiproliferative activities of *U.macropoda* A) UMPE, B) UMCHL C) UMACT D) UMMET and E) UMAQE extracts against HeLa,

Vero, HCT-15 and HepG2 cell growth detected by MTT assay after 48 hr treatment.

Figure 3.16. *In vitro* free radical scavenging activities of PE, chloroform, acetone, methanol and water extracts of *U.narum* leaves. A) DPPH radical scavenging assay; B) inhibition of lipid peroxidation; C) FRAP assay: chloroform, acetone and methanol extracts; D) FRAP assay: PE and water extracts and E) lipoxygenase inhibition assay.

Figure 3.17. *In vitro* free radical scavenging activities of PE, chloroform (CHL), acetone, methanol (MET) and water (AQE) extracts of *U.macropoda* leaves. A) DPPH radical scavenging assay B) inhibition of lipid peroxidation and C) FRAP assay and D) lipoxygenase inhibition assay.

Figure 4.1. Observation of systemic organs and the body of treated animals: A) Male, B) Female Swiss albino mice were orally given UNMET-High Dose: 400 mg/b.wt and UNPE-High Dose: 10 mg/kg b.wt. of extract for 28 days.

Figure 4.2. Body weights of A) male and B) female Swiss albino mice after administration of 200, 300, and 400 mg/kg b.wt. UNMET extract.

Figure 4.3. Body weight of A) male and B) female Swiss albino mice on the subacute oral administration of 5, 7.5, and 10 mg/kg b.wt. UNPE leaves. Extracts were administered orally on a daily basis for 28 days.

Figure 4.4. The pattern of food consumption of A) male and B) female Swiss albino mice following sub-acute administration of UNMET extract.

Figure 4.5. The pattern of food consumption of A) male and B) female Swiss albino mice following sub-acute administration of UNPE extract.

Figure 4.6. Water consumption of A) male and B) female Swiss albino mice following sub-acute administration of UNMET extract.

Figure 4.7. Water consumption of A) male and B) female Swiss albino mice following sub-acute oral administration of UNPE leaves extract.

Figure 4.8. Histological examinations of the main organs in male Swiss albino mice following sub-acute toxicity study.

Figure 4.9. Histological examinations of the main organs in female Swiss albino mice following 28 days of repeated dose sub-acute toxicity study

Figure 5.1. Solid tumor volume (cm^3) in Swiss Albino mice after UNPE administration. Animals were inoculated with 1×10^6 cells in the right hind limb.

Figure 5.2. I(A): Body weight of UNPE pretreated animals; I(B): body weight of animals that got simultaneous treatment of UNPE along with tumor induction; II(A): total WBC count of DLA induced solid tumor-bearing mice that received pretreatment of different doses of extract; II(B): total WBC count of DLA induced solid tumor-bearing mice that received simultaneous extract treatment with tumor induction.

Figure 5.3. The survival rate of ascites tumor bearing mice following UNPE extract administration.

Figure 5.4. Survival and extent of lifespan in tumor-bearing animals following UNPE extract administration.

Figure 5.5. Total ion chromatogram (TIC) of UNPE extract of *U.narum* leaves when subjected to GC MS analysis

Figure 5.6. Cytotoxic effect of fractions of UNPE extract

Figure 5.7. Antiproliferative activity of fractions of UNPE extract

Figure 5.8. HPTLC analysis of 1) UNPE extract, 2) Hexane and 3) methanol fractions visualized under A) UV 254 nm B) 366 nm and C) derivatization using spraying reagent anisaldehyde sulphuric acid

Figure 5.9. Cytotoxic effect of sub-fractions of UNPE extract MET fraction

Figure 5.10. Antiproliferative activities of sub-fractions of UNPE extract

Figure 5.11. Induction of apoptosis in HeLa cells by UNPE extracts and their sub-fractions

Figure 5.12. showing the apoptotic pattern of A) untreated control HeLa cells and ethyl acetate sub-fraction B) 50 and C) 100 µg /ml treated cells

Figure 5.13. EA sub-fraction induces cell cycle arrest in HeLa cells in vitro. Flow cytometric cell cycle analysis was performed

Figure 5.14. (A–C) Cell cycle analysis histograms.

Figure 5.15. Total Ion Chromatogram (TIC) of EA Fr of PE extract of *U.narum* leaves on GC MS analysis

Figure 6.1. shows the total ion chromatogram of the methanolic extract when subjected to UPLC-Q-TOF-MS analysis

Figure 6.2. Effect of UNMET extract on the antioxidant status of blood following sodium fluoride-induced oxidative stress

Figure 6.3. Effect of UNMET extract on the antioxidant status in the liver of mice following sodium fluoride-induced oxidative stress

Figure 6.4. Histopathology of liver tissue following NaF induced oxidative stress

Figure 6.5. Effect of UNMET on PMA induced superoxide radical generation in peritoneal macrophages.

Figure 6.6. *In vivo* anti-inflammatory effect of UNMET extract on carrageenan-induced acute inflammation in murine paw edema.

Figure 6.7. Photographs of the hind paw of mice in various experimental groups showing Evans blue retention status.

Figure 6.8. Quantitative measurement of Evans blue retained in the edematous paw of animals in the various experimental groups.

Figure 6.9. *In vivo* anti-inflammatory effect of UNMET extract on dextran-induced acute inflammation in murine paw edema

Figure 6.10. *In vivo* anti-inflammatory effect of UNMET extract on formalin-induced chronic inflammation in murine paw edema.

Figure 6.11. Photographs of animals in the study of *in vivo* anti-inflammatory effect of UNMET extract on I) carrageenan and II) formalin-induced inflammation of paw edema in mice

Figure 6.12. Effect of UNMET extract on histological changes in skin tissues of the plantar region of mouse paws.

Figure 6.13. Antioxidant potentials of *U.narum* methanolic extract fractions such as EA and AQ fractions

Figure 6. 14. Effect of UNMET-AQ fraction on LPS induced TNF- α production in RAW264.7 cells:

Figure 6.15. Expressions of TNF - α in LPS stimulated RAW264.7 cells exposed to UNMET-AQ fraction

Abbreviations

ALP	Alkaline phosphatase
AO	Acridine orange
ATCC	American type culture collection
b.wt.	Body weight
CMC	Carboxy methyl cellulose
Cox	Cyclooxygenase
DLA	Dalton's Lymphoma Ascites
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulphoxide
DPPH	1, 2 -diphenyl-2-picrylhydrazyl
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid
e.g.	Example
EA	Ethyl acetate
EAC	Ehrlich Ascites Carcinoma
EBD	Evans blue dye
EDTA	Ethylene diamine tetra acetic acid
EtBr	Ethidium bromide
FBS	Fetal bovine serum
FeSO ₄	Ferrous sulfate
FITC	Fluorescein isothiocyanate
FRAP	Ferric reducing antioxidant power assay
GAE	Gallic acid equivalents
GC-MS	Gas chromatography–mass spectrometry
GSH	Glutathione
Hb	Hemoglobin
HDL	High-density lipoprotein
HDLc	High-density lipoprotein cholesterol
HPTLC	High Performance Thin Layer Chromatography
hr	Hour
KH ₂ PO ₄	Monopotassium phosphate
LDL	Low-density lipoprotein

LPO	Lipid Peroxidation
LPS	Lipopolysaccharide
MDA	Malondialdehyde
MeOH	Methanol
Min	Minutes
Na ₂ HPO ₄	Sodium hydrogen phosphate
NaCl	Sodium chloride
NaF	Sodium Fluoride
NaHCO ₃	Sodium hydrogen carbonate
NSAID	Non-steroidal antiinflammatory drugs
PBS	Phosphate Buffered Saline
PE	Petroleum Ether
PI	Propidium Iodide
PMA	Phorbol 12-myristate 13-acetate
QE	Quercetin Equivalents
qRT-PCR	Quantitative Reverse Transcription PCR
ROS	Reactive Oxygen Species
RPMI	Rosewell Park Memorial Institute Medium
RT-PCR	Reverse transcriptase polymerase chain reaction
SD	Standard Deviation
SGOT	Serum Glutamate Oxaloacetate Transaminase
SGPT	Serum Glutamate Pyruvate Transaminase
TBA	Thiobarbituric Acid
TBARS	Thiobarbituric Acid Reacting Substance
TC	Total Count
TCA	Trichloroacetic acid
TLC	Thin Layer Chromatography
TNF-alpha	Tumor necrosis factor- alpha
TOL	Toluene
TP	Total Protein
UMACT	<i>U.macropoda</i> acetone extract
UMAQE	<i>U.macropoda</i> aqueous extract
UMCHL	<i>U.macropoda</i> chloroform extract
UMMET	<i>U.macropoda</i> methanolic extract

UMPE	<i>U.macropoda</i> petroleum ether extract
UNACT	<i>U.narum</i> acetone extract
UNAQE	<i>U.narum</i> aqueous extract
UNCHL	<i>U.narum</i> chloroform extract
UNMET	<i>U.narum</i> methanolic extract
UNMET-AQ	<i>U.narum</i> methanolic aqueous fraction
UNMET-EA	<i>U.narum</i> methanolic ethyl acetate fraction
UNPE	<i>U.narum</i> petroleum ether extract
UV	Ultra Violet
VLDL	Very low-density lipoprotein
WBC	White Blood Cells
WHO	World Health Organization

Chapter1
Review of Literature

1.1. Cancer

Cancer refers to the condition characterized by uncontrolled growth of abnormal cells that grow beyond their cellular boundaries, invade contiguous parts of the body and metastasis to other organs.

1.1.1. Global burden of cancer

Cancer is a major cause of death with an estimated 18.1 million new cancer cases reported worldwide in 2018 (World cancer fact sheet 2018, WHO). Prevalence of cancer within the five years is 43.8 million people in which lung cancer is the most diagnosed one (11.6% of the total cases) and the leading cause of cancer death (18.4% of the total cancer deaths) in both sexes. It is followed by breast cancer (11.6%) in females, male prostate cancer (7.1%), and colorectal cancer (6.1%) for incidence and colorectal cancer (9.2%), stomach cancer (8.2%), and liver cancer (8.2%) for mortality (Bray *et al.* 2018). The mortality rate of cancer was reported as 9.6 million deaths in 2018 with a greater number of all cancer deaths each year caused by lung, colorectal, stomach, liver, and female breast cancers (Bray *et al.* 2018; World cancer fact sheet 2018, WHO) (Figure 1.1).

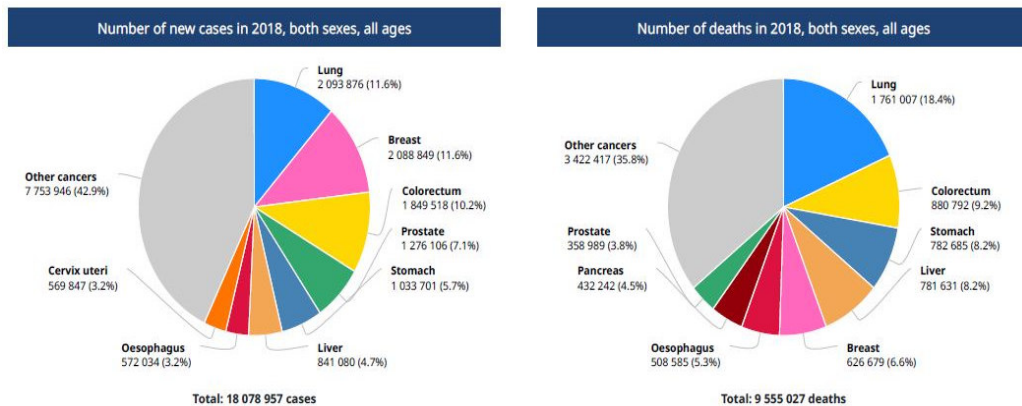


Figure 1.1. Global cancer mortality and new cases worldwide in 2018 based on GLOBOCAN 2018 report (Bray *et al.* 2018)

According to GLOBOCAN project 2018, lung cancer is the most common cancer among men (14.5% of total new cases) and breast cancer is by far the most common cancer diagnosed in women (24.2% of all new cases in women). The five most common sites of cancer among men diagnosed are the lung (14.5% of total new

cases), prostate (13.5%), colorectum (10.9%), stomach (7.2%), and liver (6.3%) whereas among women, the breast (24.2% of the total), colorectum (9.5%), lung (8.4%), cervix (6.6%), and thyroid (5.1%) as per the data from GLOBOCAN 2018. Mortality rates were estimated highest in lung cancer (22%) followed by liver (10.2%) and stomach (9.5%) among males whereas higher mortality rate was estimated in breast (15%) followed by lung (13.8%) and colorectum (9.5%) among women (World cancer fact sheet 2018, WHO). Worldwide, the incidence rate for all cancers in both sexes was about 20% higher in men (218.6 per 100,000 incidences) whereas, in women, the incidence rate is 182.6 per 100,000 with the incidence rates varying across different regions in both males and females (Bray *et al.* 2018) (Figure 1.2).

1.1.2. Territorial impressions of cancer

A territory wise global cancer incidence and mortality burden by continental level were estimated. About half of the cancer incidences seen in Asia (48.4%), and about half of this, or 23.0% of the global total, in China, and 9.8% in India. 23.4% of the incidences occur in Europe, and the remainder is in the Americas and Africa. Asia and Europe have shown an increase in the proportion of cancer-related deaths with rates of 53.9 and 20.6%, respectively; together with a decrease in the proportions exist in the countries like Oceania and North America.

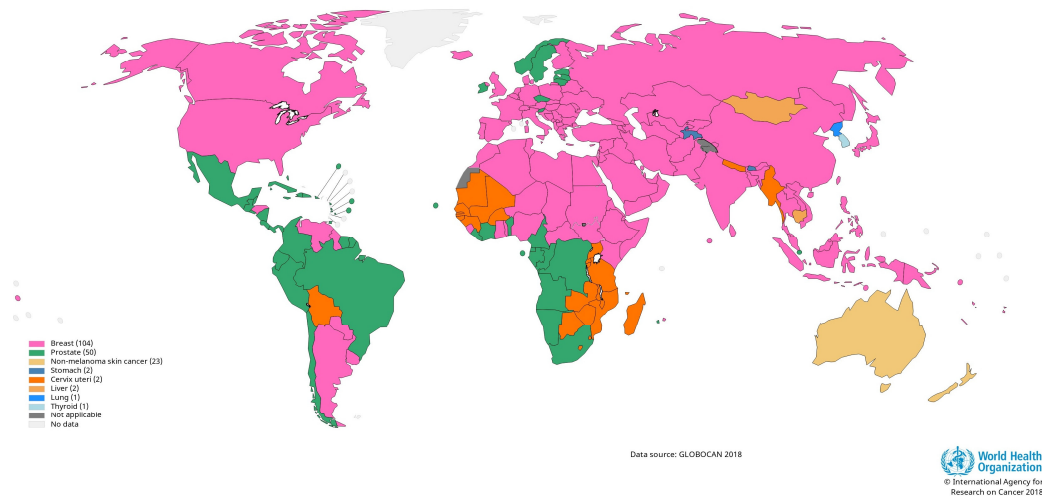


Figure 1.2. Estimated age-standardized incidence rates worldwide in 2018, in both sexes of all age group (World cancer fact sheet 2018, WHO)

If progression in major cancers is observed globally in the future, cancer prevalence will increase to 29.5 million new cases each year by 2040. This denotes an increase of 61% when compared to the year 2018 (World cancer fact sheet 2018, WHO).

1.1.3. Indian Scenario

Scientific investigations have reported that there is a rise in the number of cancer prevalence in India every year. The number of people living with cancer is estimated to be around 2.5 million (Sharma 2016). On the basis of the ICMR registry data 2016, the Indian cancer burden is estimated to be 1.45 million new cases every year and cancer-related deaths are estimated to be 5,56,400. Emerging trends of cancer incidence in India, such as the increase in lung cancer among women and colon cancer among men have been reported (Sharma 2016). Cancers at sites such as uterus, ovary, and lung are increasing among women in urban areas, whereas overall cervical cancer prevalence is reducing. The cancer incidence of colon, rectum, and prostate among men has found to be increased significantly in urban areas of Delhi, Chennai, and Bengaluru (Sharma 2016). The increasing breast and colon cancer rates indicated the association of risk factors such as lifestyles changes, urbanization, and low fruits and vegetable diets. More than 30% of cancers were associated with tobacco usage. Even though women smoking is uncommon, reports of the rising incidence of cancer especially in metropolitan areas may be due to exposure to passive smoking (Sharma 2016).

The total cancer burden is likely to increase from 979,786 cases in the year 2010 to 1,148,757 cases in the year 2020. The tobacco linked cancers in males are reported to be 190,244 in the year 2010 and it will go up to 225,241 in the year 2020 (Takiar *et al.* 2010). Female cases will be up from 75,289 in the year 2010 to 93,563 in the year 2020. For both sexes, the numbers of cancers of the alimentary tract are estimated as 107,030 and 86,606 respectively, in 2010 (Takiar *et al.* 2010). Head and neck cancer estimates are 122,643 and 53,148, respectively and lymphoid and hematopoietic cancer scenario are 62,648 and 41,591 respectively, in the year 2010. Reproductive cancers are estimated to rise from 153,850 in 2010 to 182,602 in 2020. Breast cancer is expected to rise more than 100,000 by the year 2020 (Takiar *et al.* 2010).

According to the World Cancer Report 2013, 5.37 lakh Indian women affected with cancer in 2012 as against 4.77 lakh men. In this scenario, 75% of the men affected

with cancer are with low life expectancy, while 60% mortality rate in the case of women cancer. Cancer-related death was reported to be 3.56 lakh in men and 3.26 lakh women in 2012. When compared among both sexes, breast cancer incidence is highest at 1,44,937, while the second most frequently occurring is cervix and uterus cancers with 1,22,844 cases. The third most common cancer is of the oral cavity and lip cancer with 53,842 men cases (World cancer report 2014, World Health Organization).

1.2. Carcinogenesis and risk factors

Carcinogenesis is a multistage process including initiation, promotion, and progression through which a normal cell transforms into a neoplastic one or else a benign lesion become malignant. Several exogenous and endogenous agents (called carcinogens) that cause carcinogenesis have been reported. Initiation experience irreversible multiple random mutations in cellular DNA. Reactive oxygen species, electrophilic molecules of lipid peroxidation products or metabolically converted molecules of external origin that covalently reacts with DNA to cause point or multiple mutations are initiators. The second promotional stage is a complex process which progresses over a longer period of time. Promoter molecules take advantage of mutations in DNA leading to the proliferation of cells. The third stage involves malignant growth. This malignant transition depends on genetic fingerprint of a person, aging and external agents such as physical, chemical and biological carcinogens (Ceccaroli *et al.* 2015; Hecht 2003; Grant 2014; Agarwal *et al.* 2014).

Physical carcinogens include radiations such as nonionizing (ultraviolet rays) and ionizing (x rays). Ultraviolet radiation is the major physical agent in the onset of skin cancers. It causes DNA damage and genetic mutations, which gradually leads to the development of skin cancer including melanoma. Ultraviolet radiations are of three types: UVA, UVB, and UVC with wavelengths of 315-400 nm, 280-315 nm and 100-280 nm, respectively. Almost all UVC, as well as approximately 90% of UVB radiations, are absorbed by the ozone layer in the atmosphere whereas 95% of UVA radiation reaches the earth with just 5% UVB, without absorption to the ozone layer. Numerous factors affect the amount of UV light reaching the earth such as UV light elevation, ozone depletion, latitude, altitude, and weather conditions. Phototherapy

utilizing UV radiation can also activate skin cancers. Redundant sunlight exposure and artificial tanning lamps are crucial personal attributable hazards (Narayanan *et al.* 2010). When body exposes to moderate to high doses of ionizing radiation, it will increase the risk of cancer in different organs. Atomic bomb survivors and radiotherapy treated population studies have revealed radiation as a risk factor for thyroid cancer, particularly from early life exposure (Ron 1998; Furukawa *et al.* 2013; Rose *et al.* 2012). The global tendency of increasing population exposure to medical diagnostic sources of radiation, great usage of computed tomography scans, and interventional radiology practices, have increased the threat to radiosensitive organs such as the thyroid (Schonfeld *et al.* 2011). Various cancers of the thyroid, central nervous system, salivary gland, skin, breast and blood cells (leukemia) found to be associated with radiotherapy for enlargement of tonsils or thymus gland, benign conditions of the head and neck, or benign breast lumps and tinea capitis. Women having tuberculosis who had multiple chest fluoroscopies, as well as scoliosis patients who had frequent diagnostic x-rays during late childhood and adolescence, medical diagnostic and dental x rays, have been reported to be associated with induced cancer risks (Storm *et al.* 1986; Neta *et al.* 2013; Memon *et al.* 2010). Thorotrast injections have reported developing liver cancer and leukemia (Smoron and Battifora 1972; Faber and Johansen 1967). Radium treatment in patients is found to have a high risk of bone sarcomas and possibly cancers of the breast, liver, kidney, thyroid, and bladder (Ron 2003). The link between the excess use of mobile phones and brain tumors have been reported, but the causal interpretation is contentious (World cancer report 2014, World Health Organization).

Prolonged exposure to a number of chemicals is found to be one of the major causes of cancer. This process is chemical carcinogenesis. Chemical carcinogens have both initiating and promoting activities, but the proportion of two activities for various chemicals may alter markedly (Miller and Miller 1981; Gadaleta *et al.* 2016; Vangala 2016). Aflatoxin, Ochratoxin A, fumonisin B1, (food contaminants), components of tobacco smoke, asbestos, arsenic, acrylamide, methyl methanesulfonate, ethyl methanesulfonate, polycyclic aromatic hydrocarbons such as benzo[a]pyrene, benz[a]anthracene, and dibenz[a,h]anthracene, etc., are included under chemical carcinogens. Certain exposures are linked to a specific type of cancers. Lifestyle

factors involving carcinogen exposure, for example, usage of tobacco products is well-defined for the development of cancer affecting mouth, throat, lung, and bladder. Tobacco carcinogens and their DNA adducts alter expressions of oncogenes or suppressor genes which in turn leads to cancer (Wogan *et al.* 2004). The fungus present in maize and nuts *Aspergillus flavus* and *Aspergillus parasiticus* produce a genotoxic hepatocarcinogen called Aflatoxin B1 (Liu and Wu 2010). Aflatoxin in food generates DNA adducts progressing to genetic mutations in target liver cells and form a high-risk factor for Hepatocellular carcinoma (HCC), or liver cancer. Cytochrome-P₄₅₀ enzymes metabolize AFB1 to its active intermediate AFB1-8, 9 epoxide (AFBO) which binds to liver cell DNA, resulting in DNA adducts that interact with the guanine bases of liver cell DNA and cause a mutational effect in the *p53* tumor suppressor gene. It may lead to liver cancer (Hamid *et al.* 2013). Heterocyclic amines are considered as another important class of carcinogens in foods. They are mutagens and carcinogens at multiple organ sites and are produced when food like meats are heated above 180° C for long periods. A majority of studies have related to consumption of the meat products to cancer of the gut such as colon, stomach and breast cancer. Like most other chemical carcinogens heterocyclic amines, are also not carcinogenic by itself but when metabolized by cytochrome P450 enzymes convert to chemically reactive electrophiles which react with DNA and initiate a carcinogenic response. Genetic and environmental factors influence the activity of enzymes that metabolically activate and detoxify chemicals, they can be also associated with carcinogenic risk (Wogan *et al.* 2004). Air pollution has another major threat that is found to increase cancer risk. Long-term researches have reported that there is a great association between increasing exposures to air pollution and the risk of cardiopulmonary mortality, and lung cancer mortality. Population studies have identified a number of mutagenic and carcinogenic chemical components present in the air that may cause oxidative and DNA damage that can lead to cardiovascular and reproductive abnormalities (Lewtas 2007).

Biological carcinogens include infections from certain viruses such as hepatitis B (HBV), hepatitis C (HCV) and some types of Human Papilloma Virus (HPV). HPV infection has been identified as the major cause of cervical cancer (Chuaypen *et al.* 2018; Rapti and Hadziyannis 2015). Some bacterial, as well as parasitic infections,

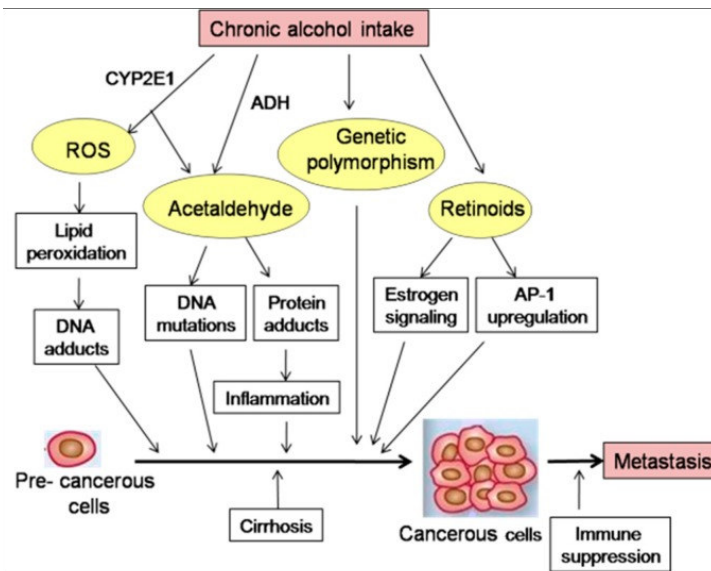
may lead to cancer development. *Helicobacter pylori* are the first bacterium to be reported as the specific lead of cancer in humans by the International agency for research on cancer (Parsonnet 1995). Recently, studies reported that two mechanisms which cause cancer by bacteria. Firstly it's by the induction of chronic inflammation and second by the production of carcinogenic bacterial metabolites. *H. pylori* cause inflammation in the distal stomach which in turn thought to cause cancer by the stimulation of cell proliferation and production of N-nitroso compounds and free radicals. Helminth infections such as schistosomiasis, opisthorchiasis, and clonorchiasis are highly carcinogenic and may induce cancer (van Tong *et al.* 2017).

Aging is another important factor in cancer development. The prevalence of cancer increases drastically with age and associated with less effective cellular repair mechanisms as a person grows older. Genomic instability, telomere disintegration, epigenetic changes, malfunctioning proteins, decreased nutrient level and incorrect metabolic processes, cellular senescence and decreased stem cell functioning are associated with the aging process (Aunan *et al.* 2017).

Recent investigations have revealed that alcohol consumption is a risk factor for oral, pharyngeal, laryngeal, esophageal, liver, colon, rectal, and breast cancer. Analysis among the Organization for Economic Co-operation and Development (OECD) belonging countries except American and Canadian Cancer Society reported that alcohol is a group 1 carcinogen and that even low-level alcohol consumption associated with the development of cancer (Amin *et al.* 2018). The actual underlying mechanism of alcohol-induced carcinogenesis has not been well defined, but some studies reported that it may be due to chemical reactions such as generation of reactive oxygen species by cytochrome P450 2E1 (CYP2E1)-mediated process, hyper estrogen levels, genotoxic effects of acetaldehyde, polymorphisms in genes encoding enzymes for ethanol metabolism and abnormal folate and retinoid metabolism. The various research described that the immunosuppressive effects of alcohol have been associated with tumor progression and metastasis process (Ratna and Mandrekar 2017) (Figure 1.3).

Physical inactivity may be a crucial factor leading to cancer. Various case-control studies were pinpointed the significant positive associations between lifetime physical inactivity and the risk of head and neck squamous cell carcinoma

(HNSCC) (Platek *et al.* 2017), renal and bladder cancer (Cannioto *et al.* 2017), and epithelial ovarian cancer (EOC) risk (Cannioto *et al.* 2016).



(Ratna and Mandrekar 2017)

Figure 1.3. Role of alcohol intake in cancer development

1.2.1. Independent risk factors contribute to oxidative stress and inflammation

Many of these risk factors of cancer (smoking, obesity, a western-style diet, ready-made drinks, and food additives, alcohol consumption, infections, etc.) are known to cause genetic instability mediated by oxidative stress in the body. Oxidative stress is characterized by the excess of reactive oxygen species (ROS), which contribute mostly in the initiation and promotional phase of carcinogenesis (Gall Troselj *et al.* 2016; Waris and Ahsan 2006). These oxidative products lead to alteration in the base composition of DNA. Changes in DNA such as rearrangement of gene duplication, miscoding, and lesion of DNA, base modification and the activation of oncogenes may be involved in the initiation of various cancers (Waris and Ahsan 2006). The most of carcinogen-induced cells are with the high level of one of the major oxidatively modified DNA base adducts (Gall Troselj *et al.* 2016). ROS also alter the cellular mechanisms such as cell proliferation, programmed cell death, senescence through oxidative stress-mediated signaling pathways which are implicated in the development of cancer (Waris and Ahsan 2006). Epidermal growth factor, fibroblast growth factor, platelet-derived growth factor, and various cytokines namely tumor necrosis factor (TNF), γ -interferon and interleukins activate intracellular tyrosine

phosphorylation cascade receptor tyrosine kinases (RTKs) and pathways as the result of ROS in cells.

Deregulation of several other signaling pathways by ROS stress have been reported to contribute to the development of cancer. One of the most important pathways is Mitogen-activated protein kinases (MAPKs) which regulate various cellular functions such as cell proliferation, differentiation, and apoptosis. Extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), and p38 kinases are the three families of MAPK proteins. Therapeutics targeted towards MAPK/ERK components have achieved varied response rates against colorectal cancer and ovarian cancer (Burotto *et al.* 2014). Yet another pathway is PI3-Kinase/Akt that modulate growth factor-mediated cell survival, transcription, protein synthesis carbohydrate and lipid metabolism (Kandel *et al.* 2002; Kennedy *et al.* 1999). Following exposure to oxidative stress, both the epidermal growth factor receptor (EGFR) and the platelet-derived growth factor receptor (PDGFR) are activated, leading to their dimerization and autophosphorylation that in turn activate Phospholipase C. Phospholipase C (PLC) is rapidly activated and plays an important role in regulating cell proliferation and differentiation. Protein kinase C (PKC) is a serine-threonine kinase involved in a signaling pathway involved in the regulation of cell growth, cell death, and responsiveness to oxidative stress (Gopalakrishna and Jaken 2000). Irregular PI3K signaling accounts for confrontation to apoptosis, angiogenesis, uncontrolled cell proliferation and the ability of cells to metastases leading to tumorigenesis. The reason for this irregular signaling is thought to occur due to mutationally overexpressed growth factor receptors, or Ras, that lead to dysfunction of upstream PI3K class I isoform (Luo *et al.* 2003). p53 signaling is a tumor suppressive pathway which regulates downstream signals leading to apoptosis, senescence and cell cycle arrest. Inactivation of p53 protein results in tumor progression. There are more than half of all cancers may involve p53-inactivating mutation mechanisms. Oxidative stress in low-level cause p53 to act as an antioxidant to eliminate oxidative stress which ensures cell survival and in a high level of oxidative stress p53 act as prooxidants that further increase the levels of oxidative stress results in cell death (Mirzayans *et al.* 2012). Risk factors such as ionizing radiation cause undergoing stress-induced premature senescence (SIPS) and not apoptosis in p53 activated human

cells, however, this SIPS cells are viable and secrete different factors that may promote cancer growth and progression (Mirzayans *et al.* 2012). Hypoxia/reperfusion, different endotoxin, ultraviolet radiation, and hyperosmolarity condition induces different cytokines and interleukins which activate the JAK/STAT pathway (Dudley *et al.* 2004). UV irradiation and TNF-alpha induce activation of p38 MAPK resulting in phosphorylation STAT1 Ser727 (Kovarik *et al.* 1999). c-Abl is a non-receptor tyrosine kinase present in the nucleus as well as in the cytoplasm, functioning for the regulation of cell growth, survival, and morphogenesis (Sirvent *et al.* 2008). The expression in the cytoplasm results in cell proliferation and survival, however activation of nuclear Abl resulted in induction of apoptosis after genotoxic stress. The canonical Wnt pathway causes nuclear localization of β -catenin, which can then interact with members of the T-cell factor/lymphoid enhancer-binding factor (Tcf/Lef) family of DNA-binding proteins to activate transcription of Wnt target genes (Mulroy *et al.* 2002; Lento *et al.* 2014). Wnts can signal through protein kinase C, Rho, or c-Jun N-terminal kinase (JNK) pathways (Veeman *et al.* 2003). Mutational deregulation of this pathway is often associated with colorectal cancers (CRC). The leptin is the adipose-derived cytokine, important in regulating appetite and body weight (Uddin *et al.* 2014). Obesity-induced an inflammatory response and elevated level of lectin influence the growth and proliferation of colorectal cancers cells through the activation of various growth and survival signaling pathways including JAK/STAT, PI3-kinase/AKT/mTOR and/or MAP kinases (Uddin *et al.* 2014). Leptin promotes cell proliferation and migration in breast cancer cells and hepatocellular carcinoma (Barone *et al.* 2012; Hill-Baskin *et al.* 2009). Obesity promotes pancreatic cancer by stimulating inflammatory signals such as IL-6, Stat3, and NF- κ B pathways and also promoting epithelial-mesenchymal transition (Gukovsky *et al.* 2013; Berger 2014).

1.2.2. Chronic oxidative stress and inflammation is the basis of cancer development

Previous research studies have revealed that prolonged oxidative stress can result in chronic inflammation, which in turn mediate most chronic degenerative diseases like cancer, diabetes, cardiovascular, neurological, and pulmonary diseases (Reuter *et al.* 2010). Our body exposed to different endogenous and exogenous factors. For example, the largest organ of the body, the skin, is found to be chronically exposed to

endogenous as well as environmental prooxidants and a well-organized protection system with enzymatic and nonenzymatic antioxidants are present in our body against this overwhelm of reactive oxidant species. The lungs are exposed to a high concentration of oxygen than any other body tissues as it's important in respiration mechanism. Overproduction of ROS in the airways results in increased oxidative stress in the airspaces, breathe sputum, lungs, and blood in patients with lung diseases. Oxidative stress produced by ROS has been found to activate a variety of transcription factors, which lead to the differential expression of inflammatory response inducing genes. ROS progresses to inflammatory response through the generation of cytokines, the modulation of signaling pathways and the activation of stress kinases such as JNK, MAPK, p38 and redox-sensitive transcription factors such as NF- κ B and AP-1 (Rahman 2003; Hussain *et al.* 2016) (Figure 1.4). In addition ROS have been documented at all stages of the inflammatory response, including the release of endogenous danger signals by damaged tissues, their sensing by innate immune receptors from the NOD-like (NLRs) and the Toll-like (TLRs) and families, and the activation of signaling pathways initiating the adaptive cellular response to such signals (Lugrin *et al.* 2014).

There are two stages of inflammatory response namely acute and chronic inflammation. The initial stage of inflammation (innate immunity) of shorter duration and a regulated form is termed as acute inflammation, mediated through the activation of the immune system of the body and is beneficial for the host body. The second stage of long persisting response named as chronic inflammation, a dysregulated form of inflammation results in the onset of various chronic illnesses including cancer (Reuter *et al.* 2010; Lin and Karin 2007).

In acute inflammation, the immune responses can be divided into vascular and cellular responses. If there is a tissue injury or infections by microorganisms, inflammatory stimuli called the vascular events to occur in the microvasculature. These rapid responses resulted in vasodilation and increase the permeability of blood vessels which in turn lead to the leakage of inflammatory mediators such as histamine, serotonin, and bradykinin into the tissue and produces edema. The interaction between integrins namely CDII/CDI8, and adhesion molecules such as CAM-1 and CAM-2 expressed on white blood cells and endothelium cells resulted in

high-affinity binding of leucocytes in the endothelium. During inflammatory responses, white blood cells and mast cells infiltrated to the damaged site resulted in respiratory burst due to ROS production. Among the white blood cells, neutrophils are the first recruited to the site which leads to an acute inflammatory response by the respiratory burst. In addition to this response, the inflammatory cells secrete C5a complement fragment, secretions of basophils such as platelets activating factor, histamine, and leukotriene B and metabolites of arachidonic acid, cytokines, chemokines, and interleukins stimulates rapid leucocytes recruitment and produce more reactive species. These mediators activate second messenger system and through a cascade of reactions, they induce the transcription factors such as nuclear factor κ B (NF- κ B), signal transducer and activator of transcription 3 (STAT3), hypoxia-inducible factor-1 α (HIF-1 α), activator protein-1 (AP-1), nuclear factor of activated T cells and NF-E2 related factor-2 (Nrf2), which in turn mediate rapid cellular inflammatory responses. Stimulation of cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), expression of inflammatory cytokines (tumor necrosis factor (TNF), interleukin-1 (IL-1), IL-6 and chemokines (IL-8 and CXC chemokine receptor 4 (CXCR4)), have been documented to play a major role in oxidative stress-induced inflammation (Abdulkhaleq *et al.* 2018; Reuter *et al.* 2010) (Figure 1.4).

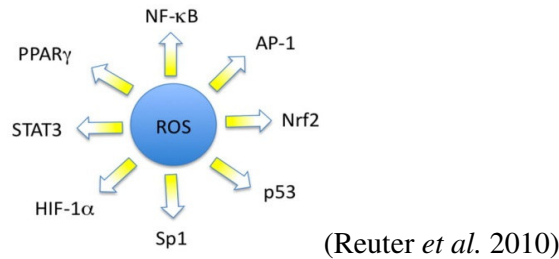
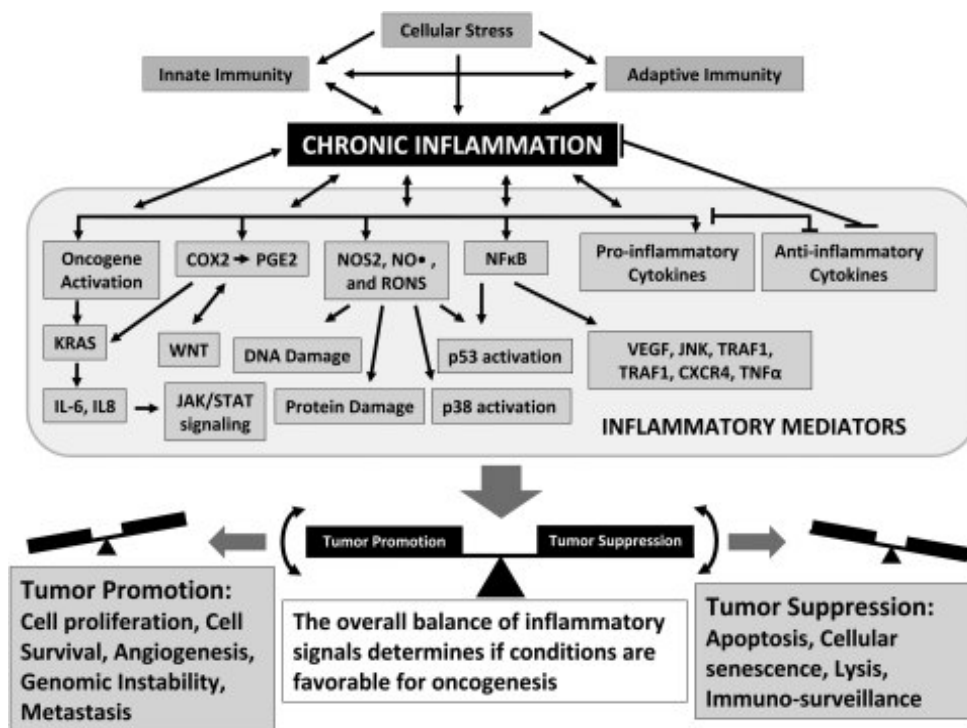


Figure 1.4. Effect of ROS on signaling pathways

Chronic inflammation is marked by monocyte and lymphocytes infiltration, the proliferation of fibroblasts, the recruitment of collagen fibers and connective tissue formation leads to granuloma formation. Reactive nitrogen species, proteases, and other reactive oxygen species in the recruited cells lead to tissue degeneration. Chronic inflammatory processes induce oxidative or nitrosative stress in the cell and stimulate lipid peroxidation (LPO) chain reaction, leads to the generation of excess reactive oxygen species (ROS), reactive nitrogen species (RNS), and DNA-reactive

aldehydes. These compounds modified DNA bases and form LPO-derived DNA adducts. These DNA adducts resulted in carcinogenesis and cancer (Bartsch and Nair 2006). Chronic inflammation induced by biological, chemical, and physical factors is related to increased risk of human cancer at various sites (Bartsch and Nair 2006; Reuter *et al.* 2010). Prolonged inflammation resulted in the secretion of inflammatory response mediators such as cytokines, free radicals, prostaglandins and growth factors which can alter genetic and epigenetic profile like point mutations in tumor suppressor genes, DNA methylation, nuclear histone acetylation/deacetylation and post-translational modifications, causing changes in critical pathways essential for the normal cellular homeostasis and resulted in the development and progression of cancer (Hussain and Harris 2007). These mediators produced by inflammatory cells also alter and inactivate mismatch repair functions which resulted in DNA damage and mutations and has been implicated in the initiation and /or promotion of inflammation-mediated carcinogenesis (Ohnishi *et al.* 2013) (Figure 1.5).



(Schetter *et al.* 2010)

Figure 1. 5. Inflammation and cancer

1.3. Possible approaches to cancer management

It is generally agreed that 90-95% of cancer can be prevented just by avoiding risk factors however genetic predisposition that accounts for the remaining 5% cancer is hard to prevent. Since all risk factors so far identified contribute to chronic oxidative stress and inflammatory conditions in the body, it is possible that maintaining innate immunity and oxidative balance or boosting them can be adopted as a preventive strategy. This can be achieved either by dietary regimen or by supplementing exogenous antioxidant, anti-inflammatory or immune stimulatory agents. Many of these agents can limit cancer recurrence as well as prevent chemotherapy-induced secondary toxicities. However, in already developed cancers, modern treatment strategy has to be followed especially curative chemotherapy based on the stages of cancer.

1.3.1. Preventive strategies

Cancer prevention is a more prevalent method to manage cancer risk. Identifying causative factors, removing them, and improving the body's immune and detoxifying system against these factors can prevent neoplastic development. Some people are genetically susceptible to cancer; they have to avoid environmental risk factors of cancers such as long exposure to ionizing radiation, carcinogenic chemicals such as benzene, etc. In the case of chemical carcinogenesis, it is very important to develop a system in cancer prevention through better tools of disposal of hazardous chemicals from the environment, stimulate the body system to detoxify environmental agents and developing more rapid and more sensitive tools to detect cancer-inducing chemicals effects for man (Rauscher 1972). It is advised that the individuals with a genetic predisposition to cancer can undergo early screening tests and timely administration of appropriate prophylaxis or treatment. As some viral infections have a risk factor for cancer progression, prevention of infection can also reduce the incidence of human cancers. Studies suggest that virus-positive cancers can be prevented by vaccination, for example, personalized peptide vaccination for HCV-positive advanced hepatocellular carcinoma (Yoo *et al.* 2017). Obesity or unhealthy diet contributes 10% of cancer death (Basen-Engquist and Chang 2011). These risk factors can be prevented by maintaining a normal body mass index, eating healthy foods with plenty of vegetables, fruits, and whole grains.

1.3.2. Different modalities of curative cancer treatment

There are different cancer treatment available and are being recurrently improved. More fruitful and better treatments are available nowadays and cancers detected at early stages are being treated successfully. This has tremendously increased the cancer survivor population. Surgical treatment, radiotherapy, chemotherapy, and hormone therapy are the major conventional types of cancer treatment. Distinct immunological treatments and targeted drug delivery method are practiced nowadays. The types of treatment depend on the types of cancer, the organ involved, grade and stage of advancement, whether it has spread, and the general health of the patient. Treatment is either one treatment or a combination of treatments depends upon the condition of the patient. However, the early diagnosis of cancer makes the treatment easier (PubMedHealth 2016).

Surgery is the most effective treatment modality for localized primary tumors. In contrast to chemotherapy and radiotherapy, surgery excise whole cancer cells and healthy surrounding to prevent the spreading of localized tumors (Urruticoechea *et al.* 2010). But surgical procedures are unable to remove cancer cells which metastasis from one primary tumor and locate in another site. So combination therapy is preferred for these metastatic cells. Combination therapy is usually surgery either followed by chemotherapy or radiotherapy.

Chemotherapy is the commonly used method for treating various diseases by the administration of chemicals. Cancer chemotherapy is the use of chemicals for the treatment of cancer which aims to stop the uncontrolled growth of cancer cells (PubMedHealth 2016). Cytotoxic or cytostatic drugs are the chemicals which destroy cancer cells or to stop the uncontrolled division of cancer cells. These drugs affect not only cancer cells, but also actively dividing healthy cells in the body. Cancer cells are more sensitive to chemotherapy as they often divide appreciably faster than normal cells. There were different cytotoxic drugs for cancer treatment. Nitrogen mustard, alkylating drugs such as cyclophosphamide and chlorambucil, folate antagonists such as aminopterin and amethopterin, methotrexate and 6-thioguanine and 6-mercaptopurine for treating leukemia, drug for solid tumors such as 5-fluorouracil (5-FU) against colorectal, head and neck cancer, alkaloids from vinca and ibenzmethylin (procarbazine) applied to leukemia and Hodgkin's disease, tamoxifen for breast

cancer are some of the drugs in cancer chemotherapy. Various cytotoxic drugs are used together in cancer therapy to obtain a better result. Polychemotherapy such as CBV (cisplatin, bleomycin, and vinblastine) for metastatic germ cancer, CMF (Cytosan, methotrexate, and fluorouracil), for treating breast cancer used more efficiently than individual drug treatment. Subsequent improvements in treatment led to liposomal therapy with decreasing some of the side effects of chemotherapy such as cardiotoxicity. First steps in nanotechnology-based liposomal drugs include liposomal doxorubicin and daunorubicin. Further advances in chemotherapy and genetics/molecular biology-based treatments have contributed to the decline in the rate of cancer-related deaths. Another pharmacological trend was the development of kinase inhibitors (Arruebo *et al.* 2011). DNA topoisomerases have a major role in DNA replication; hence, inhibiting this class of enzyme subsequently kills replicating cells. Type I topoisomerases inhibitors are also a potent chemotherapeutic drug. Development of angiogenic inhibitors is another advance approach to cancer chemotherapy with fewer side effects. The major disadvantage of chemotherapy is its adverse side effects. Treatment with some cytotoxic drugs usually causes hair loss, nausea, damage to the mouth and pharynx mucosa, diarrhea, and destruction of bone marrow leads to falling in white blood cells count. Other disadvantages of cytotoxic drugs include damages to mucous membranes of the eyes, edema, constipation, changes in finger and toenails, hypersensitivity and muscle pain. Long-term exposure includes the risk of neurotoxicity, heart and lung damage and kidney failure (Haiguang Liu 2015).

Radiation therapy is another common cancer treatment method for localized cancers. The high physical energy of radiations causes changes in the cellular structure leads to cell death. Though cancer cell divides faster than normal, they are more affected by radiotherapy. Another advancement is radiopharmaceutical therapy. Introduction of a radioactive medicine orally or intravenously kill the tumor cells, one such radioisotope therapy is radioiodine for thyroid cancer treatment. Radiation provides significant benefit to many women with breast cancer. Side effects of radiation therapy are localized to the particular area of exposure and depending on which organ affected, duration of treatment and dose of exposed radiation. Common side effects reported are affected bone marrow with fall in a white blood cell, blood platelet and hemoglobin counts, damage to the mouth, pharyngeal and intestinal

mucosa, skin damage, radiation-induced pneumonitis low rate of tissue regeneration, fatigue, headache and damage to heart and blood vessels (website, Cancer Society of Finland). It is also associated with the deleterious effects of toxicity including cardiac and pulmonary toxicity, lymphedema, and secondary malignancy (Brown *et al.* 2015). Radiotherapy is recommended either before or after the surgical treatment depending upon the nature of the tumor. Another effective method is chemoradiotherapy, a combination of chemotherapy and radiotherapy simultaneously to get a better effect. But it may increase the side effects of radiotherapy.

Immunotherapy is another approach to cancer treatment which stimulates the natural immune system or makes use of components of the body's immune system to fight against cancer cells. It is a group of treatment methods helps to stop or slow down the growth of cancer cells in different ways. These are more specific to cancer cells than chemotherapy (Dillman 2011). Various types of immunotherapies are monoclonal antibodies such as trastuzumab, rituximab, ofatumumab, cetuximab, alemtuzumab, panitumumab and bevacizumab, the anti-CTLA-4 monoclonal antibody ipilimumab, which blocks regulatory T-cells; nonspecific immunotherapies like BCG and levamisole, the cytokines interferon- α and interleukin-2; oncolytic virus therapy, the immunotoxins such as denileukin diftitox and gemtuzumab ozogamicin; nonmyeloablative allogeneic transplants with donor lymphocyte infusions, T-cell therapy and cancer vaccines (idiotypic vaccines in lymphoma and a peptide vaccine in melanoma). As it improves the immune functions of the body, there is a range of possible side effects reported such as inflammation and autoimmune diseases. Immunocancer therapy is a major focusing area of research nowadays (Dillman 2011).

Cancer stem cells (CSCs) are a distinctive group of tumor cells that control tumor initiation, progression, and repair. Though CSCs are highly resistant to chemoradiotherapy, new modalities to fight against cancer have to be implemented. Considering CSC immunogenicity and expression of CD44, ALDH, CD133, or EpCAM antigens which distinguish CSCs from healthy cells, makes immune targeting of CSCs a novel approach for cancer therapy. (Kwiatkowska-Borowczyk *et al.* 2015).

Hormonal cancer therapy, a form of targeted drug therapy works by preventing the production and effects of hormones. Some cancers such as prostate and breast cancer

have hormone sensitivity (androgen and estrogen, respectively). Tamoxifen, an estrogen antagonist that blocks estrogen synthesis is now widely used to prevent or delay the recurrence of breast cancer (Lumachi *et al.* 2015). Hormonal treatment is also used for cancer such as thyroid cancer and endometrial cancer. The human body tolerates hormone therapy better than that of chemotherapy.

Gene-based therapeutic strategies include *ex vivo* and *in vivo* cytokine gene transfer, drug sensitization with genes using prodrug administration, and the transduction of drug resistance genes into bone marrow stem cells for protection from the adverse effect of chemotherapy. Gene replacement therapy for tumor suppressor genes and inactivation of oncogene expression are recent advances for targeting the genetic lesions in the cancer cell (Roth and Cristiano 1997). Another promising approach for cancer treatments is the use of recombinant vaccines and the use of expression vector constructs that induce the inactive prodrugs into active drugs (Roth and Cristiano 1997).

1.4. Use of herbs and plant-derived compounds in the management of cancer

Various therapies are offered for cancer management using plants and plant-derived products. For centuries, plants are well known for their potential cancer prevention and anticancer properties.

1.4.1. Herbs and phytochemicals in cancer prevention

Various phytochemicals and herbal extracts from different plants, fruits, vegetables, and spices have been reported to possess potent chemopreventive role. Previous studies have shown that many plants such as *Anacardium occidentale*, *Asparagus racemosus*, *Abrus precatorius*, *Andrographis paniculata*, *Centella asiatica*, *Curcuma longa*, and *Cedrus deodara* possess cancer preventive potential (Desai *et al.* 2008). Berberine is an isoquinoline alkaloid isolated and extracted from plants such as *Berberis aetnensis* C. Presl., *Berberis aristata*, *Berberis vulgaris*, *Coptis chinensis*, *Coptis japonica*, *Coptis rhizome*, *Hydrastis canadensis*, *Phellodendron amurense*, and *Tinosora cordifolia*. It has been used in traditional Indian and Chinese medicine for many decades. Berberine is demonstrated to up regulate many pathways which lead to breaks in DNA double strand and cell cycle arrest, berberine-induced apoptosis, autophagy of cancer cells, senescence and inhibition of growth and progression of cancer cells and DNA replication (McCubrey *et al.* 2017). It has been

reported to have anti-inflammatory activities and inhibit the production and expression of IL-6, TNF-alpha, COX-2 and monocyte chemoattractant protein 1 (MCP1). It alters the effect of prostaglandin E2 (PGE2) and downregulates MMP2 and MMP9 genes Raf/MEK/ERK and NF-kappa B pathways thereby reduce the metastasis process (Tillhon *et al.* 2012).

Ginger, the rhizome of the plant *Zingiber officinale*, has been consumed in India as part of the food, spice or traditional medicine. The major pharmacologically-active component of ginger is 6-Gingerol (Wang *et al.* 2014). It is known to exhibit anti-inflammatory, antioxidant, chemopreventive and anticancer properties. Evidence for anticancer properties mediated through its cytotoxic potential by targeting multiple biological pathways involved in apoptosis, regulation of cell cycle, and inhibition of angiogenesis has been increasingly reported (Wang *et al.* 2014). [6]-Gingerol are reported to possess anti-tumor and metastatic inhibitory activity *in vitro* and *in vivo* against a variety of cancer cells such as breast cancer (Poltronieri *et al.* 2014). Both [6]-Gingerol and [10]-Gingerol induce cell cycle arrest, the elevation of intracellular Ca²⁺, caspase-mediated apoptosis and prevents proliferation in colon cancer cells induced by PMA through downregulating MAPK/AP-1 signaling (Radhakrishnan *et al.* 2014; Martin *et al.* 2017).

Curcuma longa locally called turmeric is a herbaceous plant of ginger family Zingiberaceae. Turmeric rhizome has been extensively used in India as the part of their food ingredient and in the treatment of various such as jaundice, wounds, burns, insects bites, pain, dysentery and skin problems in folklore medicine as well as in Ayurveda. Curcuminoids are the active components of *C. longa* viz. curcumin (diferuloylmethane), demethoxycurcumin, and bisdemethoxycurcumin. Among them the most active one, curcumin possesses a large number of pharmacologic effects such as anti-inflammatory, antioxidant, antiproliferative, apoptotic-inducing and antiangiogenic activities (Chainani-Wu 2003; Menon and Sudheer 2007; Ono *et al.* 2013; Bhandarkar and Arbiser 2007; Kuttan *et al.* 1985). Curcumin is the most prominent chemopreventive agent studied among compounds derived from plants.

Epigallocatechin gallate is another compound widely studied as the most potent cancer chemopreventive polyphenol present in green tea. These polyphenols inhibit

angiogenesis and metastasis. It induces apoptosis through regulating the expression of matrix metalloproteinases, VEGF, IGF-1, EGFR, cell cycle regulatory proteins and inhibits NF- κ B, PI3-K/Akt, Ras/Raf/MAPK and AP-1 signaling pathways (Shankar *et al.* 2007; Du *et al.* 2012).

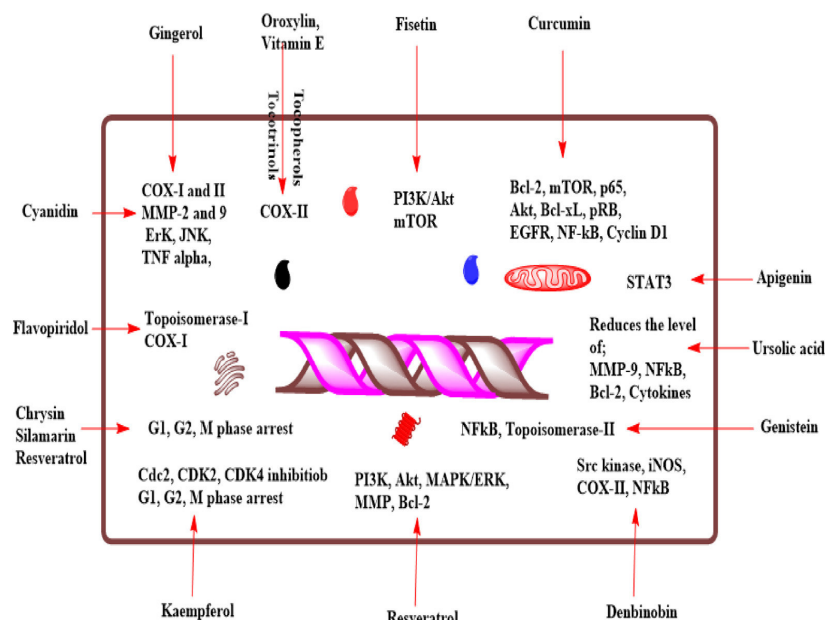
Kaempferol (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one) is a yellow flavonoid and phytoestrogen compound found in grapes, broccoli, apples, and yellow fruits, tea, strawberries, and beans. Due to its antioxidant and anti-inflammatory effect, Kaempferol has been attributed with significant therapeutic potential for cancer prevention and as an antitumor agent by regulating cell cycle mechanism, apoptosis, metastasis and angiogenesis in various cancer cell types (Kim and Choi 2013).

Cyanidin-3-glucoside, a natural phytoconstituent derived from blackberry, are reported to have chemopreventive and chemotherapeutic activity by downregulating pathways of NF- κ B and AP-1 and expression of cyclooxygenase-2 and tumor necrosis factor- α . Its pharmacological activities are found to be mediated through the inhibition of MAPK activity and it also arrests 12-O-tetradecanoylphorbol-13-acetate (TPA) induced neoplastic transformation in JB6 cells and of a human lung carcinoma cell proliferation of A549 (Ding *et al.* 2006).

Genistein is a naturally occurring isoflavonoid isolated from *Genista tinctoria* L. in 1899. Genistein acts as a chemotherapeutic agent against different types of cancer, mainly by inducing apoptosis, blocking the mechanism of the cell cycle, and angiogenesis and inhibiting metastasis in cancer cells. It also acts by targeting different molecular mechanisms via altering expression of caspase proteins, Bcl-2-associated X protein, nuclear transcription factor κ B, Bcl-2, extracellular signal-regulated kinase 1/2 (ERK1/2), MAPK, Wingless and integration 1 β -catenin (Wnt/ β -catenin), and phosphoinositide 3 kinase/Akt (PI3K/Akt) signaling pathways (Spagnuolo *et al.* 2015).

Silymarin, genipin, quercetin, luteolin and many other phytochemicals isolated from various plants are reported with a number of biological properties such as antioxidant, anti-inflammatory and cytotoxic. These classes of molecules thus can be candidates as chemopreventive and chemotherapeutic drugs either used alone or in synergism with

other drugs. The mechanism of action of some of the plant-derived compounds by activating expression of various genes, proteins, enzymes, and signaling pathways in order to prevent cancer metastasis are depicted in figure 1. 6.



(Iqbal *et al.* 2017)

Figure 1. 6. Mechanism of action of different phytochemicals on cellular pathways

1.4.2. Use of herbs and plant-derived compounds in curative cancer therapy

Plants with medicinal properties have been explored for thousands of years in folk medicines all over the world, traditional Chinese and Indian medicine. Some nations follow the plant-based treatment as their main medicinal source as per WHO report (Ernst 2000). Many plants are used for human health in developed and underdeveloped nations (Greenwell and Rahman 2015). Herbal products such as plant extracts, dry powders and parts of plants, fungi, and algae have been used as complementary medicines (MM Robinson and Zhang 2011). Pharmaceutical researches focus on plant-derived compounds as they are found to be less toxic compared to the synthetic chemotherapeutic compounds. There are enormous plant secondary metabolites with reported anticancer properties. This has led to the development of new drugs (Juarez 2014). The anticancer efficacies of a number of plants are still being under active research and some plants have shown promising results. A number of medicinal plants such as *Alstonia scholaris*, *Tinospora cordifolia*, *Ziziphus nummularia*, *Curcuma longa*, *Phyllanthus amarus*, *Annona*

muricata, *Mappia foetida*, *Withania somnifera*, and *Boswellia serrata* have shown promise in anticancer therapies and are usually used in alternative medicine to the use of conventional allopathic medicine (Desai *et al.* 2008).

Naturally-derived compounds from medicinal plants can be targets for potential anticancer treatments (Juarez 2014). Many phytochemicals have been identified for their anticancer properties include polyphenols, brassinosteroids, taxols, honokiol, plumbagin, curcuminoids, resveratrol, halofuginone, podophyllotoxin, and etoposide, etc. (Greenwell and Rahman 2015). Isolation and extraction of phytochemicals from plants started in the early nineteenth century and is a major breakthrough in the field of pharmaceutical research. Phytochemicals derived from plants have been authorized and subscribed as medicines nowadays (Young-Won Chin *et al.* 2006). One such example is the discovery of analgesic drugs such as morphine and codeine from opium (*Papaver somniferum* L.) (Fridlender *et al.* 2015).

Catharanthus roseus (*C. roseus*) (Apocynaceae) is the source of vinca alkaloids (VA), a major group of phytochemicals along with other phytochemicals used for various type of cancer like lung, breast, leukemia, testes and hepatic cancer. Important vinca alkaloid derivatives are vinorelbine, vindesine, vincristine and vinblastine (Bennouna *et al.* 2008). Vinorelbine, vindesine, vinfosiltine, and vinovelbine are the synthetic derivatives currently in use (Iqbal *et al.* 2017). These antimitotic drugs arrest cell cycle at metaphase by inhibiting the dynamics of spindle microtubules (Jordan *et al.* 1992).

One of the most known plant-derived strong anticancer agents is Paclitaxel (Taxol®). Paclitaxel has been isolated from the leaf and bark of *Taxus baccata* (*T. baccata*) and *T. Canadensis* and *Corylus avellana* and is used to treat a great range of cancers including lung, breast ovarian and prostate cancer. Taxanes induce microtubule stabilization and mitotic cell arrest, apoptosis and affect Androgen receptor (AR) signaling (Darshan *et al.* 2011). Taxanes act by binding to β -tubulin in the lumen of microtubules and alter the microtubule dynamics and lead to metaphase cell arrest. Docetaxel is a semi-synthetic analog of paclitaxel, synthesized from natural precursor 10-deacetylbaccatin III from *T. baccata* has been approved to be used as such or in combination with other compounds to treat a wide range of cancers. Another synthetic

analog under clinical trials is larotaxel, ortataxel, milataxel and tesetaxel (Xie and Zhou 2017; Iqbal *et al.* 2017)(Figure 1.7).

The camptothecins are a new class of novel chemotherapeutic agents which acts by targeting the topoisomerase I enzyme. It was first isolated from bark and stem of *Camptotheca acuminata* (Nyssaceae). Camptothecin derivatives such as irinotecan and topotecan are approved for broad antitumor activity in phase I/II preclinical studies. Irinotecan is used to treat colorectal cancer while topotecan for the treatment of ovarian and lung cancer. 9-aminocamptothecin and GI 147211C are undergoing phase I and early phase II preclinical evaluation. Other potent analogs such as lurtotecan, exatecan, rubitecan, silatecan are also in the process of preclinical studies as antitumor agents (Lansiaux and Bailly 2003).

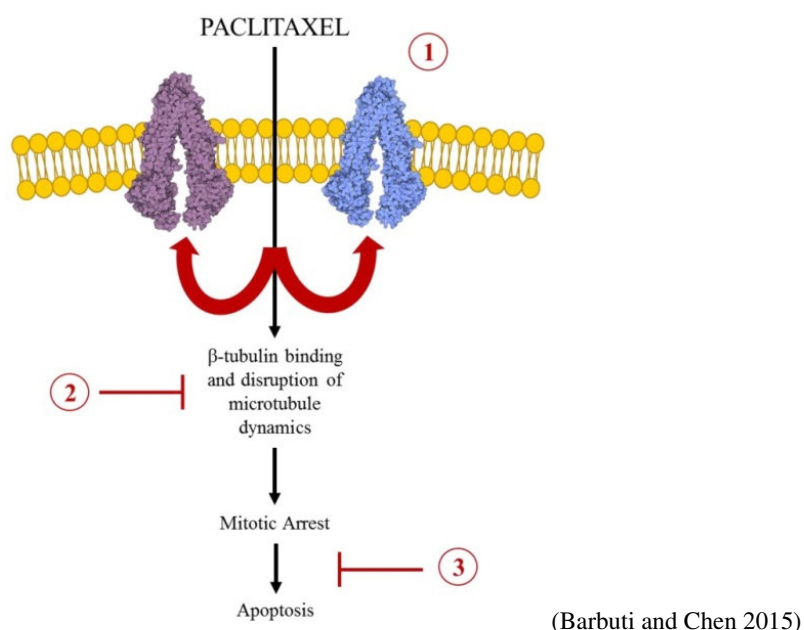


Figure 1.7. Mechanism of action of Paclitaxel on apoptosis process

Cephalotaxus spp. is reported to have anticancer efficacy. *C.griffithii* needle is found to be important phytochemicals with multiple cellular targets such as induced cell cycle arrest and apoptosis by activating both intrinsic and extrinsic apoptotic pathways and downregulated hTERT, hTR, and c-Myc expression for control of different cancers (Moirangthem *et al.* 2014). Antitumor alkaloids from *C. harringtonia* such as harringtonine, isoharringtonine, homoharringtonine, and deoxyharringtonine are found to have shown significant activity against L-1210

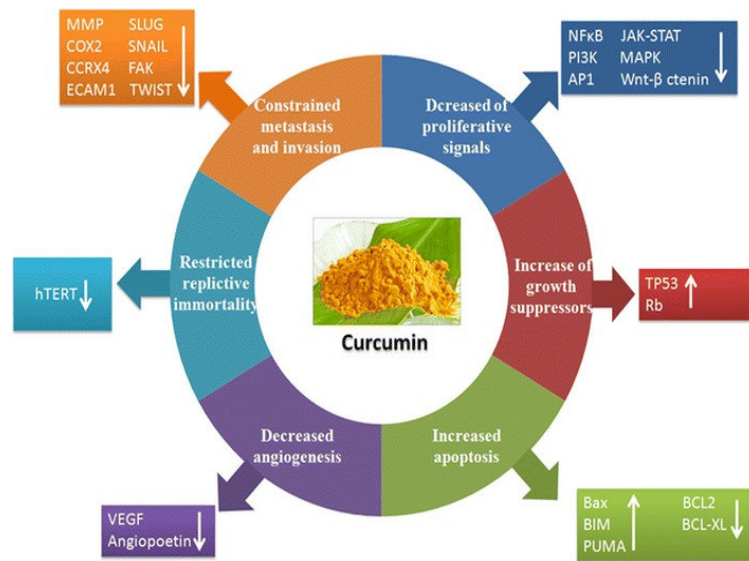
leukemia and against experimental P388 leukemia in mice (G. *et al.* 1972). A cytotoxic alkaloid, homoharringtonine inhibits protein biosynthesis by interfering with the protein elongation process leading to cell death and is recently approved by USFDA for chronic myeloid leukemia treatment. It blocks the progression of cells from G1 phase into S phase and from G2 phase into M phase. It acts by inhibiting the cells from G1 to S and G2 to M phase progression in the cell cycle. Clinical research has revealed the potential of homoharringtonine treating acute myeloid leukemia, chronic myeloid leukemia and myelodysplastic syndrome (MDS), but not effective for acute lymphoblastic leukemia and solid tumors (Alvandi *et al.* 2014; Zhou *et al.* 1995).

Several reports have been published about the anticarcinogenic effects of curcumin (isolated from *Curcuma longa*) or administration of curcumin along with chemotherapy for the treatment of several cancers including human glioblastoma, multiple myeloma, head squamous cell carcinoma, prostate, ovarian, breast, pancreatic, colorectal, and urinary bladder cancers (Juarez 2014; Singh 2007). Curcumin inhibits the growth of cancer cells by regulating several nuclear and cellular factors by mechanism of upregulation of genes expression of tumor suppressor gene p53 and Bax protein caspase enzymes (Caspase-3, 8, 9) and down-regulation of expression of genes such as Bcl-2, p65, protein kinase B (Akt), retinoblastoma protein (pRB), Sp-1 and its housekeeping gene and cyclin D1 proteins. Curcumin inhibits the STAT3 and NF- κ B signaling cascades thereby prevent cancer formation, migration, and invasion. Apoptosis was induced by the promotion of ER stress and autophagy and by inhibiting histone deacetylases (Vallianou *et al.* 2015; Iqbal *et al.* 2017). Even though curcumin has the potent efficacy and usage safety it has not yet been upgraded as a medicine because of its poor bioavailability, rapid metabolism and elimination from the body (Juarez 2014) (Figure1.8).

Podophyllotoxin is a lignan isolated from the roots and rhizomes of podophyllum species such as *Podophyllum peltatum* and *Podophyllum emodi*. Podophyllotoxin causes polymerization of tubulin and suppression of the formation of the mitotic-spindles and leads to mitotic cell arrest (Ardalani *et al.* 2017). Studies on the mechanism of action of Podophyllotoxin also suggest that their antitumor properties are due to their interaction with DNA and

inhibition of DNA topoisomerase II (Gordaliza *et al.* 2000). Etoposide (VP-16), a podophyllotoxin derivative which blocks cell growth by inhibiting DNA topoisomerase II, is currently in clinical use especially small cell lung carcinoma and testicular cancer (Damayanthi and Lown 1998).

Resveratrol is a naturally occurring polyphenol found in a wide range of plant species such as grapes, peanuts, and berries. It is the phytoalexin secreted in plants in response to fungal infection, mechanical injury and UV radiation (Carter *et al.* 2014). It has been found that tumor growth effectively reduces by the administration of resveratrol in various cancers such as breast cancer, colorectal, liver, pancreatic and prostate cancer (Carter *et al.* 2014). Resveratrol with the highest concentration in nature found in *Polygonum cuspidatum*. Resveratrol acts by promoting cell cycle arrest and apoptosis in tumor cell, prevent tumor cell proliferation by inhibiting cyclooxygenase activity and downregulating the NF- κ B pathways (Carter *et al.* 2014).



(Bose *et al.* 2015)

Figure 1.8. Different cellular targets of Curcumin

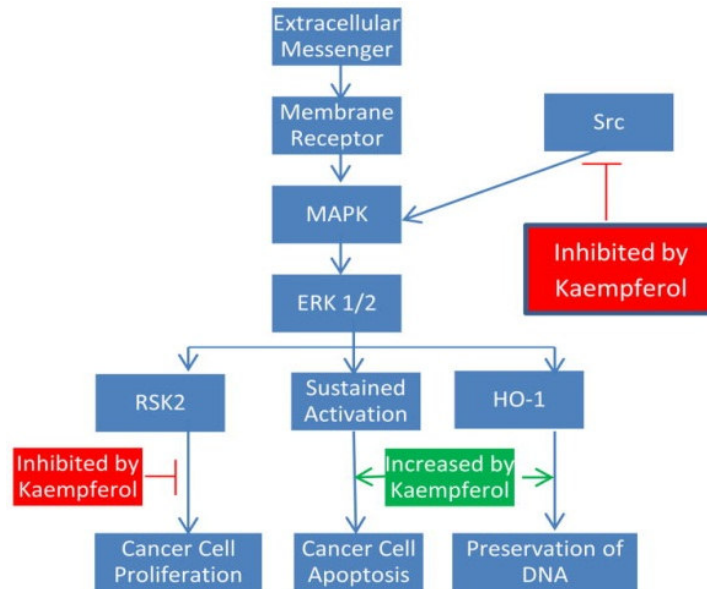
Combretastatins are natural phenols extracted from the bark of *Combretum caffrum*. Combretastatin A-4 (CA-4) and A-1 (CA-1) are demonstrated to be a microtubule-destabilizing agent that inhibits microtubule assembly by binding with tubulin similar to colchicine action. Combretastatin A-4 has been shown to be the most efficient antineoplastic agent (Pettit *et al.* 1995). Active combretastatins compounds with

strong anticancer properties are effective against leukemia, lung cancer and colon cancers (Boehle *et al.* 2001; Greene *et al.* 2012). Brassinosteroids (BRs) are steroid plant hormones ubiquitous in the plant kingdom. BRs are found to have potent growth inhibitory properties through interactions with the cell cycle machinery of several human cancer cell lines such as breast and prostate cancer without affecting normal cells (Steigerova *et al.* 2012).

Flavonoids belonging to the class of polyphenolic secondary metabolites are abundant in plants. Flavonoids present in fruits, vegetables and grains particularly of food plants (Chen and Chen 2013b; Kandaswami *et al.* 2005). Flavonoids mediate the protective effects of normal cell and disrupting the cancer cells by acting as natural chemopreventive and anticancer agents. Isoflavone intake has been reported to reduce the risk of a great variety of cancers such as estrogen-related cancers lung cancer, stomach cancer, bladder cancer or breast cancer risk, oral and laryngeal cancers, ovarian cancer and prostate cancer (Batra and Sharma 2013). Studies on the effect of flavonoids in the induction of apoptosis mechanism, inhibition of kinases involved in cell regulation process and block metastasis, downregulate the secretion of MMPs and angiogenesis and antiproliferative activity. The important compounds under this category are luteolin, quercetin, apigenin, kaempferol (Kandaswami *et al.* 2005). Kaempferol, a flavonoid and phytoestrogen compound inhibits angiogenesis by reducing the expression of angiogenesis marker vascular endothelial growth factor (VEGF) in ovarian cancer cells (Luo *et al.* 2009). Studies reported that kaempferol initiated the apoptosis process by induction of MAPK signaling route and to increase expression of heme oxygenase (HO)-1 gene, which causes an increase in the antioxidant status of cells (Chen and Chen 2013a). Kaempferol has been found to inactivating RSK2 protein by directly binding to it (Cho *et al.* 2009) (Figure 1.9).

Triterpenoids are the largest group of phytochemicals in which derivatives of isopentenyl pyrophosphate. These are synthesized from squalene which is intermediate of cholesterol biosynthetic pathway. They include diverse classes including isomalabaricanes, dammaranes, lanostanes, limonoids, oleananes, cucurbitanes, protostanes, cycloartane, lupanes, oleanane, friedelane, and tirucallanes, etc. (Setzer and Setzer 2003). Lupeol, one of dietary triterpenes, present in many fruits and medicinal plants exhibited inhibitory effect on cell growth of human

pancreatic cancer proliferating cell nuclear antigen 1 (PCNA-1) cells by inhibiting topoisomerase II and by inducing apoptotic mechanism and cell cycle arrest in G0/G1 phase by upregulating P21 and P27 and down-regulating cyclin D1(Liu *et al.* 2015b). Betulinic acid (3 β , hydroxy-lup-20(29)-en-28-oic acid) is a pentacyclic triterpenoid with antitumor properties. It triggers cytochrome c and the mitochondrial apoptotic pathway in cancer cells mainly by its cytotoxic effect. Betulinic acid is a potent activator of stress-induced transcriptional activation of nuclear factor- κ B (NF- κ B) (Fulda 2008). Activity of angiogenesis-regulating enzyme aminopeptidase N, overexpressed in several cancer condition found to be inhibited by betulinic acid and is attributed to activation of impaired functioning of the transcription factors specificity protein 1 (Sp1), Sp3, and Sp4, which in turn regulate vascular endothelial growth factor (VEGF) expression (Kasperczyk *et al.* 2005).



(Chen and Chen 2013a)

Figure 1.9. The action of kaempferol on different cellular pathways

Luteolin, 3',4',5,7-tetrahydroxyflavone in fruits, vegetables, and medicinal herbs exhibited cytotoxicity towards cancer cells through suppressing cellular pathways such as phosphatidylinositol 3'-kinase (PI3K)/Akt, nuclear factor kappa B (NF-kappaB), and X-linked inhibitor of apoptosis protein (XIAP), and stimulating apoptosis pathways and thereby induce the tumor suppressor p53(Lin *et al.* 2008).

Ellipticine, an alkaloid isolated from *Ochrosia elliptica* and *Rauwolfia sandwicensis* trees, is a potent antineoplastic agent cause cytochrome P450 (CYP) and/or peroxidase-mediated activation leads to the formation of covalent DNA adducts and alter biotransformation enzymes, thereby modulating their own metabolism resulted in genotoxic and pharmacological effects (Stiborova *et al.* 2011).

Capsaicin is a naturally occurring potent alkaloid widely present in red and chili peppers. The research data documented strongly agree that capsaicin alters various genes expression in cancer cell growth, angiogenesis and metastasis process (Clark and Lee 2016). Capsaicin induces mitochondrial membrane permeabilization and caspase activation leads to apoptosis in human KB cancer cells (Lin *et al.* 2013). Several studies show that capsaicin can relieve inflammation and inhibit activation of nuclear factor (NF)-kappa B/p65 DNA binding activity by different agents such as TNF-alpha. Level of Bcl-2 was also found to be reduced. Capsaicin arrest adult cell growth(Sancho *et al.* 2002; Zhang *et al.* 2003). Capsaicin inhibits the growth of leukemic T-cell leukemia cells by induction of G0-G1 phase cell cycle arrest and apoptosis, without affecting normal bone marrow cells. Capsaicin elevates the intracellular ROS production and oxidative stress in cancer cells and thereby induces the apoptotic mechanism (Ito *et al.* 2004).

Apigenin, chemically 4', 5, 7-trihydroxyflavone is a flavonoid present in many plant varieties such as parsley, chamomile, celery, carrot, and kumquats. The studies have been shown that apigenin suppressed prostate cancer progression by attenuation of insulin-like growth factor binding protein-3 signaling and inhibition of angiogenesis and metastasis in transgenic adenocarcinoma of a mouse prostate (TRAMP) model (Shukla *et al.* 2012) and modulation of cell proliferation, apoptosis, and angiogenesis markers, and modulation of phase I and II detoxification processes in a 7,12-dimethyl benz[a]anthracene (DMBA)-induced oral carcinogenesis in experimental model(Silvan and Manoharan 2013). Apigenin is found to up-regulating pro-apoptotic proteins and/or downregulate antiapoptotic ones, and trigger the intrinsic apoptotic pathway in prostate cancer cells. Apigenin increases the Bax/Bcl-2 ratio leads to an increase in cell apoptosis in prostate cancer cells (Shukla *et al.* 2012). Apigenin is reported to downregulate several cancer signaling pathways, including MAPK, PI3K/Akt and NF- κ B pathways and effective in cancer treatment (Madunic *et*

al. 2018). Chrysin is a plant flavone, widely present in various plants including propolis and in honey. It has been most widely used as a herbal remedy for many diseases in traditional medicine in Asian countries. Many study reports demonstrated that chrysin is very effective as bactericidal, antiallergic, antioxidant, anti-inflammatory, antiestrogenic, and antitumor properties. Studies with experimental models have shown that chrysin induces apoptosis, cell cycle arrest and inhibition of angiogenesis, tumor invasion and metastasis leads to the death of cancer cells but reported to be safe for normal cells. Chrysin exhibits these properties by affecting multiple targets of cell signaling pathways, responsible for inflammation, growth, angiogenesis, and metastasis of cancer cells (Kasala *et al.* 2015).

1.4.3. Annonaceae family: exploring potential phytochemicals

Annonaceae is one of the most diverse plant families consisting of 2016 accepted species of plants, most of them are documented as the rich source of biologically active phytochemicals with great pharmacological potentials. Several species of this family, such as *Annona*, *Asimina*, and *Uvaria* possess edible fruits which are regularly consumed by people. Annonaceae plants have been used for the treatment of arthritis pain, rheumatism, snakebite, diarrhea, jaundice, analgesic, astringent, dysentery and neuralgia in traditional medicine for centuries. Alkaloids, diterpenes, triterpenes, diterpene flavone glycosides, flavonoids, lignans, sterols, and most active annonaceous acetogenin are found to be rich in Annonaceae extracts (Attiq *et al.* 2017). These phytochemicals attributed to the broad array of its pharmacological effects. Phytochemicals from some plants in the family are being clinically evaluated for the treatment of Parkinson's disease, cardiovascular disease, and viral infections (Aminimoghadamfarouj *et al.* 2011). A great range of Annonaceae family plants is used for treating malaria and other illnesses in traditional medicine all over the tropical regions (Frausin *et al.* 2014). The Annonaceae plants have been used for wound healing, hypoglycemic, anti-inflammatory, antioxidant and antiproliferative effects (Formagio *et al.* 2013). More than 70 flavonoids have been isolated from annonacean plants and more than 50 new bioactive flavonoids are reported to possess broad pharmacological activities such as antitumor and antioxidant activities in last two decades (Hu and Wu 2007; Yang *et al.* 2000; Nam *et al.* 2017). Many members of *Annona* species (Annonaceae) are reported as good sources of potential antitumor

agents (Tundis *et al.* 2017) and derived compounds also show antitumor efficacy (Coothankandaswamy *et al.* 2010).

Phytochemistry investigations on *Annona squamosa*, commonly called sweetsops or sugar apple, has identified the annonaceous acetogenins, diterpenes, alkaloids, cyclopeptides, phytosterols, flavonoids, terpenoids, tannins, glycosides, phenolic compounds, and saponins. Essential oils of the plant have been used as a tonic, cool medicine, abortient and heart sedative in traditional medicine (Pandey and Barve 2010; Ma *et al.* 2017). Numerous studies on *A. squamosa* have found that it has anticancer, antioxidant, antidiabetic, cytotoxic, genotoxic, antitumor, antihypertensive, hepatoprotective, antiparasitic, antimalarial, insecticidal (Jaswanth A *et al.* 2010), microbicidal and molluscicidal activities (Pandey and Barve 2010; Ma *et al.* 2017).

Eight mono-tetrahydrofuran annonaceous acetogenins such as cis-annotemoyin-1, squafosacins B, C, F, and G and squadiolins A, B and C, glabranin, annotemoyins-1 and -2, bullatencin, cis-bullatencin, and uvariamicins-I, -II, and -III, were isolated from of *A. squamosa* seeds. Among these, squadiolins A and B showed potent cytotoxicity against human Hep G2 hepatoma cells even in ng/mL concentration and significant activity against human MDA-MB-231 triple negative breast cancer cells. Human Hep G2 and 3B hepatoma and MCF-7 breast cancer cells are found to be dead with the treatment of Squafosacin B. chelation Mono-THF acetogenins are also found to be good chelators of calcium ions (Liaw *et al.* 2008). Three new bis-tetrahydrofuran annonaceous acetogenins and four known annonaceous acetogenins such as squamostatin -A and D, uvarigrandin A, bullatacin, have been isolated with significant *in vitro* cytotoxicity towards five human tumor cell lines from a 95% ethanolic extract of *A. squamosa* seeds (Chen *et al.* 2011).

Annona muricata Linn. (soursop) or custard apple has a long history as a traditionally used medication for the ailments such as fever, respiratory and skin illness, hypertension, inflammation, diabetes rheumatism, arthritic pain and neuralgia in tropical regions as well as African countries (Coria-Téllez *et al.* 2016). Phytochemical investigations of extracts revealed alkaloids, coumarins, saponins, terpenoids, flavonoids, lactones, anthraquinones, tannins, cardiac glycosides, phenols and

phytosterols as its phytoconstituents. Many *in vitro* studies conducted have revealed the potential antioxidant (Padmini *et al.* 2015), differential cytotoxicity, anti-stress, anti-inflammatory, anxiolytic, antimalarial, antidepressant, gastroprotective, wound healing, hypoglycemic, anticancer, hepatoprotective, antitumor, immunomodulatory (Gavamukulya *et al.* 2017) , cyclooxygenase (COX)-1 and COX-2 inhibitory and analgesic activities (Ishola *et al.* 2014) in *A.muricata* plant.

Many phytochemicals have been identified from different plants of Annonaceae family such as flavonoids, acetogenins, alkaloids, and steroids, etc. (Leboeuf *et al.* 1980; Leboeuf *et al.* 1981; Matsushige *et al.* 2012). Sesquiterpenoids are documented to play a highly significant role in human health, as for its nutritive value as well as medicinal properties because of remarkable potential for cardiovascular disease and cancer treatments (Chadwick *et al.* 2013). The important plants of the Annonaceae family with biological activity reported already are depicted in table 1.1.

Acetogenins are the main phytochemical C35 or C37 compounds derived from the polyketide pathway of Annonaceae family which are well investigated for their potential antitumor activities different tumor cell lines. The mechanism of action of acetogenins is mainly their ability to inhibit complex I and NADH oxidases in mitochondrial electron transport chain (Tundis *et al.* 2017).

Studies have shown that synergistic interactions among Graviola leaf phytochemicals such as flavonoids and acetogenins found to impart protection against prostate cancer (Yang *et al.* 2015). The mechanism of action of acetogenins is mainly impairment of energy metabolism due to ATP depletion.

Annonacin, an acetogenin from *A.muricata* found to inhibit the mitochondrial ETC complex I and was reported to alter the energy metabolism and impart toxicity in mesencephalic dopaminergic neurons (Lannuzel *et al.* 2002; Lannuzel *et al.* 2003; Lannuzel *et al.* 2006; Potts *et al.* 2012). It underlined the reason for the onset of the neuronal dysfunction and atypical form of Parkinson disease in Guadeloupe while repeated consumption of edible fruits of *A. muricata* regularly (Lannuzel *et al.* 2002; Lannuzel *et al.* 2003; Lannuzel *et al.* 2006; Potts *et al.* 2012).

Studies of measurement of cell viability by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and determination of cell death by lactate dehydrogenase levels on Lund human mesencephalic neurons demonstrated that intake of plant powder from Annonaceae containing dietary supplements may be hazardous to health in terms of neurotoxicity (Hollerhage *et al.* 2015). Further investigations are required to avoid the neurotoxic effect of annonacean acetogenins. Some of the important compounds isolated from Annonaceae family plants are given in table 1.2.

Table 1.1. Pharmacologically important plants in Annonaceae family

Sl.No	Plants	Properties
1.	<i>Annona coriacea</i>	Antitumor (Tundis <i>et al.</i> 2017)
2.	<i>A. crassifolia</i>	Antitumor (Tundis <i>et al.</i> 2017)
3.	<i>A. dioica</i>	wound healing, hypoglycemic, anti-inflammatory, antioxidant and antiproliferative (Formagio <i>et al.</i> 2013).
4.	<i>A. hypoglauca</i>	Antitumor (Tundis <i>et al.</i> 2017)
5.	<i>A. sylvatica</i>	Antitumor (Tundis <i>et al.</i> 2017)
6.	<i>A. vepretorum</i>	antioxidant and antitumor (Araujo <i>et al.</i> 2015; Bomfim <i>et al.</i> 2016); anti-inflammatory (Silva <i>et al.</i> 2015).
7.	<i>A. crassiflora</i>	Antimutagenic (Rocha <i>et al.</i> 2016); Antiproliferative and anticholinesterase (Formagio <i>et al.</i> 2015).
8.	<i>A. muricata</i>	Antioxidant potential, differential cytotoxicity; antitumor (Tundis <i>et al.</i> 2017); (Dai <i>et al.</i> 2011) (Asare <i>et al.</i> 2015); Anti-inflammatory, cyclooxygenase (COX)-1 and COX-2 inhibitor (Ishola <i>et al.</i> 2014); Analgesic (Ishola <i>et al.</i> 2014)
9.	<i>A. pickelii</i>	Antitumor, antioxidant (Costa <i>et al.</i> 2013b; Costa <i>et al.</i> 2011).
10.	<i>A. salzmannii</i>	Antitumor, antioxidant (Costa <i>et al.</i> 2013b; Costa <i>et al.</i> 2011).
11.	<i>A. squamosa</i>	Anticancer, antioxidant, antidiabetic, cytotoxic and genotoxic, antitumor (Tundis <i>et al.</i> 2017), antihypertensive, hepatoprotective, antiparasitic, antimalarial, insecticidal (Jaswanth A <i>et al.</i> 2010)
12.	<i>Asimina triloba</i>	Antioxidant (Nam <i>et al.</i> 2017)
13.	<i>Guatteria pogonopus</i>	Antitumor (do <i>et al.</i> 2013)
14.	<i>Xylopi frutescens</i>	Cytotoxic and antitumor (Ferraz <i>et al.</i> 2013)
15.	<i>Xylopi laevigata</i>	Anticancer (Quintans Jde <i>et al.</i> 2013).

Table 1.2. Compounds isolated from different plants of Annonaceae family

Sl.No	Plant-derived compounds	Plants	Properties
1.	10-hydroxyglaucanetin	<i>A. muricata</i>	Antitumor (Coothankandaswamy <i>et al.</i> 2010)
2.	27-hydroxybullatacin	<i>A. glabra</i>	Cytotoxic (Liu <i>et al.</i> 1999)
3.	7,7-dimethylaporphine	<i>Guatteria friesiana</i>	Antiproliferative (Costa <i>et al.</i> 2016)
4.	8-hydroxy-1,4,5-trimethoxy-7-oxoaporphine	<i>Pseuduvaria trimera</i>	Anticancer activity (Sesang <i>et al.</i> 2014)
5.	8-hydroxy-3,9-dimethoxy-1,2-methylenedioxyaporphine	<i>Fissistigma poilanei</i>	Cytotoxic (Thuy <i>et al.</i> 2012)
6.	8-hydroxy-9-methoxy-1,2-methylenedioxyaporphine	<i>Fissistigma poilanei</i>	Cytotoxic (Thuy <i>et al.</i> 2012)
7.	8-hydroxyartabonatin c	<i>Pseuduvaria trimera</i>	Anticancer (Sesang <i>et al.</i> 2014)
8.	9-methoxyisomoschatoline	<i>Guatteria hispida</i>	Antioxidant (Costa <i>et al.</i> 2010)
9.	9-methoxyisomoschatoline	<i>Guatteria hispida</i>	Antioxidant (Costa <i>et al.</i> 2010)
10.	Acetogenins	<i>A. muricata</i>	Cytotoxic, calcium chelation (Liaw <i>et al.</i> 2008; Zhao <i>et al.</i> 1993); insecticidal (Zhang <i>et al.</i> 2010)
11.	Annoglaxin	<i>A. glabra</i>	Cytotoxic (Liu <i>et al.</i> 1999)
12.	Annoglaxin	<i>A. glabra</i>	Cytotoxic (Liu <i>et al.</i> 1999)
13.	Annomocherin	<i>A. cherimolia</i>	Cytotoxic (Kim <i>et al.</i> 2001)

14.	Annomontacin	<i>A. cherimolia</i>	Cytotoxic (Kim <i>et al.</i> 2001)
15.	Annomuricins	<i>A. muricata</i>	Cytotoxic (Wu <i>et al.</i> 1995)
16.	Annomuricins	<i>A. muricata</i>	Cytotoxic (Wu <i>et al.</i> 1995)
17.	Annonacin	<i>A. muricata</i> , <i>A. cherimolia</i>	Antitumor (Ko <i>et al.</i> 2011)
18.	Annonacin-a-one	<i>Asimina triloba</i>	Antitumor (Zhao <i>et al.</i> 1993)
19.	Annonaretin A	<i>A. reticulata</i>	Anti-inflammatory (Thang <i>et al.</i> 2013)
20.	Annosquacins	<i>A. squamosa</i>	Cytotoxic, antiproliferative (Chen <i>et al.</i> 2012)
21.	Anonaine	<i>A. salzmannii</i>	Antioxidant and antimicrobial (Costa <i>et al.</i> 2013a)
22.	Anonaine	<i>A. muricata</i>	Antioxidant, antimicrobial (Costa <i>et al.</i> 2013a)
23.	Asimicin	<i>Xylopiaromatica</i>	Antitumor (Colman-Saizarbitoria <i>et al.</i> 1995)
24.	Asimilobine	<i>A. salzmannii</i>	Antioxidant and antimicrobial (Costa <i>et al.</i> 2013a)
25.	Bullatacin	<i>A. muricata</i>	Antitumor (Oberlies <i>et al.</i> 1997; Morre <i>et al.</i> 1995)
26.	Caryophyllene oxide	<i>A. vepretorum</i>	Antioxidant and antitumor (Araujo <i>et al.</i> 2015; Bomfim <i>et al.</i> 2016)
27.	Dioxoaporphine	<i>Pseuduvaria rugosa</i>	Cytotoxic (Uadkla <i>et al.</i> 2013)
28.	Flavonoid	<i>A. squamosa</i>	Tumor growth-inhibitory (Yang <i>et al.</i> 2015; Hu and Wu 2007) (Li and Yu 1998)
29.	Gigantetrocinone	<i>Asimina triloba</i>	Antitumor (Zhao <i>et al.</i> 1993)
30.	Glacins	<i>A. glabra</i>	Cytotoxic (Liu <i>et al.</i> 1998)

31.	Isoannonacin	<i>Asimina triloba</i>	Antitumor (Zhao <i>et al.</i> 1993)
32.	Isocerasonine	<i>Guatteria hispida</i>	Antioxidant (Costa <i>et al.</i> 2010)
33.	Limonene	<i>A. vepretorum</i>	Antioxidant and antitumor (Araujo <i>et al.</i> 2015; Bomfim <i>et al.</i> 2016)
34.	Liriodenine	<i>A. salzmannii</i>	Antioxidant and antimicrobial (Costa <i>et al.</i> 2013a)
35.	Mitregenin.	<i>Mitrephora maingayi</i>	Insecticidal activity (Zhang <i>et al.</i> 2010)
36.	Muricins	<i>A. muricata</i>	Cytotoxic (Chang and Wu 2001); antiproliferative (Sun <i>et al.</i> 2014)
37.	Plagioneurins	<i>Disepalum plagioneurum</i>	Cytotoxic (Eparvier <i>et al.</i> 2006)
38.	Plagionicin	<i>Disepalum plagioneurum</i>	Cytotoxic (Eparvier <i>et al.</i> 2006)
39.	Pseudovarines	<i>Pseuduvaria rugosa</i>	Cytotoxic, antiproliferative (Taha <i>et al.</i> 2011; Taha <i>et al.</i> 2011)
40.	Spathulenol	<i>A. vepretorum</i>	Antioxidant and antitumor (Araujo <i>et al.</i> 2015; Bomfim <i>et al.</i> 2016)
41.	Squamolone	<i>Asimina triloba</i>	Antitumor (Zhao <i>et al.</i> 1993)
42.	Squamostanin	<i>A. squamosa</i>	Cytotoxic (Yang <i>et al.</i> 2009)
43.	Venzeenin	<i>Xylopia aromatica</i>	Antitumor (Colman-Saizarbitoria <i>et al.</i> 1995)
44.	A-pinene	<i>A. vepretorum</i>	Antioxidant and antitumor (Araujo <i>et al.</i> 2015; Bomfim <i>et al.</i> 2016)

1.4.4. Uvaria plants

It is a well-known genus of flowering plants under the Annonaceae family. According to the plant list published by Royal Botanic Gardens, Kew and Missouri Botanical

Garden, uvaria genus is having 421 known species and out of this 37 is accepted. In Latin the meaning of 'uva' is grape; the name uvaria implies the resemblance of the fruit of uvaria to the grapefruit.

The plants under uvaria and different compounds isolated from uvaria species are documented with great range of biological activities such as antimicrobial, antimalarial, anti-inflammatory, cytotoxic, antiproliferative and antitumor activities (Nkunya *et al.* 1991; Dai *et al.* 2012; Philipov *et al.* 2000; Fall *et al.* 2004; Olumese *et al.* 2016; Okwu and Iroabuchi 2009; Awodiran *et al.* 2018).

Uvaria acumulata is a plant under Annonaceae with remarkable biological properties. Boiled root decoction has been used traditionally for treating snakebite, stomach ache, painful menstruation, etc. (Tempesta *et al.* 1982). Scientific studies on *U. acuminata* Oliv chloroform extract have shown growth inhibitory effect on the P-388 lymphocytic leukemia (Tempesta *et al.* 1982). Among the twenty-eight traditionally used medicinal plants, collected from the eastern part of Tanzania have been screened for their biological activity using the Brine Shrimp bioassay and eight plant extracts out of them were documented for their potent activity. *Uvaria acuminata* root extract was found to be the most effective one among them (Massele and Nshimo 1995). Novel polyoxygenated seco-cyclohexenes compounds named as uvamalols A-C, uvarimacrophin A and uvamalols D-G, have isolated from *Uvaria macrophylla* roots by fractionation (Wang *et al.* 2005; Wang *et al.* 2003). Investigations on chemical constituents of *Uvaria grandiflora*, a bisexual flowering plant under Annonaceae family has documented the presence of two major triterpenes, suberosol, and Lupeol (Zhao *et al.* 1999). Some of the important Uvaria plants with their studied properties are given in table 1.3.

A novel lignan glycoside, ufaside, and six already reported compounds named as daucosterol, oxoanolobine, catechin, epicatechin, ergosta-4,6,8(14),22-tetraen-3-one and glutin-5-en-3-one have been isolated from the aerial parts of *U. rufa*. Investigation of cytotoxicity against human cancer cell lines such as MCF-7, MDA-MB-231, LNCaP, MKN7, SW480, KB, LU-1, HepG2, and HL-60 compounds, oxoanolobine and ergosta-4,6,8(14),22-tetraen-3-one are showed moderate activity against human lung adenocarcinoma cell line (LU-1) (Nguyen *et al.* 2015). The list of

some important compounds isolated from *Uvaria* species with their activities is depicted in Table 1.4.

Table 1.3. *Uvaria* plants studied for their biological properties

Sl. No	Plants	Properties
1.	<i>U. accumulata</i>	Cytotoxicity (Nkunya <i>et al.</i> 1991; Lasswell and Hufford 1977), growth inhibitory (R. Cole <i>et al.</i> 1976); antianemic, antimalarial (Gathirwa <i>et al.</i> 2011); (Peter <i>et al.</i> 2014)
2.	<i>U. scheffleri</i>	Cytotoxicity (Auranwiwat <i>et al.</i> 2017)
3.	<i>U. afzelii</i>	Hepatoprotective (Omoruyi <i>et al.</i> 2014), cytotoxicity, antiplasmodial (Menan <i>et al.</i> 2006)
4.	<i>U. dac</i>	Antiproliferative (Awale <i>et al.</i> 2012; Ueda <i>et al.</i> 2013)
5.	<i>U. grandiflora</i>	Cytotoxic (Pan and Yu 1997)
6.	<i>U. rufa</i>	Antituberculosis (Macabeo <i>et al.</i> 2012), Antioxidant (Buncharoen <i>et al.</i> 2016).
7.	<i>U. valderramensis</i>	Antitubercular, cytotoxic (Macabeo <i>et al.</i> 2014)
8.	<i>U. chamae</i>	Cytotoxic (Lasswell and Hufford 1977); anti-inflammatory (Okwu and Iroabuchi 2009), Antianemia, immune system inducer (Olumese <i>et al.</i> 2016)

Table 1.4. Important phytochemicals isolated from *Uvaria* species with their activities

Sl.No	Phytochemicals	Plants	Properties
1.	(-)-3-O-Debenzoylzeilenone	<i>Uvaria grandiflora</i>	Cytotoxicity (Ho <i>et al.</i> 2015)
2.	(-)-Uvaribonol	<i>U. rufa.</i>	Cytotoxicity (Auranwiwat <i>et al.</i> 2017)
3.	(-)-Uvaribonol	<i>U. cherreensis</i>	Antiplasmodial, cytotoxicity (Auranwiwat <i>et al.</i> 2017)
4.	(8',9'-Dihydroxy)-3-Farnesyldole	<i>U. acuminata</i>	Cytotoxicity (Nkunya <i>et al.</i> 1991)

5.	Acumitin	<i>U. acuminata</i>	Cytotoxicity (Ichimaru <i>et al.</i> 2004)
6.	Afzeliindanone	<i>Uvaria afzelii</i>	Hepato protective (Omoruyi <i>et al.</i> 2014)
7.	Calamistrins	<i>U. calamistrata</i>	Cytotoxic (Zhou <i>et al.</i> 1999)
8.	Chamanetin	<i>U. chamae</i>	Cytotoxicity (Lasswell and Hufford 1977)
9.	Chamuvarinin	<i>U. acuminata</i>	Cytotoxic (Fall <i>et al.</i> 2004)
10.	Chamuvarinin	<i>U. chamae</i>	Cytotoxic(Fall <i>et al.</i> 2004)
11.	Cherrevenaphthalenes	<i>U. cherrevensis</i>	Antiplasmodial, cytotoxicity (Auranwiwat <i>et al.</i> 2017)
12.	Corydine	<i>U. chamae</i>	Cytotoxicity (Philipov <i>et al.</i> 2000)
13.	Dichamanetin	<i>U. chamae</i>	Cytotoxicity (Nakatani <i>et al.</i> 2005)
14.	Diuvaretin	<i>U. acuminata</i> , <i>U. chamae</i>	Cytotoxicity (Nkunya <i>et al.</i> 1991) (Lasswell and Hufford 1977).
15.	Ergosta-4,6,8(14),22-Tetraen-3-One	<i>U. rufa</i>	Cytotoxicity (Nguyen <i>et al.</i> 2015)
16.	Grandifloracin	<i>Uvaria dac</i>	Antiproliferative, antiausterity agent(Awale <i>et al.</i> 2012; Ueda <i>et al.</i> 2013)
17.	Hamilcone	<i>U. hamiltonii</i>	Cytotoxicity (Huang <i>et al.</i> 1998)
18.	Hamiltones	<i>U. hamiltonii</i>	Cytotoxicity (Huang <i>et al.</i> 1998)
19.	Hamiltrone	<i>U. hamiltonii</i>	Cytotoxicity (Huang <i>et al.</i> 1998)
20.	Hamilxanthene	<i>U. hamiltonii</i>	Cytotoxicity (Huang <i>et al.</i> 1998)
21.	Hydroxyespintanol	<i>U. scheffleri</i>	Cytotoxicity (Ichimaru <i>et al.</i> 2010)
22.	Isochamanetin	<i>U. chamae</i>	Cytotoxicity(Lasswell and Hufford 1977)
23.	Isochamuvaritin	<i>U. acuminata</i>	Cytotoxicity (Ichimaru <i>et al.</i> 2004)
24.	Isoquercitrin	<i>U. rufa</i>	Glycation inhibition (Deepralard <i>et al.</i> 2009)
25.	Isoquercitrin 6-Acetate	<i>U. rufa</i>	Glycation inhibition (Deepralard <i>et al.</i> 2009)
26.	Isouvaretin	<i>U. chamae</i>	Cytotoxicity (Nakatani <i>et al.</i> 2005)
27.	Isouvaritin	<i>U. acuminata</i>	Cytotoxicity (Ichimaru <i>et al.</i> 2004)
28.	Kweichowenol	<i>U. kweichowensis</i>	Antitumour (Xu <i>et al.</i> 2005)
29.	Nantenine	<i>U. chamae</i>	Cytotoxicity (Philipov <i>et al.</i> 2000)
30.	Nornantenine	<i>U. chamae</i>	Cytotoxicity(Philipov <i>et al.</i> 2000)

31.	Oxepinone 3	<i>U.valderramensis</i>	Cytotoxic (Macabeo <i>et al.</i> 2014)
32.	Oxoanolobine	<i>U. rufa</i>	Cytotoxicity (Nguyen <i>et al.</i> 2015)
33.	Pinocembrin	<i>U.chamae</i>	Cytotoxic effect (Lasswell and Hufford 1977).
34.	Schefflerichalcone	<i>U. scheffleri</i>	Cytotoxicity (Ichimaru <i>et al.</i> 2010)
35.	Uvaretin	<i>U. acuminata</i>	Cytotoxicity (Nkunya <i>et al.</i> 1991; Ichimaru <i>et al.</i> 2004), Antitumor (Cole <i>et al.</i> 1976)
36.	Uvaretin	<i>U. chamae</i>	Cytotoxicity (Nakatani <i>et al.</i> 2005)
37.	Uvaricin	<i>Uvaria sp.</i> , <i>U. acuminata</i>	Antiproliferative, antitumor (Dai <i>et al.</i> 2012) (Tempesta <i>et al.</i> 1982)
38.	Uvarigrin	<i>U. acuminata</i> , <i>U.grandiflora</i>	Cytotoxic (Pan and Yu 1997) (Ichimaru <i>et al.</i> 2004)

A detailed account of the biological activity of the *Uvaria* species available in Kerala has not yet been studied. Considering the potential of the bioactive components of this plant, the anticancer studies on the plants from *Uvaria* species may have pharmaceutical importance. So less explored plants under *Uvaria* species such as *Uvaria narum* and *Uvaria macropoda* are taken for a comparative study of their biological activities and with much emphasis on their cancer preventive or curative potential.

1.5. *Uvaria narum* (Dunal) Wall.

1.5.1. Description

Uvaria narum (Dunal) Wall. (Annonaceae) is a large woody scandent shrub climber with sparsely hairy branchlets and dorsiventral dark bluish green leaves having distinct upper and lower surface. Bruised leaves smell like cinnamon. It's a dicot. Flowers are reddish, solitary, terminal or leaf-opposed, 2.5 cm in diameter, stamens with anthers concealed by the overlapping connectives, carpels, numerous, scarlet red; seeds chestnut brown. Distributed in the hilly regions of western peninsular India and Sri Lanka and grows up to an altitude of 1,200 m. Flowering and fruiting are between the period of November and June.

1.5.2. Taxonomic classification

Scientific name	:	<i>Uvaria narum</i> (Dunal) Wall.
Kingdom	:	Plantae
Division	:	Tracheophyta
Class	:	Magnoliopsida
Order	:	Magnoliales
Family	:	Annonaceae
Genus	:	<i>Uvaria</i>
Species	:	<i>Uvaria narum</i>



Figure 1.10. *Uvaria narum* (Dunal) Wall.

1.5.3. Common names

South Indian Uvaria	:	English
Kureel, Narumpanal, Kooril, Koorilvalli, Korandapazham	:	Malayalam
Neelavalli, Valeeshakhota	:	Sanskrit
Bugadee balli, bugadee hoo, gunavaara	:	Kannada
Kaala-apkara	:	Marathi
Pulliccan, pulikkan	:	Tamil

1.5.4. Phytochemistry

The GC-MS analysis of root bark essential oil of *U.narum* has revealed the presence bornyl acetate (15.2%) and patchoulone (8.1%), a tricyclic sesquiterpene ketone as major individual constituents out of 52 phytocomponents from the oil (Hisham *et al.* 1992). Triterpenes, glutinol, glutinone, taraxerol, beta-sitosterol, and annonaceous acetogenins uvariamicins-I, II and III mixture, isodesacetylurvaricin, squamocin-28-one, narumicins-I and II stereoisomeric mixture, squamocin and panalicin were reported from the hexane and ethyl acetate root bark fractions of the root barks of *Uvaria narum* Wall. and *Uvaria hookeri* King (Padmaja *et al.* 1993; Hisham *et al.* 1991a; Hisham *et al.* 1991b; Hisham *et al.* 1990). Preliminary studies showed that *U.narum* is rich in biologically important compounds such as terpenoids, triterpenoids, flavonoids, steroids, coumarins, alkaloids, tannins, cardiac glycosides, saponins proteins and carbohydrates (Florence A R *et al.* 2014; Varghese 2013).

1.5.5. Traditional uses

U.narum popularly used in ethnomedicine for the treatment of skin diseases. Different parts of the plant were found to be used in the treatment of vitiated Vata, pitta, eczema, itching, varicose vein, hemorrhoids, jaundice, inflammation, gastrointestinal problems, fever, and Herpes (Padmaja *et al.* 1993; (Varghese *et al.* 2017). This plant is reported with insecticidal activities. The root of the plant is used by traditional practitioners to treat constipation and skin diseases such as pityriasis and eczema (Padyana *et al.* 2013). The leaves are recommended in rheumatic swellings, biliousness, and typhoid. A decoction of the root bark is given to women to control fits at the time of delivery (Varghese 2013). The literature collected revealed the

mentioning of neelavalli (valeeshakhota) in various treatments in Ayurveda medicines. It is known to be a good pain reliever (Varghese 2013).

1.5.6. Pharmacological properties

1.5.6.1. Antimicrobial activities

The antibacterial activity has been tested by the disc diffusion technique showed that acetogenin compounds isolated from root barks of *Uvaria narum* Wall showed potent activity against both Gram-positive and Gram-negative organisms. These compounds are found to inhibit the growth of *Candida*, *Penicillium* and *Aspergillus* species suggesting its antifungal properties. Crude extracts of the plant are reported with potent anthelmintic activity in a concentration-dependent manner when compared to standard drug mebendazole (Padmaja *et al.* 1993). The *U.narum* leaf methanolic and ethyl acetate extracts are found to exhibit excellent broad spectrum antibacterial effects on *Pseudomonas aeruginosa* (Reddy *et al.* 2012). *U.narum* PE extracts have shown maximum activity with a large zone of inhibition against the harmful human pathogens evaluated and discovered the lead compounds that actually responsible for its antimicrobial properties (Varghese 2013). Bioactivity-guided fractionation of acetone extracts of *U.narum* has resulted in a new molecule, 2-E-(2''-oxo-5''-acetoxy cyclopent-3''-en-1''-ylidene) ethyl benzoate which is found to be potent antifungal properties (Varghese *et al.* 2017).

1.5.6.2. Antioxidant properties

The *U. narum* methanol extract and essential oil of leaf are found to have potent scavenging activity on 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical in *in vitro* studies (Reddy *et al.* 2012). Except this, no detailed information is available so far.

1.5.6.3. Hepatoprotective effect

Studies have been reported that the ethanolic leaf extract of *U.narum* ameliorates the depletion of the antioxidant system of the animals caused by CCl₄ exposure and found to protect the liver function (Patel *et al.* 2013).

1.6. *Uvaria macropoda* Hook. f. & Thomson

1.6.1. Description

These are dicotyledonous woody climbers, Lianas, young shoots tomentose. Leaves simple narrowly elliptic or oblong, acute or acuminate apex, base obtuse or rounded, glabrous, to 15 x 5 cm. Flowers solitary. Pedicels and calyx tuberculate, ferrugineus-tomentose. Petals dull-purple with yellow at base, elliptic or ovate, connate at base, tomentose on both sides. Fruitlets long-stipitate. Distributed in semi-evergreen forests of the Western Ghats, south India, and Srilanka. In Kerala, it is distributed in Kollam, Thrissur, and Kannur. The flowering and fruiting are between March and June.

1.6.2. Taxonomic classification

Scientific name	: <i>Uvaria macropoda</i> Hook. f. & Thomson
Kingdom	: Plantae
Division	: Tracheophyta
Class	: Magnoliopsida
Order	: Magnoliales
Family	: Annonaceae
Genus	: <i>Uvaria</i>
Species	: <i>Uvaria macropoda</i>



Figure 1.11. *Uvaria macropoda* Hook. f. & Thomson

1.6.3. Phytochemistry : No studies were reported

1.6.4. Traditional uses : No literature

1.6.5. Pharmacological properties: No pharmacological activities documented

Chapter 2

Materials and Methods

2.1. Materials

2.1.1. Chemicals

15-lipoxygenase	: Sigma Aldrich, USA
1-naphthol	: MERK specialities Pvt Ltd., Mumbai
2, 2-diphenyl-1-picrylhydrazyl	: Sigma Aldrich, USA
-2, 5-diphenyltetrazolium bromide	
3-(4, 5-dimethylthiazol-2-yl)	: SRL Pvt. Ltd. India
Acetic acid	: MERK specialities Pvt Ltd., Mumbai
Acetic anhydride	: MERK specialities Pvt Ltd., Mumbai
Acetone	: MERK specialities Pvt Ltd., Mumbai
Acridine orange	: MERK specialities Pvt Ltd., Mumbai
Aluminum chloride	: MERK specialities Pvt Ltd., Mumbai
Ammonium hydroxide	: NICE chemicals Pvt Ltd., Kochi
Anisaldehyde	: Sisco Research Laboratories Pvt. Ltd. Ascorbic
acid	: Sisco Research Laboratories Pvt. Ltd.
Benzene	: MERCK chemical Ltd. India.
Bismuth carbonate	: MERCK chemical Ltd. India.
Bismuth nitrate	: Sisco Research laboratories Pvt. Ltd.
Bovine serum albumin (BSA)	: MERK specialities Pvt Ltd., Mumbai
Butanol	: MERK specialities Pvt Ltd., Mumbai
Calcium chloride	: MERK specialities Pvt Ltd., Mumbai
Carboxy methyl cellulose (CMC)	: Sisco Research Laboratories Pvt. Ltd.
Carrageenan	: Sigma Aldrich, USA
Chloroform	: MERK specialities Pvt Ltd., Mumbai
Copper sulfate (CuSO ₄ .5H ₂ O)	: MERCK chemical Ltd. India.
Cyclophosphamide	: Neon laboratories Ltd., Mumbai
Dextran	: Hi-Media, Mumbai
Dextrose	: MERK specialities Pvt Ltd., Mumbai
Di potassium hydrogen phosphate	: MERK specialities Pvt Ltd., Mumbai
Diclofenac	: Torrent labs, Private Ltd., Ahmedabad
Diethyl ether	: MERK specialities Pvt Ltd., Mumbai
Dimethyl sulfoxide (DMSO)	: MERK specialities Pvt Ltd., Mumbai

Dulbecco's modified eagle medium (DMEM):	Life technologies, Bangalore
EDTA	: MERK specialities Pvt Ltd., Mumbai
Ethidium bromide (EtBr)	: Sigma Aldrich, USA
Ethyl acetate	: MERK specialities Pvt Ltd., Mumbai
Evans blue dye	: Sisco Research Laboratories, India
Ferric chloride	: MERCK chemical Ltd. India.
Ferric ferrocyanide	: MERCK chemical Ltd. India.
FeSO ₄	: MERK specialities Pvt Ltd., Mumbai
Fetal bovine serum (FBS)	: Gibco, Life technologies, Bangalore
Folin ciocalteu reagent	: MERK specialities Pvt Ltd., Mumbai
Formaldehyde	: MERK specialities Pvt Ltd., Mumbai
Gallic acid	: Sigma Aldrich, USA
Glutathione reduced (GSH)	: Sisco Research Laboratories, India
Hexane	: MERK specialities Pvt Ltd., Mumbai
Hydrochloric acid	: MERK specialities Pvt Ltd., Mumbai
Iodine flakes	: Sigma Aldrich, USA
Isopropanol	: MERCK chemical Ltd. India.
KH ₂ PO ₄	: MERK specialities Pvt Ltd., Mumbai
Lead acetate	: MERCK chemical Ltd. India.
Leishman Stain	: Sisco Research Laboratories, India
L-Glutamine	: Hi-Media laboratories, Mumbai
Linoleic acid	: Sigma Aldrich, USA
Mercuric chloride	: MERCK chemical Ltd. India
Methanol	: MERK specialities Pvt Ltd., Mumbai
Na ₂ HPO ₄	: MERK specialities Pvt Ltd., Mumbai
NaCl	: MERK specialities Pvt Ltd., Mumbai
NaHCO ₃	: MERK specialities Pvt Ltd., Mumbai
Ninhydrin	: MERCK chemical Ltd. India.
Nitroblue tetrazolium (NBT)	: SRL (SRL) Pvt. Ltd., Mumbai, India
Penicillin	: Hi-Media laboratories, Mumbai
Petroleum ether	: MERK specialities Pvt Ltd., Mumbai
Phenol	: MERCK chemical Ltd. India.
PMA	: Sigma Aldrich, USA

Potassium acetate	: MERK specialities Pvt Ltd., Mumbai
Potassium chloride (KCl)	: MERK specialities Pvt Ltd., Mumbai
Potassium ferrocyanide	: MERK specialities Pvt Ltd., Mumbai
Potassium hydroxide	: MERCK chemical Ltd. India.
Potassium iodide	: MERCK chemical Ltd. India.
Propylene glycol	: MERK specialities Pvt Ltd., Mumbai
Pyruvate	: Hi-Media laboratories, Mumbai
Quercetin	: Sigma Aldrich, USA
Riboflavin	: Sisco Research laboratories, India
RNase A	: Bangalore Genei Pvt Ltd, Bangalore,
Rosewell park memorial institute medium (RPMI):	Life technologies,
	Banglore
Silica gel for column chromatography:	MERCK chemical Ltd. India.
Silica gel for column	: MERCK chemical Ltd. India.
Silica gel G for TLC	: MERCK chemical Ltd. India.
Sodium azide	: Sisco Research Laboratories, India
Sodium carbonate	: MERK specialities Pvt Ltd., Mumbai
Sodium caseinate	: MERK specialities Pvt Ltd., Mumbai
Sodium dihydrogen phosphate	: MERK specialities Pvt Ltd., Mumbai
Sodium dodecyl sulfate	: MERCK chemical Ltd. India.
Sodium potassium tartrate	: MERK specialities Pvt Ltd., Mumbai
Sodium sulfate	: MERK specialities Pvt Ltd., Mumbai
Streptomycin	: Hi-Media laboratories, Mumbai
Sulphuric acid	: MERK specialities Pvt Ltd., Mumbai
Thiobarbituric acid	: Sisco Research Laboratories Pvt. Ltd.
TPTZ (2, 4, 6-tripyridyl-s-triazine)	: Sigma Aldrich, USA
Tri sodium citrate	: MERK specialities Pvt Ltd., Mumbai
Trichloroacetic acid	: MERK specialities Pvt Ltd., Mumbai
Tris-HCl	: MERK specialities Pvt Ltd., Mumbai
Triton X-100	: Hi-Media laboratories, Mumbai
Trypan blue	: Spectrum Pvt Ltd., India
Trypsin	: Life Technologies, UK
Vanillin	: MERCK chemical Ltd. India.

2.1.2. Diagnostic reagent kits:

Alkaline phosphatase (S.L)	- Agappe diagnostics Ltd., Ernakulam, India
Bilirubin Total-TAB	- Agappe diagnostics Ltd., Ernakulam, India
cDNA reverse transcription kit	- Applied Biosystems
Cholesterol HDL	- Agappe Diagnostics Ltd., Ernakulam, India
Cholesterol Total	- Agappe Diagnostics Ltd., Ernakulam, India
Creatinine	- Euro Diagnostic Systems Pvt. Ltd., Chennai
Hemoglobin (Hb)	- Agappe Diagnostics Ltd., Ernakulam, India
Mouse TNF-alpha ELISA Kit-	Quantikine ELISA Kit, R&D Systems Website

Enhancements

PowerUp™ SYBR™ PCR master mix-	Applied Biosystems
SGOT	- Agappe diagnostics Ltd., Ernakulam, India
SGPT	- Agappe diagnostics Ltd., Ernakulam, India
Total protein	- Agappe diagnostics Ltd., Ernakulam, India
Triglycerides	- Agappe Diagnostics Ltd., Ernakulam, India
Urea	- Euro Diagnostic Systems Pvt. Ltd., Chennai

2.1.3. Instruments

Cyclo vortex mixer	: Rotex Instruments Pvt. Ltd., India
Deep freezer (-20°C)	: Remi Laboratory Instruments, Mumbai
Deep freezer (-70°C)	: Eppendorf, Germany
Electronic weighing balance-ATX224:	Shimatzu Cooperation Ltd, India
Flow Cytometer	: Beckman Coulter, U.S.A
Fluorescent microscope	: Leica, German Radicle, Ambala
GC-MS analyser	: QP2010S Shimadzu system
High Performance TLC	: CAMAG system, Switzerland
High speed cooling centrifuge	: Remi Laboratory Instruments, Mumbai,
Horizontal Laminar flow hood	: Cleanair, Chennai, india
Hot air oven	: Rotex Instruments Pvt. Ltd., India
HPLC	: Shimadzu Corporation, Japan
HPTLC scanner	: CAMAG system, Switzerland
Incubator	: Rotex Instruments Pvt. Ltd., India

Inverted microscope	: Leica, German Radicle, Ambala
Laminar flow hood (Horizontal)	: Cleanair, Chennai, India
Magnetic stirrer	: Spinit, Tarsons Products Pvt. Ltd., Kolkata
Microcentrifuge	: Tarsons Products Pvt. Ltd., Kolkata
pH meter	: Elico Limited, Hyderabad, India
Phase contrast microscope	: Magnus, INVI, New Delhi, India
Serological centrifuge	: Remi Laboratory Instruments, Mumbai TLC
silica plates	: Merck KGaA, Darmstadt, Germany
Upright research microscope	: Meiji, Japan; Labex, Labovision
UV/Visible spectrophotometer	: P G instruments Ltd., India
UV/Visible spectrophotometer	: Systronics Ltd., India.
Veriti™ Thermal cycler	: Applied Biosystems, USA
MicroAmp Fast Optical PCR plates	: Applied Biosystems, USA
Step one plus Real-time PCR system:	Applied Biosystems, USA

2.1.4. Reagents

a) Dragendorff's reagent

Bismuth carbonate-5.2g

Sodium iodide - 4g

Dissolved in 50 mL glacial acetic acid and boiled for a few minutes. After 12 hrs the precipitated sodium acetate crystals are filtered off. 40 mL of this filtrate was mixed with 160 mL of ethyl acetate and 1 mL water. Stored in the amber colored bottle. 10mL of the stock was mixed with 20 mL of glacial acetic acid to prepare working solution.

b) Mayer's reagent

HgCl₂ - 1.358/60 mL water

KI - 5g/10 ml

Mixed 2 solutions well and made up to 100mL with water.

c) Wagner's reagent

Iodine - 1.27g

KI - 2g

Dissolved in 5mL sulphuric acid and made up to 100 mL

d) Phosphate buffered saline (PBS)

NaCl	- 8g
Na ₂ HPO ₄ .2H ₂ O	- 1.44g
EDTA	- 2g
KH ₂ PO ₄	- 0.2g

Dissolved the contents and made upto 1000 mL with distilled water and adjust the pH to 7.4

e) Trypan blue Dye

Trypan blue	-0.1g
NaCl	-0.9g

Dissolved in 100 mL of water. Keep beaker with solution in magnetic stirrer overnight. Then filter it through Whatmann No.1 filter paper.

f) Tris-HCl buffer (pH 7.4, 0.1 M)

Tris-HCl	-1.576 g
Distilled water	-100 mL

Mixed well and adjust the pH to 7.4.

g) 10% neutral buffered saline

NaH ₂ PO ₄	-4g
Na ₂ HPO ₄	-6.5g
Formalin	-100 mL
Distilled water	-900 mL

h) Hayem's fluid

Mercuric chloride	-0.25 gm
Sodium sulphate	-2.50 gm
Sodium chloride	-0.50 gm

Dissolve the contents in 100 mL of distilled water.

i) Turk's fluid

Crystal violet	-1 g/100 mL
Glacial acetic acid	-2 mL

Dissolve the contents in distilled water and volume was made up to 100 mL with distilled water. The solution was stirred in a magnetic stirrer overnight, filtered and ready for WBC count.

j) Acridine orange/Ethidium bromide (Ao/EtBr) stain

a) Acridine orange (AO) -5 mg per 1mL ethanol

b) Ethidium bromide (EtBr) -3 mg is dissolved in 1mL ethanol

Working AO/EtBr staining solution is prepared by mixing 1 μ L of AO and 1 μ L of EtBr and made up to 1 mL with PBS, kept for 2 weeks and used for the evaluation of the apoptotic morphology of cells.

2.1.5. Plant materials

2.1.5.1. *Uvaria narum* (Dunal) Wall.

2.1.5.1.1. Taxonomic classification

Scientific name	:	<i>Uvaria narum</i> (Dunal) Wall .
Kingdom	:	Plantae
Division	:	Tracheophyta
Class	:	Magnoliopsida
Order	:	Magnoliales
Family	:	Annonaceae
Genus	:	<i>Uvaria</i>
Species	:	<i>Uvaria narum</i>

2.1.5.1.2. Common names

South Indian Uvaria	:	English
Kureel, Narumpanal,		
Kooril, Koorilvalli, Korandapazham	:	Malayalam
Neelavalli, Valeeshakhota	:	Sanskrit
Bugadee balli, bugadee hoo, gunavaara	:	Kannada
Kaala-apkara	:	Marathi
Puliccan, pulikkan	:	Tamil

2.1.5.2. *Uvaria macropoda* Hook. f. & Thomson

2.1.5.2.1. Taxonomic classification

Scientific name	:	<i>Uvaria macropoda</i> Hook. f. & Thomson
Kingdom	:	Plantae
Division	:	Tracheophyta
Class	:	Magnoliopsida
Order	:	Magnoliales
Family	:	Annonaceae
Genus	:	<i>Uvaria</i>
Species	:	<i>Uvaria macropoda</i>

2.1.5.3. Collection and authentication of *U.narum* and *U.macropoda*

Uvaria narum and *Uvaria macropoda* are the plants selected for the study. *Uvaria narum* (dunal) wall. was collected locally from Thrissur, Kerala and *Uvaria macropoda* was collected from Kannur district, Kerala and identified using the herbarium sheets by Dr. C.N. Sunil, Associate Professor and research guide, Department of Botany, Sree Narayana Mangalam College (SNMH), Maliankara, Ernakulam. A voucher specimen was maintained (Voucher No. ACRC203 and ACRC209 for *Uvaria narum* (Dunal) Wall and *Uvaria macropoda* Hook. f. & Thomson, respectively).

2.1.6. Animals

Swiss albino and BALB/c mice (20–30 g) and male Wistar rats (250-300g) were used as an animal model for *in vivo* studies. They were purchased from Small Animal Breeding Station, Kerala Veterinary University, Small Animal breeding station, Mannuthy, and Kerala. Animals were housed under controlled conditions of temperature (22- 28° C) and humidity (60-70%) and were provided with standard rat chow (Sai Durga feeds and foods, Bangalore) and water *ad libitum*. The animals were housed in polypropylene cages with paddy husk bedding and top grill of stainless steel with facilities for providing food and water *ad libitum*. All the experiments were carried out with prior permission from Institutional Animal Ethics Committee, Amala Cancer Research Centre (Approval No. ACRC/IAEC/2015/6-01) according to the

rules and regulations of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Govt. of India.

2.1.7. Cell lines and its maintenance

Dalton's Lymphoma Ascites (DLA) and Ehrlich Ascites Carcinoma (EAC) cells were procured from Adayar Cancer Institute, Chennai. These cell lines were maintained in the peritoneal cavity of the BALB/c mice at Amala cancer research Centre. The cells were aspirated from the peritoneal cavity and 1×10^6 cells were injected to the peritoneal cavity of another healthy mouse around 14 days interval.

HeLa (Human cervical carcinoma), HepG2 (human hepatocarcinoma), HCT-15 (human colorectal adenocarcinoma) and Vero E6 (African monkey kidney) cell lines were obtained from the National Centre for Cell Science (NCCS), Pune, India. Each cell line was maintained in its appropriate medium supplemented with 10% heat-inactivated FBS and antibiotics, and incubated in 25 ml culture flasks at 37° C under a 5% CO₂ atmosphere. Sterile conditions were maintained throughout the experiment. The culture flasks with the cells were examined regularly for a color change of medium or contamination with changing the old medium with a fresh one. AT 70-80% confluence cells were subcultured. After removing the used medium, the cells were washed thrice with PBS. Trypsinisation was done by 1 mL trypsin (0.25% trypsin in 0.001% EDTA) solution at 37°C for cell detachment. A volume of 1 mL of fresh medium was added to inactivate the trypsin and mixed gently by a sterile pipette to make single cell suspension and equally divided into 5 different flasks with an adequate amount of media. The cells were monitored under an inverted microscope.

2.2. Methods

2.2.1. Extraction of plant samples

The leaves of *Uvaria narum* and *Uvaria macropoda* plants were dried separately in a hot air oven at 40° C and powdered using mixer grinder. Approximately 30 g of leaf powder of each plant were taken and was successively extracted separately using ten times volumes of solvents (1:10 v/v) of increasing polarity (such as petroleum ether, chloroform, acetone, methanol, and water) for 24 hrs period. It was centrifuged at 10000 rpm in a serological centrifuge and the supernatant was collected. Repeated the

procedure until extraction was completed. The supernatants were pooled, evaporated off the solvents and residue was collected after appropriate washing. The total residue was collected separately for two plants and stored in an airtight amber colored bottle at 4 ° C for further studies. The percentage yield of individual extracts with respect to the starting material was calculated. The extracts were dissolved in minimum volume DMSO for *in vitro* studies and in propylene glycol for animal studies.

2.2.2. Phytochemical screening

The leaf extracts of *Uvaria narum*, as well as *Uvaria macropoda* in different solvent system as petroleum ether, chloroform, acetone and methanol dissolved in ethanol was used to test for the presence of various phytochemicals according to the standard procedure of (Sofowora 1993; Evans *et al.* 2002; Harborne 1998 and Harborne 1973). The aqueous extract was dissolved in the water itself and used for the studies. Phytochemicals such as tannins, terpenoids, flavonoids, steroids, coumarins, emodins, sterols, alkaloids and leucoanthocyanins are tested using standard methods and compared with ethanol as a control.

2.2.2.1. Test for tannins

- a) Lead acetate Method: 1% lead acetate (2 or 3 drops) added to 2 mL of the extract resulted in a yellowish precipitate indicates the presence of tannins.
- b) Ferric Chloride Test: The aqueous extract was heated on a water bath and filtered. A 5% ferric chloride (4 mL) was added to 2mL of above-heated extract. Dark green precipitate indicated the presence of tannins.
- c) Potassium hydroxide method: Mixed 4 mL of freshly prepared 10% potassium hydroxide solution with 4 mL of concentrated extract. Dirty white precipitate formation indicated the presence of tannins.

2.2.2.2. Test for terpenoids

- a) Salkowski test: Acetic anhydride and concentrated sulphuric acid were added to 2 mL of the extract in a test tube. Presence of terpenoids was indicated by the formation of a blue-green ring.

- b) Sulphuric acid test: To 1 mL of plant extract added about 5 drops of concentrated sulphuric acid. Yellow coloration at the lower layer suggests the presence of triterpenoids.

2.2.2.3. Test for steroids

- a) Liebermann Buchard test: 1 mL of acetic anhydride was added to 1 mL of chloroform and cooled to 0° C. Concentrated sulphuric acid was added to the ice-cold mixture followed by the extract (1 mL). The solution was observed for blue, green, red or orange color that changes with time.
- b) Salkowski test: Same test as the test for terpenoids. Acetic anhydride and concentrated sulphuric acid were added to 2 mL of the plant extract. Formation of red coloration confirms the presence of steroids.

2.2.2.4. Test for glycosides

Different extracts of *U.narum* and *U. macropoda* was hydrolyzed (1mL) with dilute hydrochloric acid for 1 hour on a water bath and the hydrolysate was subjected to the following test:

- a) Borntrager's test: chloroform was added to the hydrolysate, mixed thoroughly and the chloroform layer separated after settling. An equal volume of dilute ammonia was added to the chloroform fraction. Ammoniacal layer gets pink color indicating the presence of glycosides.

2.2.2.5. Test for phenols

- a) Ferric chloride test: To 1 mL of crude plant extract in water, an equal amount of ferric chloride was added. The deep bluish color indicated the presence of phenols.
- b) Ammonium hydroxide test: To 2 mL plant extract, added 1% gelatin and 10% sodium hydroxide. White precipitate confirms the presence of phenols.
- c) Lead acetate Test: To the extract, 10% lead acetate solution is added; formation of white precipitate indicated the presence of phenols.

2.2.2.6. Test for flavonoids

- a) Sodium hydroxide test: To 2 mL of plant extract added 10% aqueous sodium hydroxide solution and filtered to give a yellow color. The disappearance of yellow color on the addition of dilute HCl indicated the presence of flavonoids.
- b) Ferric chloride test: Extract was boiled with water and filtered. To 2 mL of the filtrate added two drops of freshly prepared ferric chloride solution. Green, blue or violet colorations indicate the presence of flavonoids.
- c) Sulphuric acid test: Concentrated sulphuric acid was added to different extracts and yellow color formation indicates the presence of flavonoids.

2.2.2.7. Test for saponins

About 0.2g of the plant extract was shaken with 5 mL of distilled water and then heated to boil. Frothing indicates the presence of saponins.

2.2.2.8. Test for alkaloids

About 0.5g of the extract was stirred with 5 mL of 1% aqueous hydrochloric acid on a water bath and filtered. The filtrate (3mL) was divided into three portions and used for following tests

- a) Dragendorff's test: To the first portion, 2 or 3 drops of freshly prepared Dragendorff's reagent (freshly prepared) was added and observed for the formation of orange to brownish precipitate.
- b) Mayer's test: To the second portion, added one drop of Mayer's reagent and observed for the formation of white to cream-colored precipitate.
- c) Wagner's test: To the third portion, one drop of Wagner's reagent (section 2.1.4) was added to form a brown or reddish brown precipitate.

2.2.2.9. Test for carbohydrates

- a) Molisch's test: To the extract 2-3 drops of 1% alcoholic α - naphthol is added and mix well. 1 mL of concentrated sulphuric acid was added along the sides of the test tube. The appearance of the reddish-violet ring at the junction of two solutions indicates the presence of carbohydrates.
- b) Benedict's test: To 1mL of extract, 3 mL of Benedict's reagent was added. The appearance of an orange-red precipitate indicates the presence of carbohydrates.

2.2.2.10. Test for proteins and free amino acids

- a) Ninhydrin test: 1 mL ninhydrin reagent (0.05% in acetone) was added to 1 mL of plant extract. The appearance of purple color indicates the presence of amino acids.
- b) Xanthoproteic test: Added 1 mL of concentrated nitric acid to 1 mL of extract and boiled for 1 minute and allowed it to cool to room temperature. Development of yellow color shows the presence of aromatic amino acids.
- c) Biuret test: Equal volume of 5% sodium hydroxide and 1% copper sulfate was added to the plant extract. The appearance of violet color indicates the presence of proteins.
- d) Warming test: About 2 mL of the extract was heated in a boiling water bath. Proteins get coagulated due to heating.

2.2.2.11. Test for anthraquinones

To about 2 mL of the extract, 10 mL of benzene was added, mixed well and filtered. 5 mL of 10% ammonia solution was added. Mixed well and formation of pink color in the ammoniacal (lower) layer indicates the presence of anthraquinones.

2.2.2.12. Test for coumarins

Mixed 3 mL of 10% sodium hydroxide and 2 mL of aqueous extract. Formation of yellow color indicates the presence of coumarins.

2.2.3. TLC profiling and visualization

One-dimensional TLC analysis was performed on 20 × 20 cm silica plates (Merck KGaA, Darmstadt, Germany) with different mobile phases for different extracts. N-hexane: diethyl ether: acetic acid (35:15:0.75 V/ V/ V) for PE, benzene: ethyl acetate (5:1 V/ V) for CHL, chloroform: methanol: water (16:5:0.8 V/ V/ V) for ACT and MET and, methanol: water: acetic acid (18:9:1 V/ V/ V) for aqueous extracts were the mobile phases used. The TLC separated compounds (bands) were visualized under visible light, UV (360 nm) and iodine vapor. The R_f values of individual bands in each chromatogram were calculated according to the formula,

$$\text{Rf value} = \frac{\text{Distance traveled by the sample}}{\text{Distance traveled by the solvent front}}$$

2.2.3.1. Identification of compounds using different spray reagents

a) Vanillin reagent (for terpenoids)

Vanillin (10% in water) was mixed with ethanoic acid- concentrated sulphuric acid in the ratio 2:1 and sprayed on to the plates. Keep in a hot air oven for 15 min. Presence of terpenoids was indicated by the appearance of brown, dark green or purple colored spots in TLC.

b) Liebermann buchard reagent (for terpenoids)

One milliliter of acetic anhydride was added to 1mL of chloroform and cooled to 0 ° C. Then one drop of concentrated sulphuric acid was added to the cooled mixture. The TLC plates were sprayed with LB reagent. The terpenoids were observed as blue, green, red or orange spots that change with time in the visible light. It was also observed in UV transilluminator (254 nm).

c) Anisaldehyde - sulphuric acid reagent (AS reagent - for triterpenoids)

Anisaldehyde reagent prepared by mixing 7.5 g anisaldehyde in 10 mL glacial acetic acid and diluted with methanol (85mL) and then concentrated sulphuric acid (5mL) was added to it and mixed well. The separated chromatogram was sprayed with AS

reagent, heated at 100 °C for 5-10 min until maximal visualization of the spots was obtained. Triterpenoids are detected as blue, red- violet, orange or red spots with AS reagent. It was also observed under UV light (254 nm) to detect fluorescent terpenoids.

d) 20% aqueous sulphuric acid reagent (for terpenoids)

Aqueous sulphuric acid (20%) sprayed plate was heated to 110° C until spots were visible. The terpenoids were visualized as brown, pink, purple or yellow color on the sulphuric acid spray.

e) Dragendorff's reagent (for alkaloids)

The stock reagent was made by mixing reagent 1 (0.85g of Bismuth nitrate in a solution of 10 mL acetic acid and 40 mL of water) and reagent 2 (8g of Potassium iodide in 20 mL of water) in 1: 1 ratio. The working spray reagent was prepared by mixing 1mL of stock solution with 2 mL of fresh acetic acid and 10 mL of water. Detection of alkaloids and other nitrogen compounds is by the appearance of orange-brown spots on a yellow background in TLC plate.

f) Ferric ferrocyanide reagent (for phenolic compounds)

Ferric chloride (0.1g) and Potassium ferrocyanide ($K_3F_3CN_6$) (0.1g) was prepared freshly by dissolving in 10 mL of distilled water. Equal portions of these two solutions were mixed, sprayed to plates and heated at 110° C. Rapid change of coloration to blue indicates the presence of phenolic compounds.

g) Methanol- potassium hydroxide reagent (for anthraquinones)

TLC plate was sprayed with potassium hydroxide in methanol (10%). Change of original yellowish-brown color to purple color shows a positive test for the presence of anthraquinones.

h) Ammonia reagent (for flavonoids)

TLC plates were exposed to ammonia. The presence of flavonoids was indicated by yellow, pink, grey or brown spots depending on different types.

2.2.4. HPTLC analysis

The plant extract and its fractions were dissolved in methanol (10 mg/mL) and subjected to High-performance thin layer chromatography (HPTLC). About 10 μ L of the plant samples were loaded on to a pre-coated TLC plates (20 \times 20 cm) silica gel 60 F₂₅₄ (Merck, India) using automatic spotting device (CAMAG). The solvent system used for the separation was toluene: ethyl acetate: formic acid (8:2:0.2 v/v/v). After running of the sample in the TLC, developed plates were scanned and visualized at 366 (visible light) and 254 nm (UV range) and derivatized plates with anisaldehyde–sulphuric acid reagent (sprayed and heated for 10 min at 115°C) (550 nm) using CAMAG TLC Scanner 3. The obtained data were analyzed using CAMAG WinCat software.

2.2.5. GC-MS analysis

The crude petroleum ether extract was subjected to GC-MS analysis, using a QP2010S Shimadzu system, Rxi-5Sil MS column with 30-meter length, 0.25 mm ID and 0.25 μ m thickness and an MS Shimadzu detector. A carrier gas, Helium (99.99%) was used at a constant flow of 3 mL/minute in a splitless mode at 173 Pascal inlet pressure. An aliquot of 1.0 μ L ethanol solution of the sample was injected to the MS column with an injection temperature of 260 °C; the GC column oven temperature was 80 °C, with an increment rate of 5 °C/min. The detector was set at a temperature of 280 °C. Ion source temperature was maintained at 200 °C. The mass spectrum of compounds in the PE extract was obtained by electron ionization at 70 eV. The detector was working in the scan mode range of 50-500 amu. The total running time was 45 min. The peaks were obtained using the software, GC-MS solutions and confirmed with libraries of National Institute Standard and Technology, NIST 11 and WILEY 8 mass spectral databases.

2.2.6. UPLC-Q-TOF-MS analysis

UPLC-Q-TOF-MS analysis was performed using a 1290 Infinity UHPLC System, 1260 infinity Nano HPLC with Chipcube, 6550 iFunnel Q-TOFs with an autosampler (Hi sampler with model G4226A), nano HPLC with Chipcube (Microfluidic column (50 mm \times 2.1 mm \times 1.7 μ m (Waters, USA) and UHPLC diode-array detector (Agilent

Technologies, USA). The mobile phase used was 0.1% aqueous formic acid, 5% aqueous acetonitrile using gradient elution (0-2 min, 95% acetonitrile; 3-24 min, 0.1% aqueous formic acid and 5% aqueous acetonitrile; 25-30 min 5% methanol) and was delivered at a flow rate of 0.3 mL/minutes and maximum pressure of 1200 bars. The detector scanning range was 210- 400 nm. A Xevo G2 (Waters, USA) Quadrupole - Time-of-Flight was used to obtain the data. The MS operated at a cone voltage of 30 V and a capillary voltage of 2.5 kV. The source temperature was 135 °C and desolvation temperature was at 350 °C, respectively. The gas flow of 50 and 900 L/hr was cone gas flow and desolvation gas flow, respectively.

2.2.7. Silica gel column chromatography

The crude extract of UNPE was subjected to column chromatography in a glass column of dimensions 300 x 10 mm. Silica gel (Merck India) of mesh size 60–120 was used as the stationary phase and the solvents used were hexane and methanol for separation of different phytochemical fractions. Approximately 100 mg of the PE extract was dissolved in a minimum volume of methanol and then mixed with 500 mg of silica and, kept at 40 °C for evaporation to dryness and made to a fine powder using mortar and pestle. Before loading the sample the column bed was washed many times with methanol. The dried powdered extract mixed with silica was then loaded over the top of the column bed and separation was carried out by using many bed volume methanol followed by hexane as mobile phase. The column eluate was collected as 100 mL fractions up to a total volume of 200 mL yielding methanol and hexane fractions. Each fraction was evaporated off to remove solvents, washed and dried and the residue used for further study.

2.2.8. Short term cytotoxicity studies *in vitro*

2.2.8.1. Maintenance of cell lines (DLA and EAC) cells

The DLA and EAC cell lines were maintained in the peritoneal cavity of the BALB/c mice at Amala cancer research Centre. The cells were aspirated from the peritoneal cavity and 1×10^6 cells were injected to the peritoneal cavity of another healthy mouse around 14 days interval.

2.2.8.2. Preparation of rat spleen cells

Male Sprague-Dawley rats weighing 250-300 g were anesthetized with chloroform; the spleen was removed surgically and collected in a sterile nylon sieve over a Petri dish containing PBS. The spleen was gently pressed through the sieve using a plunger and PBS was then added to keep the cells moist. The disaggregated splenocytes were transferred to a test tube and centrifuged at 2000 rpm for 5 minutes. Repeated the washing and centrifugation step until the cell suspension become debris free. The cell pellet was resuspended in PBS and counted using a hemocytometer to adjust the cell count.

2.2.8.3. Cytotoxicity analysis using Trypan blue dye exclusion method

This cytotoxicity assay using trypan blue dye based on the principle that intact plasma membranes in live cells exclude the dye, whereas dead cells got deeply stained as they do not exclude the dye. Cells (Daltons Lymphoma Ascites cells, Ehrlich's Ascites cells, and splenocytes) were collected from the peritoneal cavity of mice, washed thoroughly with PBS to remove debris and remove the supernatant after centrifugation at 1500 rpm for 3 min. Repeated the washing and centrifugation until all the debris gets removed. The cell pellet was suspended in 1 ml PBS and the cell number was adjusted to 1×10^6 cells/mL. Drug to be tested were added at various concentrations (10-100 $\mu\text{g/L}$) and incubated for 3hrs at 37 °C in an incubator. The tube with no extract added was used as a control. The viability of cells was checked using Trypan blue method (Talwar 1983). 100 μL of 1% trypan blue dye was added and allowed to stand for 2 min. The live and dead cells were counted separately and determine the percentage cell by the formula.

$$\% \text{ cell death} = \frac{\text{Number of dead cells}}{\text{Total number of cells (Live + dead)}}$$

2.2.9. Antiproliferative study

2.2.9.1. Maintenance of cell lines

HeLa (Human cervical carcinoma), HepG2 (human hepatocarcinoma), HCT-15 (human colorectal adenocarcinoma) and Vero E6 (African monkey kidney) cell lines were obtained from the National Centre for Cell Science (NCCS), Pune, India. Each

cell line was maintained in its appropriate medium supplemented with 10% heat-inactivated FBS and antibiotics, and incubated in 25 ml culture flasks at 37 °C under a 5% CO₂ atmosphere. Sterile conditions were maintained throughout the experiment. The culture flasks with the cells were examined regularly for a color change of medium or contamination with changing the old medium with a fresh one. At 70-80% confluence, cells were subcultured. After removing the used medium, the cells were washed thrice with PBS. Trypsinisation was done by 1 mL trypsin (0.25% trypsin in 0.001% EDTA) solution at 37 °C for cell detachment. A volume of 1 mL of fresh medium was added to inactivate the trypsin and mixed gently using a sterile pipette to make single cell suspension and equally divided into 5 different flasks with an adequate amount of media. The cells were monitored under an inverted microscope.

2.2.9.2. Long-term *in vitro* cytotoxicity study using MTT assay

Antiproliferative activity of different extracts of *U.narum* and *U.macropoda* leaves (dissolved in DMSO) were evaluated in HeLa, HepG2, HCT-15, and Vero cell lines, using the MTT assay (Mosmann 1983). It determined the number of viable cells. This assay measures colorimetrically the reduction of 3-(4, 5-dimethylthiazol2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial enzyme succinate dehydrogenase. The tetrazolium dye enters the cells and passes into the mitochondria where it is reduced to insoluble deep purple formazan crystals in live cells which then solubilized with an organic solvent such as isopropanol, can be measured spectrophotometrically. Since MTT reduction can only take place in metabolically active cells the test is used as a measure of the viability of the cells. Cells of concentration 1×10^5 cells/mL were seeded in 12 well plates and incubated at 37 °C under a 5% CO₂ and 95% humid atmosphere. After reaching 80% confluence, the medium was replaced with fresh medium containing different concentrations (5-100 µg/mL) of individual extracts. Cells were then allowed to grow for 48 hr following which the spent medium was replaced with fresh medium containing 100 µL/mL of MTT (5 mg/mL in PBS). Following a further incubation of the plate at 37 °C for 4hr, the formazan crystals were dissolved in 1 mL of the solubilization solution (50: 0.43: 5 mL (V/V/V) of isopropanol, concentrated HCl, and Triton X-100, respectively). The absorbance of samples was read against blank at 570 nm after 15 min incubation in

37° C in UV/Vis. Spectrophotometer. All experiments were performed at least three times, and the average of the percentage absorbance was plotted against concentration. The concentration of the extract required to inhibit 50% of the growth of cell (IC₅₀) was then calculated (Van *et al.* 2011; Alley *et al.* 1988).

$$\% \text{ cell viability} = (\text{Absorbance of treated}/\text{Absorbance of control}) \times 100$$

2.2.10. *In vitro* antioxidant study

2.2.10.1. DPPH radical scavenging activity

The assay was done by the method of (Picerno *et al.* 2011)

Principle

Determination of antioxidant activity was based on scavenging of the stable 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical. DPPH free radical (Purple color) shows an absorption maximum at 517 nm. The discoloration of purple to white is due to the formation of the reduced DPPH from the pairing of odd electron of DPPH radical with hydrogen from a free radical scavenging antioxidant molecule.

Procedure

About 1.5 mL of freshly prepared DPPH (0.025 g/L in methanol) was incubated in the presence of extracts at different concentrations (20-100 µg/mL) for 20 min at room temperature in dark and the absorbance was read against methanol blank at 517 nm in UV/ Visible spectrophotometer. Vitamin C was used as a positive control. The percentage inhibition of free radicals are calculated using the formula

$$\text{DPPH scavenging activity} = (\text{Absorbance of control} - \text{Absorbance of the test}) / \text{Absorbance of control} \times 100$$

2.2.10.2. Ferric reducing ability (FRAP) assay

The antioxidant capacity of different extracts of *U.narum* and *U. macropoda* were determined according to the procedure of (Pulido 2000) with some modifications.

Principle

The method determines the ferric reducing ability (FRAP). At low pH, a ferric tripyridyl triazine (Fe III -TPTZ) complex is reduced to ferrous (Fe II) form resulted in an intense blue color solution with an absorption maximum at 595 nm.

Procedure

900 μ L of freshly prepared FRAP reagent (2.5 mL of 10 mmol/L TPTZ (2,4,6-tripyridyl-s-triazine) in 40 mmol/L HCl, 2.5 mL 20 mmol/L FeCl₃.6H₂O solution and 25 mL 300 mmol/L acetate buffer, pH 3.6) was mixed with different concentration of extracts (1-30 μ g/mL) and made the volume up to 1.0 mL using distilled water. The reaction system was incubated at 37 °C for 20 minutes. Readings were taken against distilled water blank at the absorption maximum of 595 nm in a spectrophotometer. The percentage increase in the ferric reducing activity was calculated compared with control.

2.2.10.3. Inhibition of lipid peroxidation

Inhibition of lipid peroxidation *in vitro* was done by a colorimetric method (Biswas *et al.* 2010).

Principle

Wistar rat was sacrificed by cervical dislocation procedure. The liver tissue was removed and homogenized with 40 mM Tris-HCl buffer (pH 7.0) and centrifuged at 2000 \times g for 15 minutes. Cleared liver homogenate (0.5 mL) was added to 100 μ L of each of 0.15 M KCl, 15 mM FeSO₄, 6 mM ascorbic acid, and *U.narum* and *U. macropoda* plant extract at different concentrations (20-100 μ g /mL) and kept for incubation at 37 °C for 1 hr. Ice cold TCA was added to the reaction mixture and mixed thoroughly. The sample was centrifuged at 2000 \times g for 20 minutes to remove the protein which is precipitated. To 2.0 mL of the collected supernatant added 1.0 mL TBA (0.8%) followed by heating at 90 °C for 20 minutes in a water bath. After cooling, absorbance was measured at 532 nm in a spectrophotometer. Percentage inhibition was calculated from the optical density values of control and treated tubes. Ascorbic acid was used as a positive control for the reaction.

2.2.10.4. Lipoxygenase inhibition

This assay is based on the activity of 15-lipoxygenase on oxygenation of linoleic acid. The inhibition of the lipoxygenase enzyme was determined by the method of (Dolev *et al.* 1967).

Procedure

The reaction mixture with lipoxygenase (50U), linoleic acid (100 nmoL) and different concentrations of extract (20-100 $\mu\text{g}/\text{mL}$) in a total volume of 1.0 mL phosphate-buffered saline, pH 7.4. The reaction mixture was mixed and incubated at room temperature for 1 hr. The conjugated diene formed was measured spectrophotometrically at 234 nm. Reactions were carried out in three consecutive experiments. Vitamin C was used as a positive control.

2.2.10.5. Estimation of total phenolic content

The total phenolic contents of different extracts of *U.narum* and *U. macropoda* were analyzed spectrometrically by Folin-Ciocalteu method with some modification (Ainsworth and Gillespie 2007).

Principle

A phenolic compound donates electrons in an alkaline medium to form a blue chromophore constituted by a phosphotungstic/ phosphomolybdenum complex in Folin-Ciocalteu reagent. The reduced product is measured with a spectrophotometer at 765 nm.

Procedure

In the reaction system, 50 mg of extract in methanol and gallic acid standard solutions (1-10nmo/mL) was mixed with 2.0 mL of 10% Folin-Ciocalteu reagent. The reaction mixture was thoroughly mixed and incubated in dark for 6 minutes. After the incubation period the reaction neutralizes with 4 mL 7.5 % (w/v) sodium carbonate solution. After being incubated further for 30 min at room temperature, the absorbance was measured at 765 nm using a spectrophotometer. Results were expressed as mg gallic acid equivalents (GAE) per gram of plant extract.

2.2.10.6. Estimation of total flavonoid content

The aluminum chloride colorimetric assay is used for the determination of total flavonoid content (Chia-Chi Chang 2002).

Principle

AlCl₃ forms acid stable complexes with the C-4 keto groups and either the C-3 or C-5 hydroxyl group of flavonoid compounds. The colored product formed was measured at 415 nm with a spectrophotometer.

Procedure

The reaction mixture contains different extracts of *U. narum* and *U. macropoda* leaves in 0.5 mL methanol (0.5 mg of each PE, chloroform, acetone, methanol, and water), 1.5 mL of methanol, 100 µL of 10% aluminum chloride, 100µL of 1M potassium acetate and 2.8 mL of distilled water. The total reaction system was incubated at room temperature for 30 minutes. Absorbance maximum was measured at 415 nm with a spectrophotometer. The standard curve for total flavonoids was prepared by taking quercetin as standard (50–250 mg/L). The total flavonoid content was expressed as milligram of quercetin equivalents (QTE) per gram of plant extract.

2.2.11. *In vivo* antioxidant activity

2.2.11.1. Protective effect of the UNMET extract in sodium fluoride-induced oxidative damage

Experimental design

Thirty-six Swiss albino female mice were divided into six groups of six animals each. Group I kept as normal without any treatment, Group II animals were given NaF (600 ppm) alone in drinking water was the control, Group III animals received 1% propylene glycol+ NaF (600 ppm) in drinking water was vehicle control, Group IV animals given NaF (600 ppm), were treated with standard ascorbic acid (10 mg/kg b.wt., p.o.) daily. Group V: UNMET extract at low dose (200 mg/kg b.wt. p.o.) and group VI animal UNMET extract high dose (400 mg/kg b.wt., p.o.) along with NaF (600 ppm). The animals in group II, III, IV, and V were pretreated for seven days with

respective doses of drugs. All the animals except in group I received NaF (600 mg/L) through drinking water from 8th day onwards. All animals were sacrificed on the 15th day following overnight fasting. Blood was collected through heart puncture in EDTA coated tubes. The blood was used for estimation of hemoglobin, glutathione, and activities of superoxide dismutase (SOD) and catalase. The liver was dissected out, weighed and washed in saline. Liver homogenates (25% in 0.1M Tris buffer, pH 7) of the samples were prepared by homogenization which was used for the estimation of lipid peroxidation (LPO) and total protein. This homogenate further centrifuged at 10,000 rpm for 30 min at 4 °C. The glutathione levels and activity of SOD and catalase were also estimated.

2.2.11.1.1. Preparation of hemolysate

Blood was collected through heart puncture and kept in EDTA coated tubes. The hemolysate was prepared by centrifugation at 1000 x g for 15 min. The upper layer was removed and the packed RBC were collected and washed thoroughly with phosphate buffer saline (0.01 M, pH 7.4). The hemoglobin concentration of the packed RBC was determined by Drabkin method. A known volume of red blood cells was lysed with hypotonic phosphate buffer. Centrifuged the reaction mixtures at 3000 × g for 15 min in a cooling centrifuge for removing the red blood cell debris and collected hemolysate were immediately used for biochemical analysis. It was used for estimation of SOD, Catalase and GSH.

2.2.11.1.2. Preparation of tissue homogenate

The liver tissue was collected after sacrificing animals and rinsed thoroughly in ice-cold saline (0.9% NaCl) and weighed in an analytical balance. The tissue was homogenized (in 25% in 0.1M Tris buffer, pH 7) using a Polytron homogenizer. One part of the homogenate was directly used for the determination of total protein and tissue TBARS. The other part of homogenate was centrifuged for 60 min at 10,000 rpm in a cooling centrifuge for removing cell debris, nuclei, and mitochondria. This supernatant collected was used for estimation of total protein, superoxide dismutase (SOD), catalase, glutathione (GSH) and total protein.

2.2.11.1.3. Estimation of glutathione (GSH) content in blood and tissue

Determination of GSH content in blood and tissue was done by the method of (Moron *et al.* 1979).

Principle

GSH is measured by its reaction with DTNB, which gives a yellow colored compound and determine colorimetrically with maximum absorption at 412 nm.

Procedure

A volume of 0.1 mL of the sample, (hemolysate and tissue homogenate) was mixed with 125 μ L of TCA (25%) and cooled on ice for 5 min. Then added 0.6 mL of 5% TCA and centrifuged at 2000 rpm for 10 min. 300 μ L of the supernatant collected was made up to 1mL with 0.2 M sodium phosphate buffer (pH 8.0) and 2.0 ml of freshly prepared 0.6 mM DTNB. The OD was read at 412 nm. The GSH was calculated by plotting the standard graph. The results are expressed as nmol/g Hb or nmol/ mg tissue protein.

2.2.11.1.4. Estimation of superoxide dismutase (SOD) activity in blood and tissue

SOD enzyme activity was determined according to the method of (McCord and Fridovich 1969)

Principle

Superoxide generated by the photochemical reduction of riboflavin in the reaction system reduces nitroblue tetrazolium to blue colored complex. The effect of SOD present in the tissue /serum removes superoxide radical thus inhibiting the formation of reduced NBT. This forms the basis of the assay.

Procedure:

Reaction system consisted of 100 μ L of the sample (hemolysate or tissue sample), 100 μ L of 1.5 mM NBT, 200 μ L of 0.0015 KCN in 0.1 M EDTA, 50 μ L of 0.12 mM riboflavin and volume 2.550 mL 67 mM K-Na phosphate buffer (pH 7.8). The reaction mixture devoid of test sample served as control. The initial absorbance of the reaction mixture was read at 560 nm and recorded as initial reading. After 15 min

illumination under an incandescent lamp, the absorbance of the solutions was taken as the final reading. Percentage inhibition can be calculated as the percentage change of A_T (absorbance of the test) with that of control (A_C). The activity can be obtained by the formula,

$$\text{SOD activity in blood (U/g Hb)} = \% \text{ inhibition} \times 1750 / 100 \times 50 \times \text{Hb}$$

$$\text{SOD activity in tissue (U/mg protein)} = \% \text{ inhibition} / 50 \times \text{mg protein (in } 100 \mu\text{L)}$$

2.2.11.1.5. Determination of catalase (CAT) activity in the blood and tissue

Catalase activity in the blood was determined by the method of (Aebi *et al.* 1965) and (Beers and Sizer 1952), respectively.

Principle

The breakdown of hydrogen peroxide by catalase can be observed as the decrease in absorbance in the ultraviolet range, 240 nm spectroscopically.

Procedure

The determination of tissue catalase: The absorbance of dilute peroxide solution (34 μL 30mM peroxide/ 10 mL phosphate buffer, pH 7) against buffer blank was taken. It is considered as the initial OD. The decrease in light absorption of peroxide solutions was measured at 25 °C in the 15-sec interval for 1 min after the addition of 0.1 mL of the clarified tissue sample. The difference in absorbance was termed as ΔA (change in OD per minute). The activity of catalase in tissue can be calculated as

$$\text{Catalase activity (U/mg protein)} = \Delta A / \text{min} \times 1000 \times 3 / 43.6 \times \text{mg protein}$$

Catalase in hemolysate: About 10 μL of hemolysate was diluted to 5 mL with phosphate buffer, pH 7. To 2mL of the above solution, was added 1 mL phosphate buffer as a reference tube and instead of the buffer, 1 mL H_2O_2 (30mM) is added in the test solution in a quartz cuvette. The decrease in light absorption of peroxide solutions was measured at 25 °C in the 15-sec interval for 1 min after the addition of 0.1 mL of hemolysate.

The difference in absorbance is termed as ΔA . The activity of catalase in hemolysate was calculated as

$$\text{Catalase activity (U/g Hb)} = \Delta A/\text{min} \times 1000 \times 3/43.6 \times \text{g Hb}$$

2.2.11.1.6. Estimation of lipid peroxidation in tissue

The extent of lipid peroxidation in tissue was determined as thiobarbituric acid reacting substances formation by the method of Ohkawa *et al.* 1979.

Principle

Lipid carbonyls produced during peroxidation of polyunsaturated fatty acids (PUFAs) react with thiobarbituric acid (TBA) reagent under acidic conditions in the reaction mixture to form a pink colored product with an absorption maximum at 532 nm.

Procedure

Reaction mixture contain 400 μL of tissue homogenate, 0.2 mL of 8% SDS, 1.5 mL of 20% acetic acid (pH 3.5) and 1.5 mL of 0.8% TBA and 400 μL distilled water. After mixing well, the reaction mixture was incubated at 95 °C for 1hr in a boiling water bath. The tubes were then cooled and 1 mL of distilled water was added. Mixed well and centrifuged at 2000 rpm for 5 min. The absorbance of the supernatant was recorded at 532 nm against the blank. The concentration of MDA was calculated from the standard graph plotted at concentrations ranging from 1-10 nmol and expressed as nmol of MDA equivalent/mg protein.

2.2.11.1.7. Estimation of total protein in blood and tissue

A total protein present in the blood and tissue was determined by the colorimetric method (Lowry *et al.* 1951).

Principle

The amino acids such as tyrosine and tryptophan present in proteins reduce the phosphomolybdate and phosphotungstate components of Folin-Ciocalteu reagent in an alkaline medium and produce a bluish purple color with an absorbance maximum at 660 nm.

Procedure

Tissue homogenate (25%) and blood (10 μ L) was diluted to 990 μ L of distilled water and added 5 mL of alkaline copper sulfate (50 mL of 2% sodium carbonate (Na_2CO_3) in 0.1N NaOH and 1 mL of 0.5% CuSO_4 in 1% sodium potassium tartarate). Mixed well and incubated at room temperature for 10 min. Folin-Ciocalteu reagent (0.5 mL) in water (1:1) was added. Mixed well and incubated at room temperature for 30 min. The optical density was then measured at 660 nm. Protein content was calculated from the standard graph prepared using bovine serum albumin (BSA, 10-100 μ g/mL).

2.2.11.2. Effect UMET extract on PMA-induced superoxide radical generation in peritoneal macrophages in mice

Twenty four swiss albino female mice were divided into 4 groups of 6 animals each. Group I was control. Group II: vehicle control, for group III and IV, UNLD (200 mg/kg b.wt.), and UNHD (400 mg/kg b.wt.), respectively were administered. On the fifth day, after 1hr of drug administration, sodium caseinate was injected to elicit peritoneal macrophages and activated *in vivo* by injecting PMA (100 mg/animal, i.p.). Peritoneal fluid was collected after 3hr of PMA injection and macrophages were collected by centrifugation (RPM). The cell count was adjusted to 1×10^6 cells/mL by suspending in PBS. The inhibitions of superoxide generation in macrophages were determined using the cell suspension by NBT reduction method. Cells (500 μ L) were mixed with 300 μ L NBT (0.2%), 100 μ L dextrose (5%) and HBSS (50 μ L) and incubated for 45 min in room temperature. After incubation centrifuged at 1500 rpm for 10 min and cell pellet were incubated in a boiling water bath with 2 mL for pyridine for 10 min. The supernatant was collected and optical density was measured at 515nm. The absorbance values of treated animals were compared with untreated control to find out the percentage inhibition.

2.2.12. Toxicity study *in vivo*

2.2.12. 1. Acute toxicity studies

Acute toxicity was determined as per the Organization for Economic Co-operation and Development (OECD) guideline 423 (OECD 2001), where a single high dose of 2,000, 1000, 500, 250, 100 mg/kg b.wt. were administered orally after overnight fasting. Animals in the vehicle control group (three male and three female mice) received 1% propylene glycol orally. Behavioral changes and indications such as aggressiveness or weakness, change in water and food consumption, discharges from eyes and ear, diarrhea, noisy breathing, and mortality were monitored for the first six hours after the treatment period and then daily for a period of 14 days. The changes in body weight of the surviving animals were recorded once a week.

2.2.12.2. Repeated dose sub-acute toxicity study

Swiss albino mice (25-30gm) were divided into 8 groups (5 males + 5 females animals per each dose) categorized as an untreated group; vehicle control (1% propylene glycol) treated group; 200 mg/kg b.wt. UNMET treated (LD); 300 mg/kg b.wt. UNMET (MD); 400 mg/kg b.wt. UNMET (HD), 5 mg/kg b.wt. PE extract of *U.narum* leaves treated (UNPELD); 7.5 mg/kg b.wt. UNPE extract (MD) and 10 mg/kg b.wt. UNPE extract (HD). The dosages of the extracts were determined based on acute toxicity studies. Extracts were administered daily at a specific time orally for 28 days. The animals were observed for toxic manifestation, clinical and behavioral symptoms like diarrhea, neuromuscular problems, mortality and behavioral symptoms. The changes in body weight, food consumption, and water consumption were recorded for each group every three days during the course of the experiment. At the end of the 4th week, all the animals were sacrificed following overnight fasting by ether anesthesia. Collection of blood was done by direct heart puncture. Blood collected in EDTA coated tubes were analyzed for hematological parameters such as hemoglobin content, total WBC count, total RBC count, platelets and differential count. Blood collected in test tubes without EDTA was kept for clotting, centrifuged at 3000 rpm for 10 minutes and serum was separated. This collected serum was used for determining liver function tests such as serum aspartate transaminase (SGOT/AST), Serum glutamate pyruvate transaminase (SGPT/ALT), alkaline phosphatase

(ALP) and total bilirubin, lipid profiling such as total cholesterol, triglycerides, HDL, LDL and VLDL levels and kidney function tests such as creatinine and urea using commercial available diagnostic reagent kits. Electrolytes such as sodium, potassium, chloride and bicarbonate ions were also determined. The brain, liver, stomach, kidney, intestine, heart, lungs, spleen and ovary/testes were excised and weighed and relative organ weight was calculated with respect to the final day body weight and organ somatic index calculated. Portions of these individual tissues were removed, fixed in 10% neutral buffered formalin, trimmed and tissue sections of 4 μ thickness were taken onto glass slides. The sections were then rehydrated and stained with hematoxylin and eosin. Following dehydration in graded alcohol histological details were observed under a light microscope (200 x) and photographed.

2.2.12.2.1. Determination of hematological parameters

2.2.12.2.1.1. Estimation of hemoglobin (Hb)

The hemoglobin content in the blood was determined by the method of (Drabkin and Austin 1935).

Principle

Hemoglobin in the blood reacts with potassium ferricyanide and potassium cyanide in the cyanmeth reagent and form cyanmethemoglobin. It is a stable colored product which has an absorbance maximum at 546 nm. The absorbance of cyanmethemoglobin is directly proportional to the hemoglobin present in the blood.

Procedure

About 20 μ L of fresh heparinized blood was mixed with 5 mL of the cyanmeth reagent and incubated for 5 min at room temperature. The absorbance of the standard solution (provided in the reagent kit) equivalent to 60 mg/dL hemoglobin was also estimated along with the sample.

The hemoglobin content in blood was calculated using the formula:-

$$\text{Hemoglobin (g /dL)} = (\text{Absorbance of the sample/Absorbance of Standard}) \times 60 \times 0.251$$

2.2.12.2.1.2. Determination of red blood cells (RBC)

The red blood cells are counted using the method of (Cheesbrough and McArthur 1978).

Principle

The blood sample is diluted (200 times) with red cell diluting fluid (Hayem's). This does not remove the white cells but allows the rouleaux formation and helps to count red cells to be counted under 400X magnification.

Procedure

Draw blood upto mark 0.5 and diluting fluid upto mark 101 in the RBC pipette. Mix well by rotating the pipette for 2-3 minutes between palms. Charge the solution to Neubauer counting chamber without air bubbles and allow the cells to settle down for 3 minutes. Count the cells under high power (400X) in 80 tiny squares in the center part of the chamber. The RBC count was determined by the formula

$$\text{RBC count (cells/mm}^3\text{)} = \text{Number of cells counted} \times \text{dilution factor} \times \text{depth factor} / \text{Area counted}$$

$$\text{RBC count (cells/mm}^3\text{)} = \text{Number of cells} \times 10000$$

2.2.12.2.1.3. Determination of Total leucocytes count (TC)

The total leucocyte count was estimated by the method of Cheesbrough and McArthur 1978.

Principle

When blood diluted with acetic acid (in the Turk's fluid) resulted in lysis of red blood cells leaving all the nucleated white blood cells to remain intact and stained with crystal violet stain in the fluid.

Procedure

The blood was drawn upto the 0.5 marks in WBC pipette and diluted with Turk's fluid upto the mark 11. Mixed well by keeping the pipette in a horizontal position between palms. Allowed the cells to settle by keeping it for 3 minutes. The diluted blood was then dispensed into a Neubauer counting chamber and kept to settle. Then the number of white blood cells in the corner squares was counted by using the low power objective. The total WBC count was determined by the formula

$$\text{Total WBC (cells/mm}^3\text{)} = \text{Number of cells counted} \times 50$$

2.2.12.2.1.4. Differential leucocyte count (DC)

Differential leucocyte count is done for the determination of the relative number of different type of leucocytes present in the blood by the method of (Wintrobe and Greer 2009).

Principle

A stained blood smear with Leishman stain was microscopically examined in order to determine the percentage of each type of leukocyte present and assessed the erythrocyte and platelet morphology. Under Leishman staining, neutrophil was observed as with purple colored nuclei with pink cytoplasm, Eosinophil with a purple nucleus, faint pink cytoplasm and orange-red granules, Basophils with a purple nucleus and dark blue granules and monocyte as the purple color nucleus and pink cytoplasm. Lymphocytes are with dark blue nucleus with light blue cytoplasm. Platelets are observed as violet colored granules.

Procedure

The blood smear was prepared with a drop of blood using a slide and a spreader. Allowed to dry. The staining was done with Leishman stain (0.15g/100 mL methanol). Covered the slide with stain for 2 to 3 min. Then added an equal amount of distilled water diluted stain and keep it for 2 to 3 min. After washing and air dry, observed under the microscope in oil immersion objective. Examined the slide and moved the slide in a zigzag manner to count different leucocytes. The percentages of different leucocytes were calculated by counting up to 100 cells.

2.2.12.2.1.5. Determination of Platelet count (PLT)

The platelets are counted using the method of (Cheesbrough and McArthur 1978).

Procedure

The procedure of platelet counting was the same as that of RBC count. Venous blood is used for platelets counting. Using an RBC pipette, prepared a 1:200 dilution as for RBC counting with dilution fluid (1% ammonium oxalate). Mixed gently for 2 minutes, charged the Neubauer's counting chamber and observed under microscope 40X objective. The platelets count is calculated by the formula

$$\text{Number of platelets per mm}^3 = n \times 10,000, \text{ where } n \text{ is the total number of cell counted}$$

2.2.12.2.1.6. Determination of packed cell volume (PCV)

The PCV was determined by the method of (Wintrobe and Greer 2009)

Principle

When anticoagulated blood in Wintrobe tube was centrifuged at a standard speed, erythrocytes sediments at the bottom of the tube. This sedimented red cell column is known as hematocrit or packed red cell volume and expressed as a percentage fraction of the whole blood.

Procedure

The anticoagulated blood after mixing thoroughly was filled in the Wintrobe Microhaematocrit tube using a Pasteur pipette. The tubes were centrifuged at 2500 g for 30 min. Mix blood sample thoroughly. The length of the red cell column was noted and the percentage of the red column to the total volume of the blood sample was calculated.

2.2.12.2.1.7. Determination of Erythrocyte Sedimentation Rate (ESR)

ESR was determined using the Westergren tube method (Westergren 1957).

Principle

Anticoagulated blood in Westergren's tube (to the 200 mm) left undisturbed in a vertical position. The level of the column of red blood cells was observed at the beginning (0 hr) and after 1 hr of the experiment. The distance in mm the column traveled was the ESR, expressed as mm/hr.

Westergren's method

Withdrawn 2 mL of venous blood in a dry sterile syringe and placed exactly 1.6 mL of blood in the tube containing 0.4 mL 3.8% sodium citrate solution. Mix thoroughly. Fill the Westergren's E.S.R. tube upto 0 marks and place it in the stand. The tube must be kept exactly in a vertical position. Reading may be made at the end of half-an-hour and another at the end of one hour and two hours. The ESR, distance of drop-in erythrocytes was expressed as millimeters per 1 hour.

2.2.12.2.2. Estimation of serum markers

2.2.12.2.2.1. Estimation of serum electrolytes

2.2.12.2.2.1.1. Determination of sodium and potassium in the blood

Principle

Flame photometry method is the most commonly used for determination of sodium and potassium. If an alkali metal in solution is aspirated into a low-temperature flame

in an aerosol form, it will alter excitation by the flame; emit a discrete frequency of light which is selected by an optical filter and reached in the photodetector in the flame photometer. The light emission is proportional to the concentration of the alkali in the sample (Deal 1954).

Procedure

The stock solutions of sodium and potassium standards were prepared. Sodium(1000 mEq/L) and potassium stock standard (100 mEq/L) solutions were prepared and from this, the sodium-potassium mixed working standards were prepared as of 120 /2 mEq/L (S1), 140 / 4mEq/L (S2), 160/6 mEq/L(S3). Four reaction systems – test (serum), standard1, standard2 and standard 3 were maintained. For each tube pipetted out 10 ml of distilled water. A volume of 100 μ L of serum and 100 μ L of serum and working standards S1, S2, S3 solutions were added to respective tubes. The solutions were mixed thoroughly and transferred to bulbs for flame photometric determination of samples. Flame emission intensities were determined and with a calibration curve prepared, the concentration of the sample was determined.

2.2.12.2.2.1.2. Determination of chloride in the blood

Chloride estimation was done by the modified method of (Schoenfeld and Lewellen 1964; Levinson 1976) using electrolyte diagnostic reagent kit and Photometer system.

Principle

Chloride reacts with a solution of mercuric thiocyanate and ferric nitrate to form red-brown ferric thiocyanate and stable mercuric chloride. The intensity of the red-brown color developed is proportional to the concentration of chloride present in the serum.

Procedure

Four test tubes namely blank, control, standard, and test, set and added 2 mL of chloride reagent. Then, to the blank 10 μ L of deionized water was added and to standard, 10 μ L of standard chloride reagent (100 mmol/L) was added. About 10 μ L plasma from control and experimental groups was added to control and test tubes, respectively. The contents of the tubes were mixed thoroughly and incubated at room

temperature for 2 min. The absorbance of standard, control, and test was measured at 505 nm against blank using Photometer 4010 System. The concentration of the chloride is calculated by the formula

Chloride in mmol/L = Absorbance of sample/ Absorbance of standard x100

2.2.12.2.2. Estimation of serum glutamate oxaloacetate transaminase (SGOT) activity

The SGOT/ AST activity was estimated by the IFCC kinetic method (Thefeld *et al.* 1974; Bergmeyer *et al.* 1976).

Principle

Serum glutamate oxaloacetate transaminase catalyzes the transamination reaction of L-aspartate and α -ketoglutarate to form oxaloacetate and L-glutamate. The oxaloacetates formed then react with NADH^+H^+ to form NAD^+ and L-Malate in the presence of malate dehydrogenase enzyme. The decrease in absorbance can be spectrophotometrically determined at 304 nm.

Procedure

Reagents kits were obtained from Agappe Diagnostics Ltd. Reagent 1 (Single step enzymatic reagent) consists of L-Aspartate (260 mmol/L), LDH (>1500 U/L), MDH (>900 U/L) and Tris buffer pH 7.8 (88 mmol/L) and reagent 2 consists of α -ketoglutarate (12 mmol/L) and NADH (0.24 mmol/L). The working reagent was prepared by mixing 4 volumes of reagent 1 with 1 volume of reagent 2. It was stable for 30days at 2-8 °C. The assay was done by adding 1000 μL working reagent and 100 μL serum samples, mixed well and incubated for 1 min at 37 °C. Change in absorbance can be measured per min interval for 3 min. The activity of the enzyme was calculated by the formula

$$\text{SGOT activity (U/L)} = (\Delta_{\text{OD}}/\text{min}) \times 1745$$

2.2.12.2.2.3. Estimation of serum glutamate pyruvate transaminase (SGPT) activity

The SGPT/ ALT activity was estimated by a colorimetric method (Thefeld *et al.* 1974; *Clin.Chem.Acta.* 1980).

Principle

Serum glutamate pyruvate transaminase /Alanine aminotransferase catalyzes the transamination of L-alanine and α -Ketoglutarate to form pyruvate and L-glutamate. In the assay system, the pyruvate formed react with NADH^+H^+ to form NAD^+ and lactate in the presence of Lactate dehydrogenase enzyme. The decrease in absorbance can be spectrophotometrically determined at 304 nm.

Procedure

It is a high linearity kinetic procedure using kits obtained from Agappe Diagnostics Ltd. Reagent 1 (Single step enzymatic reagent) consists of L-Alanine (600 mmol/L), LDH (>1500 U/L) and Tris buffer (110 mmol/L) and reagent 2 consists of α -ketoglutarate (16 mmol/L) and NADH (0.24 mmol/L). The working reagent was prepared by mixing 4 volumes of reagent 1 with 1 volume of reagent 2. It was stable for 30 days at 2-8 °C. About 1000 μL working reagent and 100 μL serum samples mixed well and incubated for 1 min at 37 °C. Change in absorbance measured per min interval for 3 min. The activity of the enzyme was calculated by the formula.

$$\text{SGPT activity (U/L)} = (\Delta_{\text{OD}} / \text{min}) \times 1745$$

2.2.12.2.2.4. Estimation of alkaline phosphatase (ALP) activity

The ALP activity was estimated by the method of kinetic reaction (Schlebusch *et al.* 1974; *Biochem* 1972).

Principle

Alkaline phosphatase (ALP) in serum converts para-nitrophenyl phosphate to inorganic phosphate and p-nitrophenol at pH 10.2. The change in absorbance of the resulting solution was measured at 405 nm.

Procedure:

The assay was carried out by reagent kits obtained from Agappe Diagnostics Ltd. About 20 μL serum was added to 1 mL of working reagent. Working reagent was prepared by mixing four volumes of Reagent-1 (Diethanolamine buffer (pH -10.2) - 125 mmol/L and magnesium chloride - 0.625 mmol/L) with one volume of Reagent-2 (P-nitrophenyl phosphate - 50 mmol/L). The tubes were mixed thoroughly incubated for 1 minute at 37 °C. The change in absorbance per minute for 3 minutes was measured against blank at 405 nm. The ALP activity was calculated by the formula

$$\text{ALP activity (U/L)} = (\Delta_{\text{OD}} / \text{min}) \times 2750$$

2.2.12.2.2.5. Estimation of total bilirubin

The Bilirubin levels in blood were estimated by Walters and Gerarde method (Walters and Gerarde 1970).

Principle

The sulfanilic acid reacts with sodium nitrite to form diazotized sulfanilic acid. Bilirubin reacts with the diazotized sulfanilic acid in the presence of TAB form azobilirubin with red color which is measured at 546 nm.

Procedure

A volume of 50 μL serum sample was added into 20 μL activator total and bilirubin reagent (Sulfanilic acid- 28.9 mmol/L, TAB- 9 mmol/L). A reagent blank was kept the same as above without serum. Mixed well and incubated for 5 min at room temperature. The absorbance was measured at 546 nm and total bilirubin was calculated by using the formula:

$$\text{Total bilirubin} = \text{OD of test} - \text{OD of reagent blank} \times 29.00$$

2.2.12.2.2.6. Estimation of creatinine

Serum creatinine was determined by Jaffe's kinetic method (Bonsnes and Taussky 1945).

Principle

The picric acid in reagent converts to sodium picrate in an alkaline condition which in turn reacts with creatinine to form an orange colored complex which is measured at a wavelength of 520 nm.

Procedure:

The working reagent was prepared by mixing equal volumes of picric acid reagent and alkaline buffer. Two reaction systems namely standard and test were maintained. About 50 μL of test serum and 50 μL of creatinine standard (2 mg/dL) were added into 1 mL of working reagent in the test and standard tubes, respectively. Mixed well and noted the absorbance of standard and test at 520 nm after 30 seconds (A_0) and 90 seconds (A_1). The amount of creatinine present in the serum was calculated using the formula

$$\text{Serum creatinine (mg/dL)} = (\Delta A \text{ of Test} / \Delta A \text{ of Standard}) \times 2$$

2.2.12.2.2.7. Estimation of serum urea

Serum Urea levels were estimated according to Berthelot enzymatic method (Fawcett and Scott 1960; Young *et al.* 1975).

Principle

Urea is converted quantitatively into ammonia and carbon dioxide by the action of urease. In the modified method, an ammonium ion reacts with α ketoglutarate and NADH results in the formation of glutamate and NAD^+ . The rate of oxidation of NADH to NAD^+ is measured at 340 nm. The decrease in absorbance is proportional to the concentration of urea in the serum sample.

Procedure

Prepared test tubes labeled as "S" Standard and "T" Test sample. To all tubes pipetted 1 mL urease and sodium salicylate reagent and 10 μL of standard (40 mg/dL) and sample to S and T tubes, respectively. After incubation at 37° C, read and recorded absorbance A_1 and A_2 exactly 30 sec and 60 sec at 340 nm. Calculate the change in

absorbance ΔA by subtracting $A_2 - A_1$. The amount of urea present in the serum was calculated using the formula

$$\text{Urea (mg/dL)} = (\Delta A \text{ of Test} / \Delta A \text{ of Standard}) \times 40$$

2.2.12.2.3. Determination of lipid profile

2.2.12.2.3.1. Estimation of serum total cholesterol (TC)

Total cholesterol in serum was estimated by CHOD-PAP method using a commercial kit from Agappe Diagnostics Ltd (Allain *et al.* 1974).

Principle

Colorimetric determination of total cholesterol according to the following reaction. Cholesterol ester reacts with water by cholesterol esterase to form cholesterol and fatty acids. Cholesterol in the presence of cholesterol oxidase and molecular O_2 forms 4-Cholesten-3-one and H_2O_2 . Peroxidase $2H_2O_2$ formed reacts with phenol and 4-aminoantipyrine by peroxidase enzyme produce red-colored quinone which is measured at 505 nm.

Procedure

A volume of $10 \mu\text{L}$ serum in test tube T and $10 \mu\text{L}$ of standard (200 mg/ dL) in test tube labelled as S was added along with 1 mL of working reagent (Phenol-24 mmol/L, Sodium cholate - 0.5 mmol/L, cholesterol esterase $>180 \text{ U/L}$, cholesterol oxidase $> 200 \text{ U/L}$, peroxidase $>1000 \text{ U/L}$ and 4-aminophenazone - 0.5 mmol/L in 50 mmol/L of Pipes buffer, pH 7.0). Mixed well and incubated at 37°C for 5 min and determine absorbance against reagent blank at 505 nm.

Concentration of cholesterol (mg/dL) = Absorbance of sample/ Absorbance of standard x 200

2.2.12.2.3.2. Estimation of serum triglycerides (TG)

Serum Triglycerides was estimated by CHOD-PAP method using commercial kit purchased from Euro Diagnostics Ltd.

Principle

When triglycerides (in serum) incubated with lipoprotein lipase forms glycerol and free fatty acids. Glycerol is then phosphorylated by ATP to glycerol-3-phosphate and ADP by glycerol kinase. The glycerol -3- phosphate formed is then oxidized by glycerol phosphate dehydrogenase to di hydroxyl acetone phosphate (DAP) and hydrogen peroxide (H₂O₂), This H₂O₂ formed reacts with 4-Aminoantipyrine and p - chlorophenol by peroxidase enzyme to form a violet colored complex which is measured colorimetrically at 546 nm.

Procedure

About 10 μ L serum in test tube T and 10 μ L of standard (200 mg/ dL) in test tube labelled as S was added along with 1 mL of triglyceride reagent (Pipes buffer, pH 7.00,- 5 mmol/L, TOPS- 5.3 mmol/L, potassium ferrocyanate 10 mmol/L, magnesium salt 17 mmol/L, 4-aminoantipyrine 0.9 mmol/L, ATP 3.15mmol/L, lipoprotein lipase > 1800 U/L, glycerol kinase > 450 U/L, glycerol – 3- phosphate oxidase > 3500 U/L, peroxidase > 450 U/L). Mixed the above tubes well and incubated for 5 min at 37 °C and determined absorbance against reagent blank at 546 nm. The concentration of triglycerides was calculated using the formula,

Triglyceride (mg/dL) = Absorbance of sample/ Absorbance of standard x 200

2.2.12.2.3.3. Estimation of serum HDL cholesterol (HDLc)

HDLc in serum was determined by using a commercial kit from Euro Diagnostics Ltd. (Naito 1985).

Principle

The colorimetric method based on the HDL solubilizing detergent in the kit and the HDL-c thus released; react with the cholesterol esterase, cholesterol oxidase, and chromogens to give color. The non-HDL lipoproteins such as LDL, VLDL, and chylomicrons are inhibited because of absorption of the detergents on their surfaces. The intensity of the color formed is proportional to the HDL-c concentration in the serum sample.

Procedure

A volume of 10 μL serum was added to 450 μL of R1 (DSB mT, <1mM; Cholesterol oxidase <1000 U/L, pH 7). The same volume of R1 was added to 3 μL of HDLc/LDLc Calibrator, Lyophilized human serum. These two tubes with a blank tube were incubated for 5 min at 37°C. Then added 150 μL of R2 (Cholesterol esterase < 1500 U/L, 4-Aminoantipyrine < 1 mM , Detergent < 2%, Ascorbic oxidase < 3000 , Peroxidase < 1300 U/L, pH 7) in to all the tubes such as blank, test and calibrator. This reaction mixture was mixed well and incubated at 37° C for 5 min and read the absorbance of calibrator and sample at 650 nm against a reagent blank immediately after-incubation.

HDL in the sample was calculated using the following formula:

$$\text{HDLc in Sample (mg/dL)} = \Delta A \text{ Sample} / \Delta A \text{ Calibrator} \times \text{Calibrator Concentration}$$

2.2.12.2.3.4. Estimation of low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL)

The LDL value was calculated by using Friedewald's formula (Friedewald *et al.* 1972),

$$\text{LDLc (mg/dL)} = \text{Total Cholesterol} - \text{HDLc} - (\text{Triglycerides}/5)$$

VLDL level was also calculated by using formula,

$$\text{VLDL (mg/dL)} = \text{Triglycerides (in mg/dl)} / 5$$

2.2.13. Antitumor studies using an animal model

2.2.13.1. Determination of antitumor activity using DLA cell induced solid tumor model

Swiss albino female mice (20-25 g) were divided into 8 groups comprising 6 animals each. Group I: Control - Untreated; Group II: Prevehicle control (1% propylene glycol (p.o.) treated animals 5 days before tumor induction); Group III: vehicle control (1% propylene glycol treated (p.o.) animals simultaneously with tumor induction); Group IV: Standard (cyclophosphamide, 15 mg/kg b.wt., p.o.); Group V & VI: 5 and 10 mg/kg UNPE extract (p.o.) 5 days before tumor induction; Group VII & VIII: 5 and 10 mg/kg UNPE extracts (p.o.) simultaneously with tumor induction. All treatments were done orally. DLA cells were aspirated from the peritoneal cavity of tumor-bearing mice washed thrice and suspended in PBS to a known volume. Cell suspension, 0.1 mL containing 1×10^6 cells was injected intramuscularly into the right hind limb of Swiss albino mice. Drug (vehicle, standard and extracts) administration was continued for 10 consecutive days after tumor induction and diameter of the tumor was measured using a Vernier caliper in two perpendicular planes at every three days intervals following tumor induction. The tumor volume was calculated using the formula, $V = 4/3\pi \cdot a^2 \cdot b/2$ where 'a' is the minor diameter and 'b' is the major diameter (Ma *et al.* 1991). The weight of the animal, as well as the total WBC count, was determined - every third day following tumor induction for 4 weeks. Percentage inhibition was calculated by the formula, Percentage inhibition = $C - T/C \cdot 100$, where 'T' and 'C' represent the number of days that treated and control animals survived.

2.2.13.2. Determination of survival of EAC induced ascites tumor-bearing animals

Swiss Albino female mice (20-25 g) were divided into 8 groups comprising 6 animals each. Group I: Control - Untreated; Group II: Prevehicle control (1% propylene glycol treated (orally) animals 5 days before tumor induction); Group III: vehicle control (1% propylene glycol (p.o.) treated animals simultaneously with tumor induction); Group IV: Standard (cyclophosphamide, 15 mg/kg b.wt. p.o.); Group V and VI: 5 and 10 mg/kg *U. narum* leaf PE extract (orally administered) respectively, 5 days before tumor induction; Group VII and VIII: 5 and 10 mg/kg *U. narum* leaf PE extract (p.o.)

respectively, simultaneously with tumor induction. Animals in all the groups were injected 1×10^6 EAC cells/animal intraperitoneally (0.1 mL) to induce ascites tumor. The treatments (vehicle, standard drug, and extracts) were done for 10 consecutive days. The death pattern of animals due to tumor burden was noted and the percentage of increase in lifespan was calculated using the formula, % increase in lifespan = $(T - C)/C \times 100$ where, 'T' and 'C' represent the number of days that treated and control animals survived, respectively.

2.2.14. Acridine orange/Ethidium bromide (AO/EtBr) dual staining

HeLa cells were seeded on a 6 well plate at a density of 2×10^5 cells/mL in DMEM and allowed to grow till 80% confluence. The cells were then exposed to different concentrations (10-100 $\mu\text{g/mL}$) of the extract in fresh DMEM and incubated further for 24 hrs. Then, the media was removed and the cells were washed with ice-cold PBS. The cells were collected after trypsinization and by centrifuging at 2000 rpm for 10 minutes. The cell pellet was then re-suspended in PBS (50 μL). Resuspended cells were mixed with equal volume AO/EtBr dual staining solution (Preparation of AO/EtBr stain is described in chapter 2 section 2.4.1). The cell morphology was analyzed under a Leica fluorescent microscope with red and green filters and photographed.

2.2.15. Annexin V-FITC staining

HeLa cells were plated in 6 well plates at a density of 2×10^5 and incubated for 12 hrs following which 50 and 100 $\mu\text{g/mL}$ of ethyl acetate fraction was added and incubated for further 24 hrs. Then, the cells were trypsinized and washed with pre-chilled PBS and resuspended with 100 μl of 1X binding buffer containing 5 μL of Annexin V-FITC and 5 μL of propidium iodide for 15 min in dark. Then 400 μL of binding buffer was added and cells were filtered through a cell strainer and analyzed using FACS Aria, BD, USA scanner.

2.2.16. Cell cycle analysis by flow cytometry

HeLa cells were cultured in DMEM with 10% FBS in 5% CO₂ humidified incubator. After reaching 70% confluence of cells, media was removed and cells were washed with 3 mL PBS and trypsinized with 1X trypsin. After detachment of cells, FBS containing media was added to inhibit the trypsin action and the cell suspension was centrifuged and pelleted at 1500 rpm for 2 min. The cell pellet was then washed twice with PBS to remove any cell debris. The cells were resuspended in 1 mL DMEM and counted using hemocytometer. Cells having density 2×10^5 were seeded in 6-well plate and incubated for 24hrs for cell attachment and to attain their morphology. After 24 hrs cells were washed with PBS and exposed with ethyl acetate fraction at 50 and 100 $\mu\text{g/mL}$ concentration for 24 hrs and cells were then washed ones with PBS, trypsinized and washed again with ice-cold PBS. The pellets were resuspended and fixed with ice-cold 70% ethanol and incubated in ice for 45 min. The fixed cells were again washed with ice-cold PBS twice and resuspended in 100 μL of PBS containing RNase A (1 mg / mL) for 30 min at 37 °C. About 10 μL of propidium iodide (1 mg/mL) was added followed by an incubation of 15 min in dark and analysis was carried out by BD FACS Aria system

2.2.17. *In vivo* anti-inflammatory studies

2.2.17.1. Acute inflammation study using carrageenan-induced paw edema

The experimental mice (BALB/c, female weighing about 25-30g) were divided into 5 groups (6 animals/group). Group I was kept as a control -carrageenan alone (1% w/v) and Group II was vehicle control -1% propylene glycol, p.o.. Group III: standard diclofenac (25 mg/kg b.wt.) and Group IV and V were UNLD (200 mg/kg b.wt.), and UNHD (400 mg/kg b.wt.) treated p.o., respectively. All group except group I pretreated with respective drugs for 4 days. On the 5th day, 1h after the administration of drugs, acute inflammation was induced by subplantar injection of 0.02 ml of 1% w/v carrageenan in 0.1% carboxymethylcellulose (CMC) in the right hind paw of every mouse (Winter *et al.* 1962). The Paw thickness was measured using a vernier caliper, 1 hr prior to and for every hour up to 6th consecutive hr after carrageenan administration. The percentage inhibition was calculated as % inhibition= $[(V_T - V_0)$

control – (V_T - V₀) treated]/ (V_T - V₀) control × 100, where V₀ is the initial paw thickness and V_T is the paw thickness in the different time interval.

2.2.17.1.1. Measurement of extravasations of Evans blue dye (EBD) in carrageenan-induced paw edema in mice

EBD is used as a marker for determining the plasma membrane permeability *in vivo*. Paw edema was induced as the same procedure of acute inflammatory study with carrageenan in BALB/c female mice. All the groups except group I were administered by respective drugs. After 5 days of pretreatment, subplantar injection of 0.02 ml of 1% w/v carrageenan in 0.1% carboxymethyl cellulose (CMC) was given in the right hind paw to induce acute inflammation. After 3.5 hr of carrageenan injection, EBD (25 mg/kg b.wt.) was injected into the tail vein of all animal groups and photographs of paws were captured in 30 min of EBD injection. Mice were sacrificed by cervical dislocation after 4 hr of carrageenan injection. Paws were dissected and an equal weight of paw tissues was sliced carefully from each animal. Proper homogenization was done using solutions of 1mL of acetone and 1% sodium sulfate (4:1 ratio) and incubated in the same solution for 24 hr at 37°C to allow EBD extraction from tissues. Supernatant solutions were collected after centrifugation (at 2000 rpm for 10 min). Absorbance was measured at 620 nm using spectrophotometer (Mehdi Mahmoodi *et al.* 2009). The concentration of EBD was calculated from EBD standard graph. The percentage inhibition of extravasation by the drug was calculated as % Inhibition = $(A_c - A_t) / (A_c) * 100$, Where A_c is the absorbance of solutions of the control group, and A_t is the absorbance of solutions of treated mice either with the standard or plant extract (Gupta *et al.* 2015).

2.2.17.2. Acute inflammation study using dextran-induced paw edema

The experimental mice (BALB/c female weighing about 25-30g) were divided into 5 groups (6 animals/group) and grouped as above. All group except group I pretreated with respective drugs for 4 days. On the 5th day, 1h after the administration of drugs, inflammation was induced by 0.02 ml of 1% dextran in 0.1% carboxymethylcellulose (CMC) subplantar injection in the right hind paw of all animal. The paw thickness was measured using a vernier caliper, 1 hr prior to and for every hour up to 6th

consecutive hr after dextran injection. The percentage inhibition was calculated as above (Maity *et al.* 1998).

2.2.17.3. Chronic inflammation study using formalin-induced paw edema

The groups except the control group were pretreated with respective drugs for 7 days. On the 8th day, 1h after drug administration, chronic inflammation was induced by subplantar injection of 0.02 ml of 2% formalin (freshly prepared) on the right hind paw in all animal groups (Chang and Lewis 1989). The paw thickness was measured using a vernier caliper, 1 hr prior to and for every 6 days.

2.2.17.4. Measurement of Lipopolysaccharide-induced mouse TNF- α production in RAW624.7 cells

RAW624.7 cells were cultured in DMEM with 10% FBS in 5% CO₂ humidified incubator. When the cells reached 70-80 % confluence, the media was removed and washed with 1X PBS. Cells were then trypsinized with 1X trypsin and after detachment of cells; FBS containing media was added to inhibit the trypsin action. Then the cell suspension was centrifuged at 1500 rpm for 2 min and the cell pellet was washed twice with PBS to remove any cell debris. The cells were resuspended in 1mL DMEM media and cell count adjusted cells were seeded at 1×10^4 cells/ well of 96 well plate. The next day, test materials were added at a concentration of 50 and 100 $\mu\text{g}/\text{mL}$ in DMSO and incubated for 12hrs. After incubation, 1 $\mu\text{g}/\text{mL}$ LPS was added and incubated for 12hrs, thus a total of 24 hrs of incubation. After 24hrs of drug, treatment media was removed and centrifuged at 1500rpm for 1minute to remove the cell debris and stored at -80°C .

Added 50 μL of assay diluent (from Mouse TNF-alpha Quantikine ELISA Kit, R&D Systems Website Enhancements) to each well. Add 50 μL of standard, control, or sample to each well. All the wells are covered with a plate sealer and incubate at room temperature for 2 hours. After the incubation, each well was aspirated and washed, repeating the washing for a total of 5 washes. Conjugate (100 μL) was added to each well and covered with a new plate sealer, and incubate at room temperature for further 2 hours. Then aspirated the wells and washed for 5 times. Added 100 μL substrate solutions to each well. Incubate at room temperature for 30 minutes in dark. After

incubation added 100 μ L of stop solution to each well. Read the optical density at 450 nm within 30 minutes in ELISA reader. Set wavelength correction to 540 nm or 570 nm.

2.2.17.5. Quantitative real-time PCR (qPCR) analysis

cDNA synthesis/ Reverse transcription

Total RNA from RAW624.7 cells exposed to LPS and UNMET-AQ fraction (50 and 100 μ g/mL) was isolated by the TRIzol method and quantitatively determined using Nanodrop method. For cDNA synthesis 1.5 μ g equivalent of total RNAs was reverse transcribed using the High capacity cDNA reverse transcription kit (Applied Biosystems) in the Veriti Thermal cycler. The reaction volume for one sample was adjusted as 10X RT buffer-2.5mL, 10X primer-2.5mL, dNTP-1mL, Reverse transcriptase-1.25mL, RNase inhibitor-0.25mL and water -5mL. This reaction mixture mixed with 1.5 mg of RNA (1:1) making up to 25mL of final reaction volume. The PCR run in the condition of 25 °C for 10mins, 37 °C for 120 mins, 85 °C for 5mins and then 4 °C for ∞ .

Real-time Quantitative PCR analysis

The synthesized cDNA was diluted with nuclease-free water and loaded into 96-well Micro Amp Fast Optical PCR plates (2.5 mL/ well). 7.5mL of PCR Master Mix (Power SYBR Green PCR master mix-5mL, Primer (1:10 diluted, Forward and Reverse)-0.5mL, Nuclease-free water- 2mL) was added to well and the plate was centrifuged at 1500 rpm for 2 mins and loaded into Step One plus real-time PCR system.

The primer sequences for the analysis are

mGAPDH	Forward 5' TGCCCCCATGTTTGTGATG 3'
mGAPDH	Reverse 5' TGTGGTCATGAGCCCTTCC 3'
mTNF-alpha	Forward 5' GTCCCCAAAGGGATGAGAAGTT 3'
mTNF-alpha	Reverse 5' ACAGGCTTGTCACCTCGAATTTTG 3'

GAPDH was used as an endogenous control gene. The PCR The run was performed under the following conditions. Initial holding stage for 10 min at 95 °C followed by 40 cycles of amplification with denaturation at 95 °C for 15 sec and 60 °C for 1 min and melt curve stage with 40 cycles annealing at 95 °C for 15 sec and 60 °C, + 3 °C , 95 °C for 15 secs. The fold change in the expression of the target gene was done by $2(-\Delta\Delta CT)$ method after normalization with internal control and housekeeping gene.

2.2.18. Histological examination

After the experimental period, the animals were sacrificed; respective organs were excised using a sterilized blade and washed with normal saline (0.9% NaCl) for removing any traces of blood debris. The tissues were fixed using 10% neutral buffered formalin. The tissues were dehydrated using increasing grades of alcohol and were cleared in xylene. Paraffin-embedded samples were subjected to microtome sectioning and 3-4 μm thick sections are obtained. The paraffin from the section was removed using xylene and rehydration was done using decreasing concentration grades of alcohol. The rehydrated sections were stained with hematoxylin (nuclear stain consists of potassium or ammonium (alum) - 100 g, mercuric oxide- 2.5 g, 10% alcoholic hematoxylin-50 mL, glacial acetic acid-20 mL, distilled water-1000 mL) and eosin (cytoplasmic stain with Eosin Y- 1 g, 70% ethanol -100 mL, glacial acetic acid- 5 mL) and sections in slides were observed under Magnus INVI phase contrast microscope at 200 X magnification and photographed.

2.2.19. Statistical analysis

All *in vitro* assays were performed at least thrice in duplicate tubes and data represented are mean \pm SD six values. Statistical evaluation of the data was done by one way ANOVA using graph pad software (Instat 9.0). Results were considered statistically significant when $p < 0.05$.

Chapter 3

*Preliminary screening for phytochemical constituents,
cytotoxicity and antioxidant properties of Uvaria narum
and Uvaria macropoda*

3.1. Introduction

Plants have been extensively used by traditional practitioners for the prevention and cure of various human ailments worldwide. In India, Ayurveda system of medicine effectively uses individual plant extracts as well as different herbal formulations for treatment purpose. Scientific studies on many of these medicinally used plants have identified important phytochemicals of therapeutic value (Yuan *et al.* 2016; Cragg and Pezzuto 2016).

Approximately 50% of the drugs prescribed in modern medicine are plant derived. It includes chemotherapeutic drugs like vincristine, vinblastine, taxol, podophyllotoxin, etc. which are being used successfully (Almatar and Makky 2015; Donehower and Rowinsky 2018). Drugs such as silymarin, acetylsalicylate, tamoxifen, etc. are of plant origin and they have successfully used in liver diseases, pain and inflammation and cancer treatment, respectively (Rakelly de Oliveira *et al.* 2015; Levitsky and Dembitsky 2015; Saller *et al.* 2001; Yeomans 2011). In addition, several individual plants extracts and pure compounds are at various stages of clinical trials. Phyto-pharmacological research is still exploring newer pharmacologically important plants.

In cancer management, cytotoxic molecules that interfere with cell division or that resist oxidative and inflammatory changes have been considered very important. Cytotoxic compounds may either directly affect cell division or metabolism and induce apoptosis (programmed cell death) and be effective in cancer therapy (Jamkhande and Wattamwar 2015; Slaménova and Horvathová 2013). Uncontrolled production of intracellular reactive oxygen species (ROS) leads to oxidative stress which subsequently alters metabolic processes and cellular death through biochemical and physiological lesions (Siti *et al.* 2015; Loft and Poulsen 1996).

Antioxidant molecules play a major role in the prevention of cancer by scavenging free radicals, reducing oxidative stress and thereby reducing the inflammatory response in different organs. Recent studies have focused on antioxidants from natural products for their noteworthy role in nullifying the destructive effects of ROS (Moghadamtousi *et al.* 2015). A number of major antioxidant phytochemicals have been reported in different plants, fruits, vegetables, and spices with the potent cancer chemopreventive role.

Some of the important phytochemicals including curcumin, saponins (Paris saponins, ginsenosides, and soy saponins), resveratrol, polysaccharides (apple polysaccharides and mushroom glucans), and quercetin are identified with their remarkable chemopreventive effects (Li *et al.* 2015). Therefore, molecules that prevent free radical reactions and associated oxidative changes as well as inflammatory molecule production may play significant roles in preventive treatment strategies. These molecules can be useful to prevent recurrence of cancer and also act as adjuvants to cytotoxic drugs by modulating their action or preventing secondary toxicity.

Plants belonging to Annonaceae family are well-known for its widespread use in traditional medicine. The genus *Uvaria* under this family is the most explored and thoroughly studied. Phytochemical studies on Annonaceae family plants have documented annonaceous acetogenins, diterpenes, alkaloids, anonaine, samoquasine A, squamocin-I, squamocin-B, squamocenin, motrilin, kaurenoic acid, cyclopeptides roemerine, norcorydiene, corydine, norisocorydine, carvone and linalool as the main phytoconstituents (Ma *et al.* 2017).

Uvaria species under the Annonaceae family is known to contain bioactive molecules having numerous pharmacological activities (Kongstad *et al.* 2015). *Uvaria narum* and *Uvaria macropoda* are less studied species and available locally and was selected for pharmacological evaluation pertinent to cancer. The intention was to identify and purify possible cytotoxic or anti-cancer components from these plants to the maximum extent possible.

In this chapter, we have discussed the polarity based preparation of extracts from *Uvaria narum* and *Uvaria macropoda* leaves and screening of these extracts for cytotoxic and antioxidant properties. This was done to identify the plant species containing the most active phytochemicals. Leaves of the plants were selected because the leaf is easily accessible without disturbing its natural habitat. Since plants such as *U. macropoda* are not widely distributed, the selection of leaf is more suitable to protect the plant.

Preliminary phytochemical assessment to reveal the phytochemicals in different extracts was carried out by qualitative and quantitative (for phenols and flavonoids) biochemical assays and TLC methods. *In vitro* cytotoxicity was assessed using the

trypan blue exclusion method and antiproliferative analysis by MTT assay. Antioxidant activities were determined using various *in vitro* radical scavenging, reducing and lipid peroxidation assays.

3.2. Materials and methods

3.2.1. Preparation of Uvaria plants extracts

The collection and authentication of leaves of *U. narum* and *U. macropoda* were done as mentioned in section 2.1.5.3, Chapter 2. Different extracts were prepared using petroleum ether, chloroform, acetone, methanol, and water using the serial extraction method as per the method described in section 2.2.1, Chapter 2. The solvent-free extracts were dissolved in ethanol for phytochemical analysis. For *in vitro* cytotoxicity studies, extracts were dissolved in DMSO.

3.2.2. Phytochemical screening of *U. narum* and *U. macropoda* leaves extracts

Phytochemical screening of various extracts of *U. narum* and *U. macropoda* leaves for the presence of terpenoids, steroids, alkaloids, phenolic compounds, tannins, flavonoids, saponins, and glycosides were carried out according to standard procedures (Sofowora 1993; Evans and Trease 1989; Harborne 1998; Harborne 1973) as mentioned in chapter 2 section 2.2.2.

3.2.3. TLC profiling and visualization

One-dimensional TLC analysis was performed on precoated silica plates (Merck KGaA, Darmstadt, Germany) with different mobile phases for different extracts such as N-hexane: diethyl ether: acetic acid (35:15:0.75 V/ V/ V) for PE extract; benzene: ethyl acetate (5:1 V/ V) for CHL extract ; chloroform: methanol: water (16:5:0.8 V/ V/ V) for ACT extract and MET extract and methanol: water: acetic acid (18:9:1 V/ V/ V) for water (AQE) extracts. The phytoconstituents separated by TLC were visualized under visible light, UV (254 nm) and iodine vapor. The R_f values of individual bands in each chromatogram were calculated and phytochemical classes were identified using general spraying reagents described in chapter 2 section 2.2.3.

3.2.4. Short term cytotoxicity studies *in vitro*

3.2.4.1. Cell lines

Dalton's Lymphoma Ascites (DLA), Ehrlich's Ascites Carcinoma (EAC) cells and normal rat splenocytes were taken for cytotoxicity study. Dalton's Lymphoma Ascites (DLA) and Ehrlich's Ascites Carcinoma (EAC) cells were maintained in the peritoneal cavity of Swiss albino mice as mentioned in Chapter 2, sections 2.2.8.1. Spleen cells were isolated from normal Wistar rats as per Chapter 2, sections 2.2.8.2.

3.2.4.2. Short term *in vitro* cytotoxicity trypan blue exclusion assay

In short-term *in vitro* cytotoxicity analyses, the cells (DLA, EAC and spleen cells) were suspended in 1 mL PBS and the cell number was adjusted to 1×10^6 cells. Different extracts of *U.narum* and *U.macropoda* were added at various concentrations (10-100 $\mu\text{g/mL}$) in a minimum volume of vehicle and incubated for 3 hr at 37 °C. Cell death was checked using the trypan blue exclusion method as described in chapter 2 section 2.2.8.3.

3.2.5. Antiproliferative study

3.2.5.1. Maintenance of cell lines

HeLa (Human cervical carcinoma), HepG2 (human hepatocarcinoma), HCT-15 (human colorectal adenocarcinoma) and Vero E6 (African monkey kidney) cell lines were obtained from the National Centre for Cell Science (NCCS), Pune, India. Each cell line was maintained in its appropriate medium under standard sterile conditions as described in chapter 2 section 2.2.9.1.

3.2.5.2. Long-term cytotoxicity study using MTT assay

Antiproliferative activity of different extracts of *U.narum* and *U.macropoda* leaves at various concentrations (10-100 $\mu\text{g/mL}$ in DMSO) were evaluated in HeLa, HepG2, HCT-15, and Vero cell lines, using the MTT assay as described in chapter 2 section 2.2.9.2.

3.2.6. Antioxidant activity of *U.narum* and *U.macropoda* extracts

3.2.6.1. Reducing potential of the extract

3.2.6.1.1. DPPH radical scavenging activity

Determination of antioxidant activity was based on scavenging of the stable 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical (Picerno *et al.* 2011). Vitamin C was used as the positive control (Refer chapter 2 section 2.2.10.1.)

3.2.6.1.2. FRAP assay

The ferric reducing ability of the extracts was measured according to the procedure described by (Pulido 2000) with some modifications. The percentage increase in the ferric reducing activity was then calculated as described in chapter 2 section 2.2.10.2.

3.2.6.2. Effect on lipid peroxidation

3.2.6.2.1. Inhibition of lipid peroxidation

Inhibition of lipid peroxidation in liver tissue homogenate by the plant extracts was determined spectrophotometrically from the optical density values of control and extract treated tubes (Biswas *et al.* 2010) as per the procedure detailed in chapter 2 section 2.2.10.3). Ascorbic acid was used as a positive control.

3.2.6.2.2. Lipoxygenase activity

This assay is based on a decrease in oxygenation of linoleic acid by 15-lipoxygenase in the presence of different concentrations of extracts (20-100 $\mu\text{g/mL}$). Formation of conjugated diene was measured spectrophotometrically at 234 nm. Vitamin C was used as positive control (Dolev *et al.* 1967). Refer to chapter 2 section 2.2.10.4.

3.2.6.3. Quantitative estimation of antioxidant compounds

3.2.6.3.1. Estimation of total phenolic content

The total phenolic content of different extracts was analyzed spectrometrically using a modified Folin-Ciocalteu method (Ainsworth and Gillespie 2007) as mentioned in chapter 2, section 2.2.10.5.

3.2.6.3.2. Estimation of total flavonoid content

Total flavonoid content was ascertained using aluminum chloride colorimetric assay (Chia-Chi Chang 2002) (chapter 2 section 2.2.10.6)

3.2.7. Statistical analysis

All *in vitro* assays were performed at least thrice in duplicate and values represented are mean \pm SD. Statistical evaluation of the data was done by one way ANOVA using graph pad software (Instat 9.0). Results were considered statistically significant when $p < 0.05$.

3.3. Results

3.3.1. The percentage yield of *U. narum* and *U. macropoda* leaf extracts

The percentage yield of *U. narum* leaf extracts prepared using solvents of increasing polarity is given in table 3.1. The yield of UNPE, UNCHL, UNACT, UNMET and UNAQE (w/w) extracts were found to be 7 ± 0.83 , 1.34 ± 0.34 , 2.84 ± 0.46 , 5 ± 1.12 and $3.8 \pm 0.94\%$ of starting material, respectively. The maximum yield was found to be in petroleum ether extract. In the case of *U. macropoda* leaf, the percentage yield of different extracts was calculated with respect to the starting material similar to the procedure followed for *U. narum*. The obtained percentage yield of UMPE, UMCHL, UMACT, UMMET and UMAQE (w/w) extracts were 3.2 ± 0.75 , 1.2 ± 0.92 , 3.08 ± 1.02 , 2.52 ± 0.75 , $2 \pm 0.68 \%$, respectively.

Table 3.1. The percentage yield of different extracts of *U. narum* and *U. macropoda* leaves with respect to starting material.

Plants	PE	CHL	ACT	MET	AQE
<i>U. narum</i>	7.0 ± 0.83	1.34 ± 0.34	2.84 ± 0.46	5.00 ± 1.12	3.8 ± 0.94
<i>U. macropoda</i>	3.2 ± 0.75	1.20 ± 0.92	3.08 ± 1.02	2.52 ± 0.75	2.0 ± 0.68

Values are expressed as mean ± SD

3.3.2. Phytochemical analysis

Phytochemical analysis of *U. narum* leaf extract revealed the presence of various phytochemical compounds as depicted in Table 3.2. Different classes such as terpenoids, steroids, flavonoids, tannins, alkaloids proteins, amino acids, and carbohydrates were present in these extracts. However, in *U. narum* leaves, the aqueous extract did not show the presence of tannins and alkaloids.

Table 3.2. Phytochemical screening of different extracts of *U. narum* leaves

Phytochemicals	UNPE	UNCHL	UNACT	UNMET	UNAQE
Terpenoids	+++	+	+	+	+
Steroids	+++	+	+	+	+
Tannins	+	-	+	+	-
Flavonoids	+	+	+++	+++	++
Saponins	-	-	-	+	+
Leucoantocyanins	-	-	-	-	-
Alkaloids	+++	++	++	+	-

Phytochemicals	UNPE	UNCHL	UNACT	UNMET	UNAQE
Phenol	+	+	+++	+++	++
Carbohydrates	+	+	+	+	+++
Protein	-	-	-	-	+
Emodins	-	-	-	-	-
Antraquinones	-	-	-	-	+
Coumarins	+	+	+	-	-

Presence+/- absence -; + + + Intense; + + Moderate; + Slight

Phytochemical analysis of different extracts of *U. macropoda* leaf (Table.3.3) revealed the presence of various classes of bioactive phytochemicals such as terpenoids, steroids, tannins, flavonoids, saponins, leucoanthocyanins, alkaloids, phenol, carbohydrates, protein, emodins, anthraquinones, and coumarins.

Table 3.3. Phytochemical screening of different extracts of *U. macropoda* leaves

Phytochemicals	UMPE	UMCHL	UMACT	UMMET	UMAQE
Terpenoids	+	+	+	+	+
Steroids	+	+	+	+	+
Tannins	+	-	+	+	+
Flavonoids	+	+	+	+	+
Saponins	-	-	-	++	-

Phytochemicals	UMPE	UMCHL	UMACT	UMMET	UMAQE
Leucoantocyanins	-	-	-	-	-
Alkaloids	+	+	+	+	-
Phenol	+	+	+	+	+
Carbohydrates	-	-	+	+	+
Protein	-	-	-	-	+
Emodins	-	-	-	-	-
Antraquinones	-	-	-	-	-
Coumarins	+	+	+	+	+

Presence+/- absence -; + + + Intense; + + Moderate; + Slight

3.3.3. Thin layer chromatography analysis

3.3.3.1. Thin layer chromatography analysis of *U.narum*

UNPE extract on TLC analysis revealed the presence of five visible bands (and two additional bands under UV light (254 nm) (with the Rf values; 0.01, 0.15, 0.33, 0.43, 1.0, 0.67, 0.87 and, respectively). The same bands were visualized when the TLC plate was developed using iodine vapor. Various spraying reagents were used for the identification of the phytochemicals on TLC plates. The bands with Rf values 0.33, 0.43, 0.57 and 0.97 were found to be positive for steroid and terpenoids class of compounds when sprayed with vanillin-sulphuric acid and anisaldehyde sulphuric acid spray reagents. Among seven steroid compounds obtained in the vanillin-sulphuric acid spray, 3 were fluorescent which were detected upon spraying Liebermann-Burchard reagent. In conclusion, color reactions of these various reagents showed that petroleum ether extract was rich in terpenoids and phytosterols. This extract also gave a mild positive result for alkaloids (Figure 3.1).

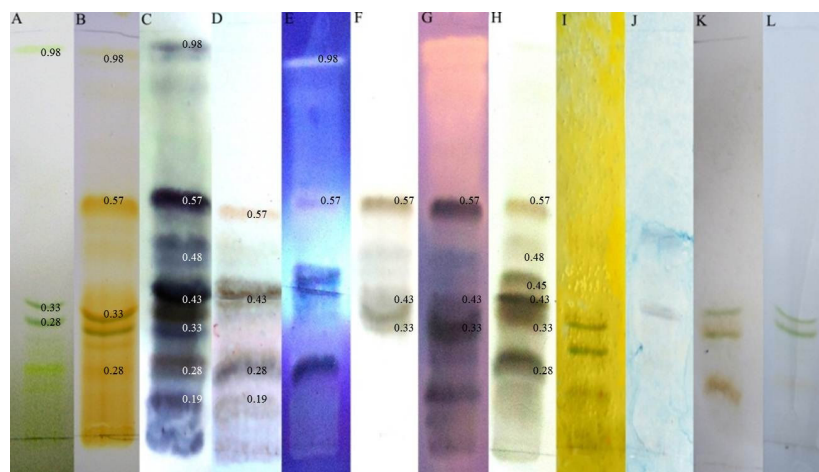


Figure 3.1. TLC of the UNPE extract obtained by developing sample (10 μ g/ml) using mobile phase, n-hexane: diethyl ether: glacial acetic acid (35:15: 0.75) and observed under (A) visible light and (B) iodine vapour and with spray reagents such as (C) vanillin (D) Liebermann-Burchard reagent, (E) Liebermann-Burchard UV (F) anisaldehyde (G) anisaldehyde UV, (H) 20% sulphuric acid (I) Dragondorff's, (J) ferric ferrocyanide, (K) methanol- potassium hydroxide and (L) ammonia.

Thin layer chromatography analysis of the UNCHL extract revealed the presence of four visible bands (with Rf values 0.06, 0.28, 0.35, 0.94 respectively) with the solvent system benzene: ethyl acetate (5:1) and four additional fluorescent bands under UV light (254 nm) (with Rf values 0.31, 0.53, 0.64, 0.66) and two extra bands (0.22, 0.97) under iodine vapors (Figure 3.2). Among these compounds, bands with Rf values 0.31 and 0.64 were positive for terpenoids as observed with anisaldehyde-sulphuric acid and vanillin-sulphuric acid spray reagents. Liebermann-Burchard spray for steroids identified a band with Rf value 0.97 which is a fluorescent steroid. Colour developed in the chromatogram revealed the presence of terpenoids and phytosterol in chloroform extract.

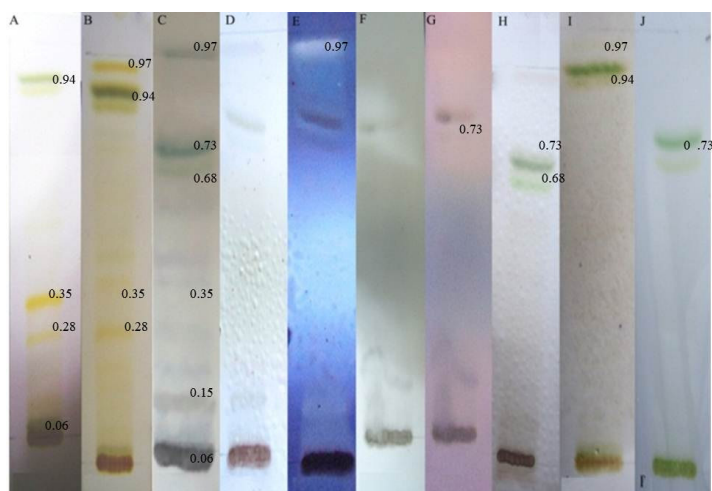


Figure 3.2. Thin layer chromatogram of the UNCHL extract obtained by developing sample (10 $\mu\text{g/mL}$) using mobile phase, benzene: ethyl acetate (5:1), and observed under (A) visible light, (B) iodine vapour, and sprayed with (C) vanillin reagent, (D) Liebermann reagent, (E) Liebermann-Burchard UV (F) anisaldehyde reagent, (G) anisaldehyde UV, (H) 20% sulphuric acid reagent, (I) methanol potassium hydroxide reagent and (J) ammonia reagent

A total of 17 bands were obtained in TLC chromatogram of UNACT. Out of these, five bands were detected under visible light (with Rf values 0.071, 0.18, 0.32, 0.47 and 0.94 respectively), and seven bands (with Rf values 0.16, 0.24, 0.32, 0.47, 0.59, 0.65, and 0.94) were visualized when exposed to iodine vapor and along with these bands, three additional bands were seen under UV (254 nm) (Figure 3.3). Compounds with Rf values 0.02, 0.24, 0.32, 0.59, 0.65 and 0.94 were visualized with vanillin-sulphuric acid and anisaldehyde- sulphuric acid spray reagents indicating the presence of terpenoids. Four of them (0.32, 0.59, 0.65 and 0.94) were fluorescent terpenoids. Steroids positive bands with Rf values 0.02, 0.16, 0.24 and 0.32 were obtained with Liebermann-Burchard spray reagent. Bands with Rf values 0.15, 0.46, and 0.89 were positive for flavonoids in ammonia reagent and bands with Rf values 0.18, 0.33 and 0.94 were identified as anthraquinone positive using methanol-potassium hydroxide as the spraying reagent (Figure 3.3).

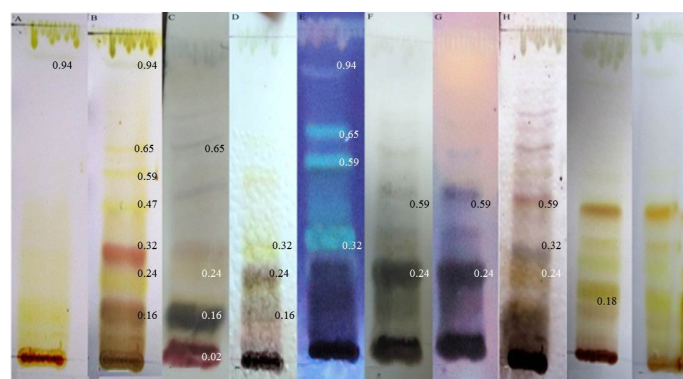


Figure 3.3. Chromatogram of the UNACT extract obtained by developing sample (10 $\mu\text{g/mL}$) using mobile phase chloroform: methanol: water (16:5:0.8 V/ V/ V), and observed under (A) visible light and (B) iodine vapour and spraying with (C) vanillin reagent, (D) Liebermann-Burchard reagent, (E) Liebermann-Burchard UV (F) anisaldehyde reagent, (G) anisaldehyde UV, (H) 20% sulphuric acid reagent, (I) methanol potassium hydroxide reagent and (J) ammonia reagent.

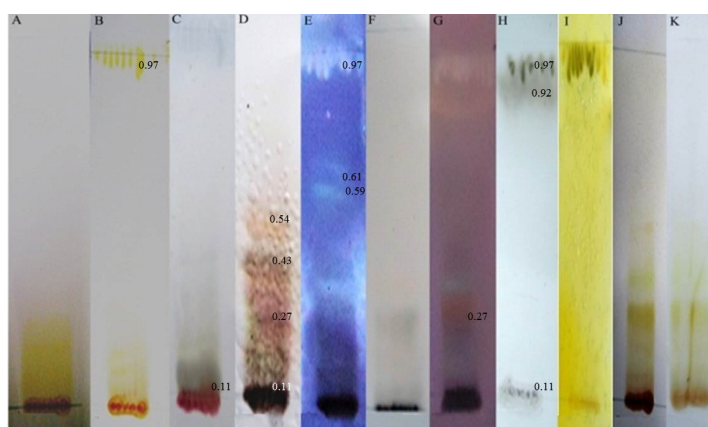


Figure 3.4. TLC of the UNMET extract obtained by developing sample (10 $\mu\text{g/mL}$) using mobile phase chloroform: methanol: water (16:5:0.8 V/ V/ V), and observed under (A) visible light and (B) iodine vapour and spraying with (C) vanillin reagent, (D) Liebermann-Burchard reagent, (E) Liebermann-Burchard reagent (UV) (F) anisaldehyde reagent, (G) anisaldehyde (UV), (H) 20% sulphuric acid reagent, (I) dragonodorff's reagent, (J) methanol- potassium hydroxide reagent and (K) ammonia reagent

UNMET extract developed with solvent system chloroform: methanol: water (16:5:0.8 V/ V/ V), revealed the presence of four bands under visible light (with Rf

values, 0.11, 0.33, 0.43 respectively), four bands fluorescing under UV (with Rf values 0.39, 0.54, 0.81, 0.97) and two iodine positive bands (Rf values 0.27, 0.91) in thin layer chromatogram. Terpenoid positive bands having Rf values 0.43, 0.54 and 0.92 were visualized when sprayed with vanillin-sulphuric acid and anisaldehyde sulphuric acid reagents. Steroid positive bands with Rf values 0.11, 0.27, 0.43 and 0.54 were visualized in Liebermann-Burchard (LB) reagent spray. There were fluorescent terpenoids with Rf value 0.59, 0.61 and 0.97 that were seen at 254nm on using LB spray. Bands with Rf values 0.15, 0.46 and 0.89 positive for methanol-potassium hydroxide reagent were identified as anthraquinones. Ammonia reagent spray detected compounds with Rf values 0.18, 0.33 and 0.94 indicating the presence of flavonoids in the methanolic extract (Figure 3.4).

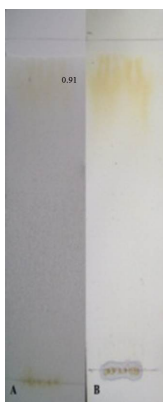


Figure 3.5. TLC of the UNAQE by developing sample (10 $\mu\text{g/mL}$) using mobile phase methanol: water: acetic acid (18:9:1 V/ V/ V), and observed under (A) visible light and (B) iodine vapor.

UNAQE under TLC analysis showed one a single band under visible light (with Rf values 0.91), three additional fluorescent bands (with Rf values 0.39, 0.86, 0.96) under UV light (254 nm) and no bands in the presence of iodine vapors with methanol: water: acetic acid (18: 9:1 V/ V/ V) as mobile phase. There were fewer phytochemicals present in the aqueous extract compared to the other four extracts when developed with the spraying reagents (Figure 3.5).

3.3.3.2. Thin layer chromatography analysis of *U.macropoda*

UMPE extract upon thin layer chromatographic analysis resulted in the identification of five bands on exposure to visible light (Rf values 0.09, 0.136, 0.227, 0.273, 0.318

and 0.955), twelve fluorescent bands under UV light (254nm) (with Rf values 0.01, 0.05, 0.11, 0.16, 0.20, 0.26, 0.3, 0.32, 0.37, 0.4, 0.5, 0.57 and 0.95) and nine bands when developed in iodine vapour (with Rf values 0.111, 0.178, 0.222, 0.333, 0.444, 0.556, 0.722, 0.889 and 0.944). Among the 9 bands detected using iodine spray, 3 of them were visualized as fluorescent bands in UV. The bands with Rf values 0.139, 0.191, 0.209, 0.304, 0.391, 0.452, 0.539 and 0.956 were found positive for terpenoids class of compounds when sprayed with anisaldehyde-sulphuric acid reagents and bands at Rf values of 0.279, 0.327, 0.356, 0.577 and 0.962 were positive for steroid with the vanillin-sulphuric acid reagent. Six terpenoid and steroid compounds were observed in UMPE extract using the above reagent sprays. Three bands with Rf values 0.120, 0.193 and 0.241 were found to be positive for Dragendorff's reagent revealing the presence of alkaloids (Figure 3.6).

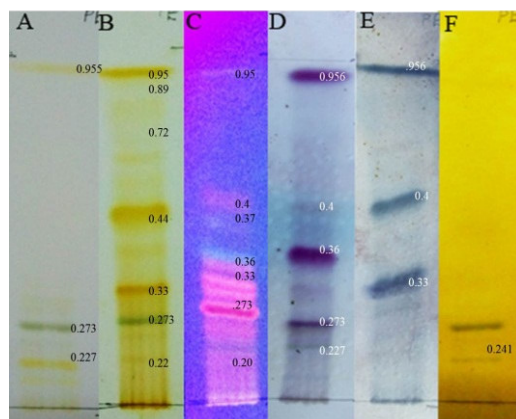


Figure 3.6. Thin layer chromatogram of the leaf UMPE obtained by developing sample (10 $\mu\text{g/mL}$) using mobile phase, n-hexane: diethyl ether: glacial acetic acid (35:15: 0.75) and observed under (A) visible light, (B) iodine vapour (C) UV light (254nm), and with spray reagents (D) anisaldehyde reagent, (E) vanillin reagent and (F) Dragendorff's reagent

Thin layer chromatography analysis of the UMCHL leaves revealed the presence of nine bands under visible light (with Rf values 0.083, 0.208, 0.317, 0.4, 0.483, 0.733, 0.792, 0.833, 0.892 and 0.917), ten fluorescent bands on exposure to UV light (254 nm) (Rf values of 0.035, 0.049, 0.092, 0.139, 0.326, 0.403, 0.479, 0.556, 0.729, 0.764, 0.833, 0.868 and 0.944) and nine bands when developed with iodine vapors (0.161, 0.081, 0.132, 0.201, 0.278, 0.382, 0.625, 0.806, 0.863, and 0.968). Bands with Rf

values 0.38, 0.743, 0.810, 0.857, 0.905 and 0.952 were found to be positive for terpenoids by anisaldehyde–sulphuric acid spray reagent and 0.117, 0.471, 0.894 and 0.941 Rf bands with vanillin-sulphuric acid showed the presence of steroids. Seven bands were visualized when sprayed with Dragendroff’s reagent (Figure 3.7), thus showing the presence of alkaloids in the extract.

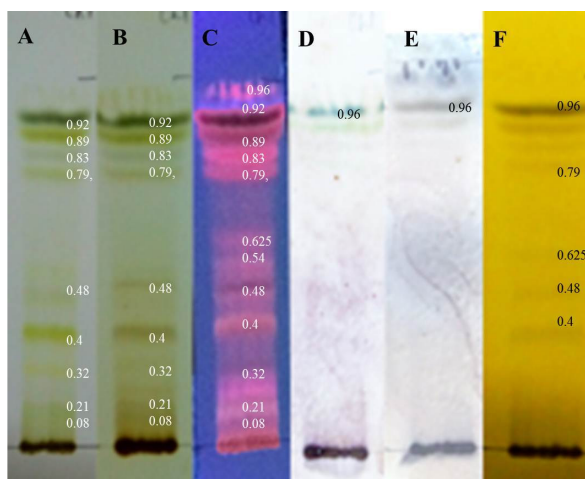


Figure 3.7. TLC of the UMCHL extract obtained by developing sample (10 $\mu\text{g/ml}$) using mobile phase, benzene: ethyl acetate (5:1), and observed under (A) visible light, (B) iodine vapour (C) UV light (254nm), and with spray reagents (D) Anisaldehyde reagent, (E) vanillin reagent and (F) Dragendorff's reagent.

TLC analysis of UMACT leaf revealed the presence of three visible bands with Rf values 0.064, 0.128 and 0.897, five fluorescent bands under UV (254 nm) with Rf values 0.16, 0.36, 0.56, 0.92 and 0.96 and five bands when exposed to iodine vapors (Rf values 0.117, 0.153, 0.518, 0.753 and 0.989). Six bands with Rf values 0.130, 0.261, 0.391, 0.435, 0.565 and 0.93 were obtained while spraying anisaldehyde-sulphuric acid reagent and 0.130, 0.435, 0.685 and 0.93 with vanillin-sulphuric acid spray reagent showed positive for terpenoids. Compound with Rf value 0.94 was found to be positive for alkaloid (Figure 3.8).

UMMET extract on thin layer chromatography revealed one (Rf value 0.11), three (Rf values 0.033, 0.144, 0.756 and 0.978) and three positive bands (Rf values 0.316, 0.105 and 0.989) under visible light, UV light and in the presence of iodine vapors, respectively. In anisaldehyde sulphuric acid reagent spray, terpenoids with Rf values

0.1, 0.33, 0.57 and 0.94 were detected. Three bands showed positive results with an ammonia reagent spray indicating the presence of flavonoids. An alkaloid positive band with Rf value 0.988 was obtained when sprayed with Dragendroff's reagent spray (Figure 3.9).

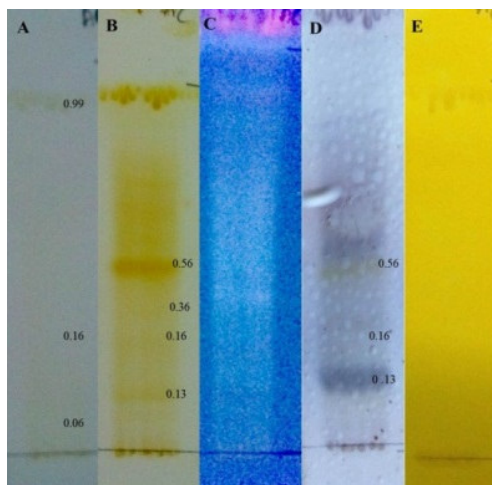


Figure 3.8. TLC of the UMACT extract obtained by developing sample (10µg/ml) using mobile phase chloroform: methanol: water (16:5:0.8 V/ V/ V), and observed under (A) visible light, (B) iodine vapour (C) UV light (254nm), and with spray reagents (D) Anisaldehyde reagent and (F) Dragondorff's reagent.

AQE extract of *U.macropoda* upon TLC analysis showed one band in UV light (Rf value 0.043) and three bands when exposed to iodine vapor (Rf values 0.038, 0.25 and 0.325). No bands were observed under visible light (Figure 3.10).

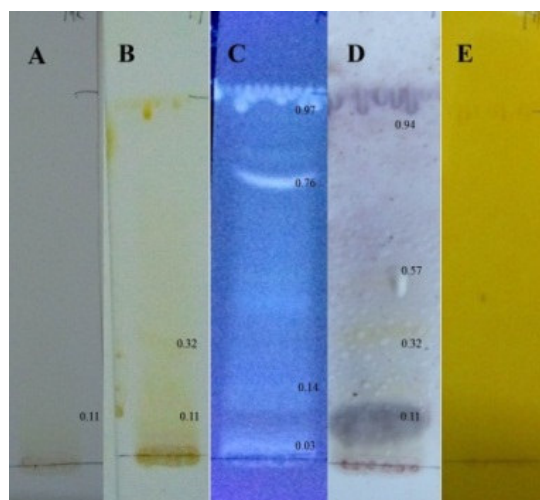


Figure 3.9. TLC of the UMMET extract obtained by developing sample (10 $\mu\text{g}/\text{mL}$) using mobile phase chloroform: methanol: water (16:5:0.8 V/ V/ V), and observed under (A) visible light, (B) iodine vapor (C) UV light (254 nm), and with spray reagents (D) anisaldehyde reagent, (E) vanillin reagent and (F) Dragendorff's reagent.

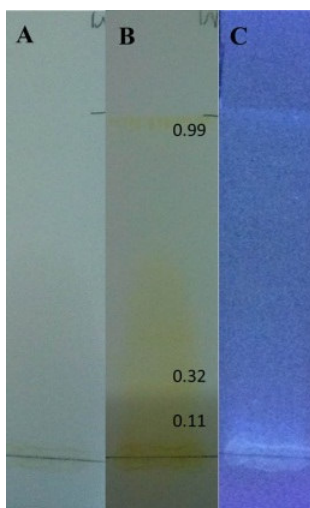


Figure 3.10. TLC of the UMAQE extracts obtained by developing sample (10 $\mu\text{g}/\text{ml}$) using mobile phase butanol: acetic acid: water (60:15:25 V/ V/ V) and observed under (A) visible light, (B) iodine vapor (C) UV light (254nm)

3.3.4. Short term *in vitro* cytotoxicity of extracts on various cell lines

3.3.4.1. Effect of *Uvaria narum* extracts using trypan blue method

Petroleum ether extract of *U.narum* leaf (UNPE) at various concentrations (10-100 $\mu\text{g}/\text{mL}$) did not show any appreciable cytotoxicity towards spleen cells (Figure 3.11 and Table 3.4). The highest concentration of 100 $\mu\text{g}/\text{mL}$ caused cytotoxic effects in approximately 36.75%, and hence IC_{50} value was not calculated. On the other hand, UNPE extract showed a dose-dependent increase in the cytotoxicity towards DLA and EAC cells. The IC_{50} value of UNPE extract for DLA was found to be 19 ± 0.57 $\mu\text{g}/\text{mL}$ and that for EAC cells was 38 ± 0.74 $\mu\text{g}/\text{mL}$.

UNCHL showed dose-dependent cytotoxicity towards all the three cell lines. The IC_{50} for UNCHL extract was 15 ± 1.2 , 17 ± 0.99 and 9.1 ± 0.36 $\mu\text{g}/\text{mL}$ with EAC, DLA, and splenocytes, respectively (Figure 3.11 and Table 3.4). The UNACT extract was

found to be significantly cytotoxic to spleen cells, DLA and EAC cells with IC₅₀ concentrations of 29 ± 3.21, 19 ± 1.17 and 29 ± 0.92 µg/mL, respectively. Significantly high toxicity towards all the three cell lines was shown by the methanolic extract also. The half maximum cell death for UNMET extract was observed to be 1.9 ± 0.53, 3.6 ± 0.95 and 15 ± 1.56 µg/mL concentration for EAC, DLA, and splenocytes, respectively. The aqueous extract, however, was found to be less toxic to these cell lines (Figure 3.11 and Table 3.4).

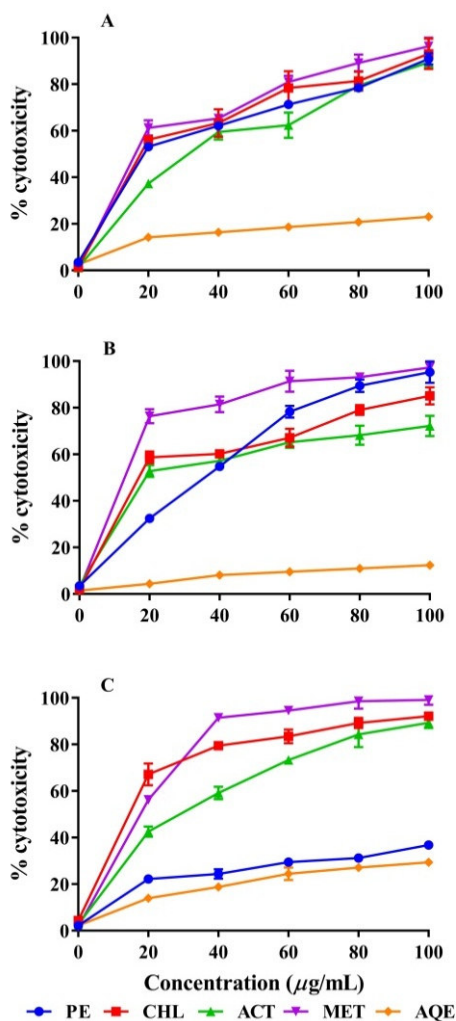


Figure 3.11. Cytotoxicity of *U. narum* leaf extracts towards (A) DLA, (B) EAC and (C) spleen cells. Various concentrations (10-100 µg/mL) of individual extracts were added to cell suspension in 1 mL PBS and incubated for 3hr at 37 °C. The cell death was determined by the Trypan blue method. Values are mean ± SD.

3.3.4.2. Effect of *Uvaria macropoda* extracts using trypan blue method

Uvaria macropoda extracts were found to be less cytotoxic to three of the cell lines compared to the effect of *U. narum* leaf extracts. All the extracts except the chloroform extract were found to be less toxic with an IC₅₀ concentration greater than 100 µg/mL. Chloroform extracts showed dose-dependent toxicity towards DLA cells with an IC₅₀ value of 57.60 ± 1.51 µg/mL. However, UMCHL extracts were observed to be less toxic towards EAC and splenocytes with IC₅₀ values greater than 100 µg/mL (Figure 3.12 and Table 3.4).

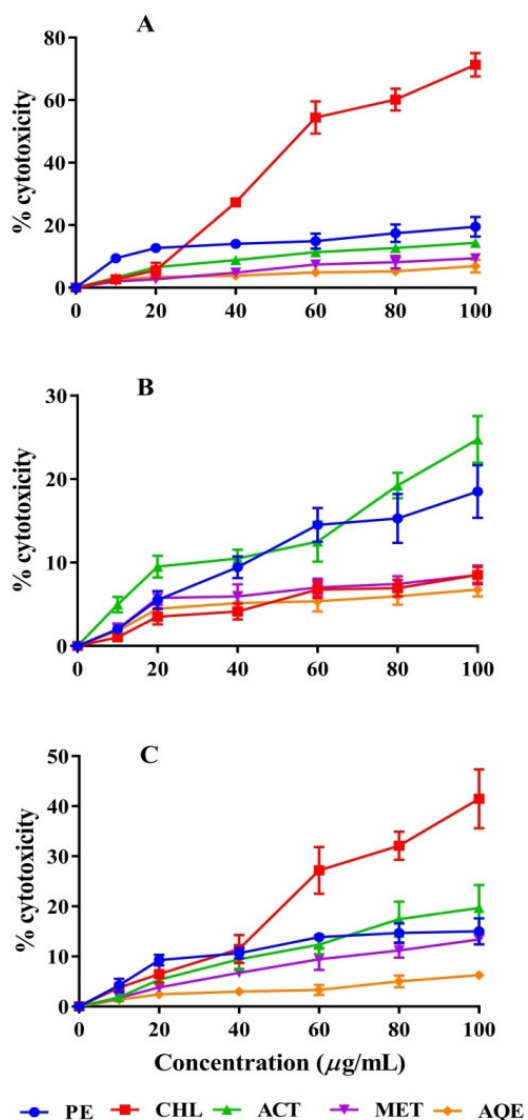


Figure 3.12. *In vitro* cytotoxicity of *U. macropoda* leaf extracts on (A) DLA, (B) EAC and (C) spleen cells. Various concentrations (10-100 µg/mL) of individual extracts were added to cell suspension in 1 mL PBS and incubated for 3hrs at 37 °C. The cell death was determined by the Trypan blue method. Values are mean ± SD.

Comparison of the IC₅₀ value of different extracts of *U. narum* and *U. macropoda* revealed that the *U. narum* extracts exhibited more cytotoxicity towards the three cell lines when compared to *U. macropoda* leaf extract. Among these extracts, UNPE extract showed differential toxicity towards DLA and EAC cell lines and was non-toxic to normal spleen cells (Table 3.4).

Table 3.4. IC₅₀ values of *U.narum* and *U.macropoda* leaf extracts on *in vitro* cytotoxicity study on DLA, EAC and spleen cells by trypan blue method.

Extract	IC ₅₀ (µg/mL)		
	DLA cells	EAC cells	spleen cells
<i>U.narum</i>			
PE	19.0 ± 0.57	38.0 ± 0.74	>100
CHL	15.0 ± 1.2	17.0 ± 0.99	9.1 ± 0.36
ACT	19.0 ± 1.17	29.0 ± 0.92	29.0 ± 3.21
MET	1.9 ± 0.53	3.6 ± 0.95	15.0 ± 1.56
AQE	>100	>100	>100
<i>U.macropoda</i>			
PE	>100	>100	>100
CHL	57.60 ± 1.51	>100	>100
ACT	>100	>100	>100
MET	>100	>100	>100
AQE	>100	>100	>100

3.3.5. Anti-proliferative activity of extracts on various cell lines

3.3.5.1. Effect of *U.narum* extracts using MTT assay

U.narum extracts exhibited remarkable antiproliferative effect over HeLa, HepG2, and HCT-15 cell lines. In HeLa (Human cervical carcinoma) cells, all the five extracts showed antiproliferative activity in a dose-dependent manner. UNPE exhibited inhibition in cell growth with an IC₅₀ of 20.10 ± 0.97 µg/ mL whereas with the chloroform, acetone, methanol and water extracts of *U.narum* the documented IC₅₀ values were 48.25 ± 1.2, 61.55 ± 1.07, 43.77 ± 0.42 and 97.98 ± 3.34 µg/mL, respectively (Figure 3.13 and Table 3.4). The IC₅₀ of UNPE extract in HepG2 (Human hepatocellular carcinoma cell) cell line was 15 ± 0.53 µg/ mL. All other extracts such

as chloroform, acetone, methanol, and water had IC_{50} values 78.80 ± 2.09 , 60.67 ± 1.48 , 83.95 ± 2.08 and $>100 \mu\text{g/mL}$, respectively (Figure 3.13 and Table 3.4). The antiproliferative effect in HCT-15 cells (Human colorectal adenocarcinoma cells) by the extracts was dose-dependent. UNPE, chloroform, acetone, methanol and water had an IC_{50} values of 5.29 ± 0.49 , 40.21 ± 2.37 , 61.43 ± 2.47 , 55.42 ± 3.01 and $>100 \mu\text{g/mL}$, respectively (Figure 3.13 and Table 3.4). In the case of Vero cells (kidney epithelial cells of African green monkey), UNPE extract showed an IC_{50} value of $43.72 \pm 1.35 \mu\text{g/ mL}$ and chloroform, acetone, methanol, and water extracts showed IC_{50} values of 26.21 ± 1.91 , 41.42 ± 2.63 , 38.05 ± 3.02 and $41.83 \pm 1.64 \mu\text{g/ mL}$, respectively (Figure 3.13 and Table 3.4). Water extract was found to be non-toxic to the cells used in the study.

The results suggest that the UNPE extract is more potent with selective toxicity towards HeLa, HCT-15 and HepG2 cells. Morphological changes observed following treatment with the extracts are depicted in Figure 3.14. In the untreated HepG2 cells, large numbers of vacuoles were observed. However, during treatment with UNPE and UNCHL extracts, these vacuoles were found to be reduced. The morphological changes were observed in UNPE, UNCHL and UNACT extract treated cancer cells.

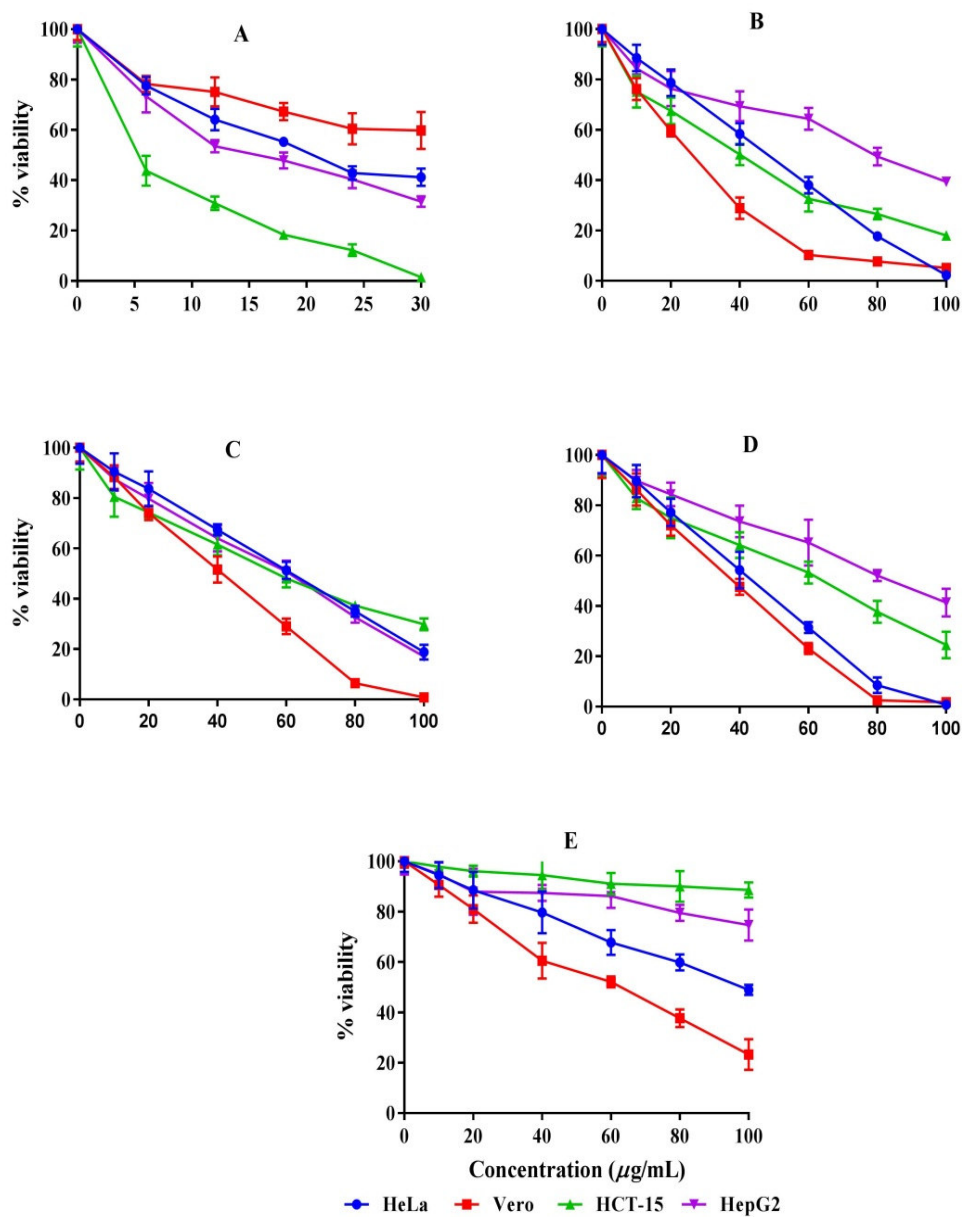


Figure 3.13. Antiproliferative activities of *U.narum*. A) UNPE, B) UNCHL, C) UNACT, D) UNMET and E) UNAQE extracts against HeLa, Vero, HCT-15 and HepG2 cell growth detected by MTT assay after 48 hr treatment. Values represent means \pm SD.

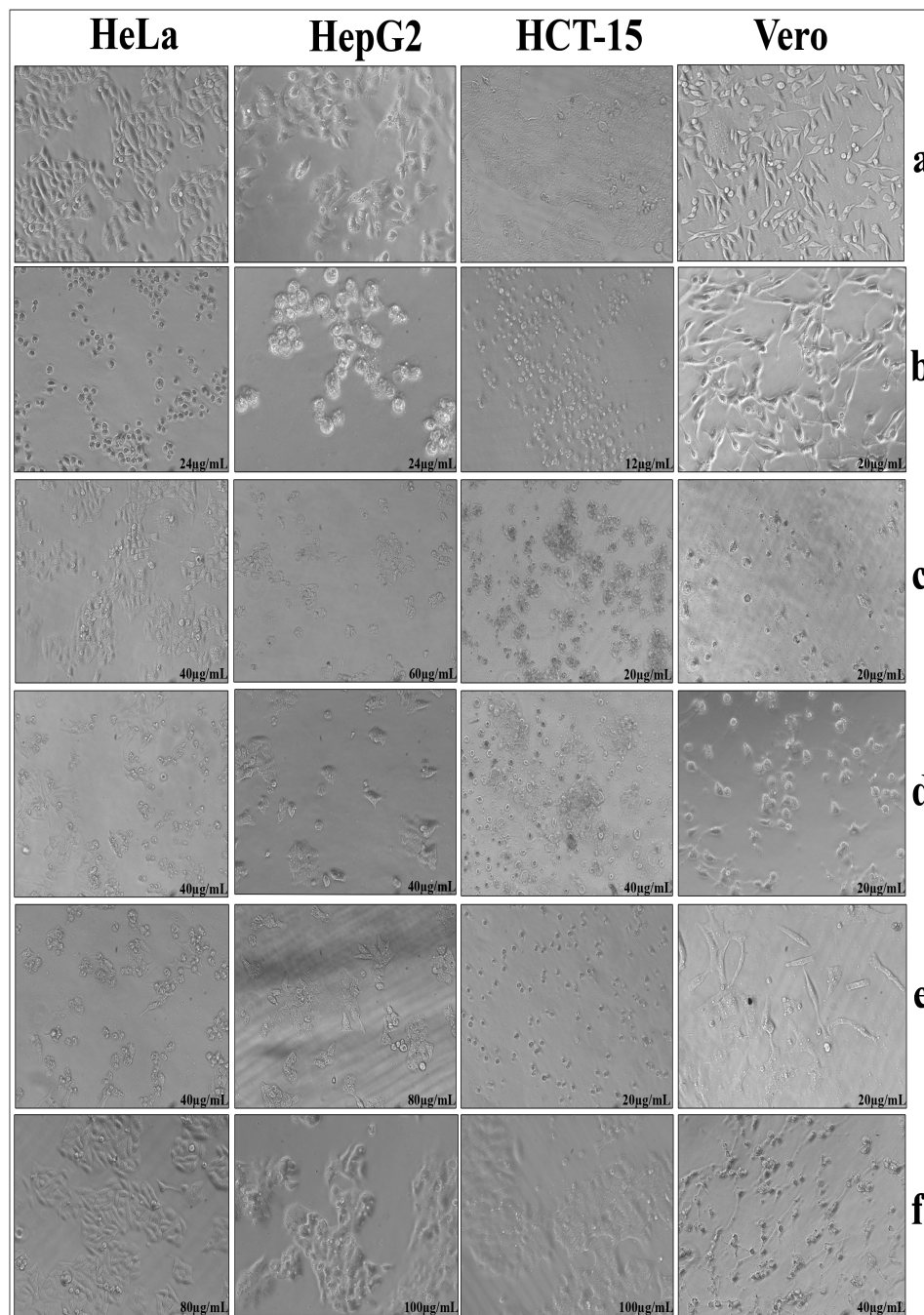


Figure 3.14. Morphological changes observed under phase contrast microscope 200X in HeLa, HepG2, HCT-15, and Vero cells, after 48 hr treatment with different extracts of *U.narum* leaves. a) Control b) UNPE c) UNCHL d) UNACT e) UNMET and f) UNAQE.

3.3.5.2. Effect of *U.macropoda* on various cell lines using MTT assay

Antiproliferative activity of different extracts of *U.macropoda* leaf was analyzed using the MTT assay. All the five extracts of *U.macropoda* were found to be less toxic to HeLa as well as HepG2 cells with IC₅₀ values greater than 100 µg/mL (Figure 3.15 and Table 3.5). UMPE, UMCHL and UMACT extracts exhibited antiproliferative property against HCT-15 cells with IC₅₀ values of 97.25 ± 2.97, 39.92 ± 1.49, and 99.12 ± 4.92 µg/ mL, respectively. UMMET and UMAQE extracts showed less cytotoxicity with IC₅₀ values of more than 100 µg/ mL (Figure 3.15 and Table 3.4).

UMPE extract exhibited antiproliferative activity against Vero cells with an IC₅₀ value of 91.49 ± 3.48 µg/ mL and that of chloroform, acetone, methanol, and water extracts were found to show IC₅₀ values greater than 100 µg/ mL (Figure 3.15 and Table 3.4). As in the *U.narum* water extract, water extract of *U. macropoda* was also found to be non-toxic in the cells studied. The results suggest that the antiproliferative activity of different extracts of *U.macropoda* towards HeLa, HCT-15 and HepG2 cells was less compared to that of *U.narum* extracts.

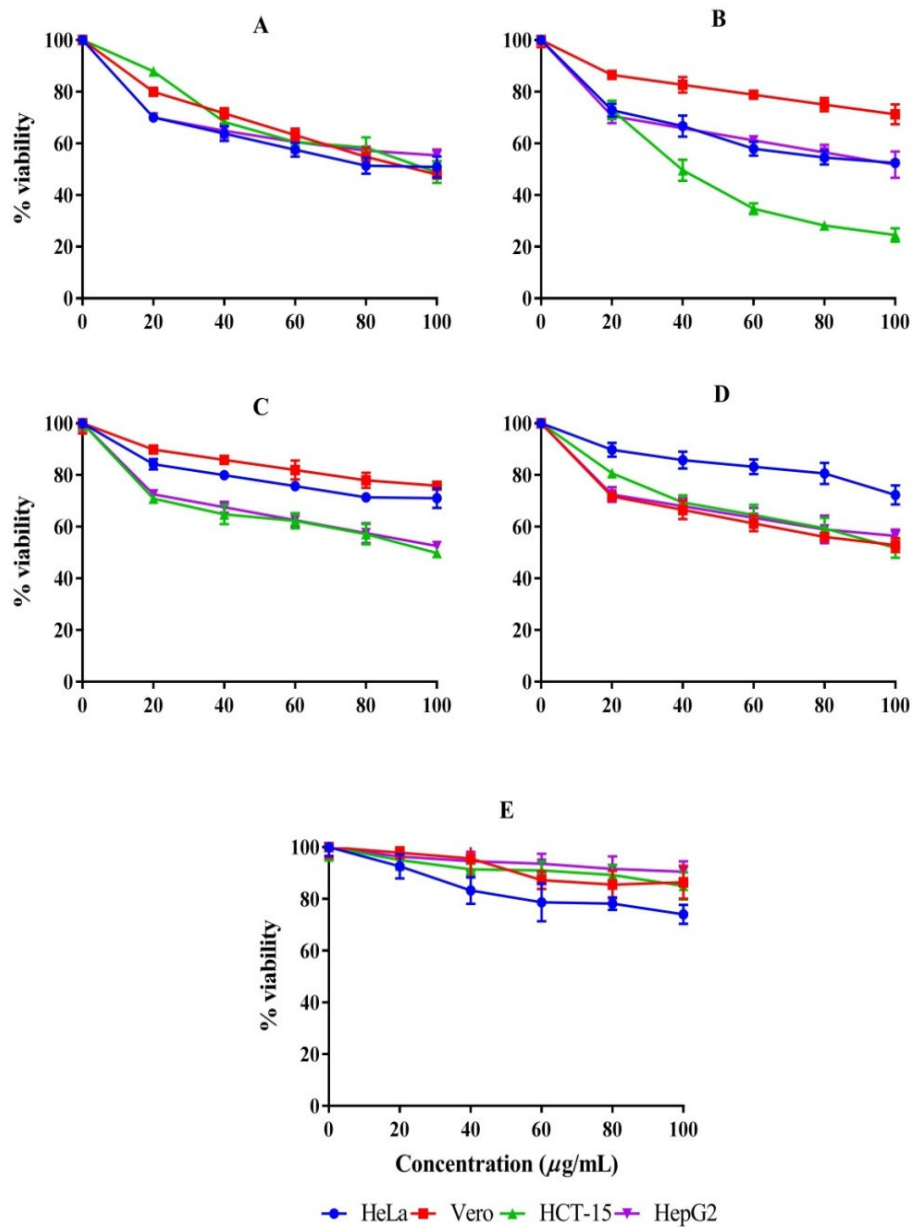


Figure 3.15. Antiproliferative activities of *U. macropoda* A) UMPE, B) UMCHL C) UMACT D) UMMET and E) UMAQE extracts against HeLa, Vero, HCT-15 and HepG2 cell growth detected by MTT assay after 48 hr treatment. Values represent means \pm SD.

Table 3.5. IC₅₀ values of *U.narum* and *U.macropoda* leaf extracts on *in vitro* antiproliferative study on HeLa, Vero, HCT-15 and HepG2 cells by MTT assay.

Extracts	IC ₅₀ (μg/mL)			
	HeLa	Vero	HCT-15	HepG2
<i>U.narum</i>				
PE	20.10 ± 0.97	43.72 ± 1.35	5.29 ± 0.49	15 ± 0.53
CHL	48.25 ± 1.2	26.21 ± 1.91	40.21 ± 2.37	78.80 ± 2.09
ACT	61.55 ± 1.07	41.42 ± 2.63	61.43 ± 2.47	60.67 ± 1.48
MET	43.77 ± 0.42	38.05 ± 3.02	55.42 ± 3.01	83.95 ± 2.08
AQE	97.98 ± 3.34	41.83 ± 1.64	>100	>100
<i>U.macropoda</i>				
PE	>100	91.49 ± 3.48	97.25 ± 2.97	>100
CHL	>100	>100	39.92 ± 1.49	>100
ACT	>100	>100	99.12 ± 4.92	>100
MET	>100	>100	>100	>100
AQE	>100	>100	>100	>100

3.3.6. *In vitro* antioxidant activity of various extracts of *U.narum* and *U.macropoda*

The different solvent extracts from *U.narum* leaves exhibited varying degrees of antioxidant activity *in vitro*. Among the different plant extracts analyzed, acetone and methanolic extracts showed significant antioxidant potential. *U. macropoda* extracts also exhibited antioxidant activity in a dose-dependent manner.

3.3.6.1. Reducing potential of extracts

3.3.6.1.1. DPPH radical scavenging activity

The stable free radical, DPPH was effectively scavenged by different leaf extracts of *U. narum*. A dose-dependent reduction of DPPH free radical was observed within a concentration range of 10-100 μg/mL of the extract added to the reaction. An extract

with a lower IC₅₀ value indicates its higher antioxidant potential. UNACT as well as UNMET extracts showed strong antioxidant activity with IC₅₀ values 19.07 ± 1.76 and 18.65 ± 0.93 µg/mL, respectively followed by UNCHL, UNPE and UNAQE (IC₅₀ values 80 ± 4.27, 96 ± 3.15 and >100 µg/mL, respectively) (Figure 3.16A). In all these extracts, when the concentration of the extract increased, the radical scavenging efficacy also increased. The maximum percentage of radical-scavenging activity was observed in the UNMET followed by UNACT extract (Figure 3.16A). IC₅₀ for Vitamin C in this assay was found to be 2.8 ± 0.14 µg/mL.

DPPH radical scavenging activity of leaf extracts of *U.macropoda* are depicted in Figure 3.17A. The scavenging ability of different solvent extracts of *U.macropoda* were concentration-dependent with IC₅₀ values 91.42±2.43, >100, 73.54±3.65, 97.64±4.13 and >100 µg/mL for UMPE, UMCHL, UMACT, UMMET and UMAQE extracts, respectively. The UMACT extract exhibited the strongest DPPH radical scavenging activity compared to other extracts.

3.3.6.1.2. Reducing power of the extracts using FRAP assay

The antioxidative property has been reported to be related to reducing power of the extracts. All the extracts of *U.narum* exhibited ferric reducing antioxidant capacity in a dose-dependent manner (Figure 3.16C and D). However, the rate of FRAP activity was found to vary with the extracting solvent used. It was seen that the UNPE and water extracts possessed moderate FRAP values with EC₅₀ of 20.97 and 60 µg/mL, respectively (Figure 3.16D) while UNMET and UNCHL extracts exhibited maximum FRAP followed by UNACT extracts with EC₅₀ values of 4.13 ± 0.73, 5 ± 0.24, and 7.68 ± 1.03 µg/mL, respectively (Figure 3.16C).

The antioxidant potential of different extracts of *U .macropoda* leaves was estimated for their ability to reduce TPTZ–Fe (III) complex to TPTZ–Fe (II). The ferric reducing ability of the extracts revealed that UMACT and UMMET extracts of *U .macropoda* had potent FRAP activity (with EC₅₀ values 46.23± 1.45 and 38.41± 3.41 µg/mL, respectively). All other extracts showed low reducing power with EC₅₀ values >100 µg/mL (Figure 3.17C).

3.3.6.2. Effect of extracts on lipid peroxidation

3.3.6.2.1. Inhibition of lipid peroxidation by extracts

Peroxidation of polyunsaturated lipids in the membrane causes changes in the permeability of the membrane and thereby leads to cellular damage. The action of extract reduces the Fe^{2+} induced significant lipid peroxidation (LPO) in tissue homogenates. Among the various extracts prepared from *U.narum* leaves chloroform and methanolic extracts showed the highest inhibition of lipid peroxidation with IC_{50} values 77 ± 2.49 and $76 \pm 1.27 \mu\text{g/mL}$, respectively. The other extracts such as UNPE and acetone also showed the inhibitory effect with IC_{50} values 96 ± 2.42 and $88 \pm 4.13 \mu\text{g/mL}$. The water extract was found to be less effective in showing an IC_{50} of more than $100 \mu\text{g/mL}$ (Figure 3.16B). Ascorbic acid showed a 50% inhibition of lipid peroxidation at $39.30 \pm 2.97 \mu\text{g/mL}$.

Among the extracts of *U.macropoda*, UMACT extract showed the highest percentage inhibition of LPO in liver homogenate with an IC_{50} value of $77.42 \pm 1.90 \mu\text{g/mL}$. IC_{50} values determined were 80 ± 4.91 , 78 ± 3.72 , 78.14 ± 2.13 and $91 \pm 3.21 \mu\text{g/mL}$ for UMPE, UMCHL, UMMET and UMAQE, respectively (Figure 3.17B).

3.3.6.2.2. Lipoxygenase inhibition

The inhibitory effect of lipoxygenase catalyzed the reaction by the *U.narum* extracts revealed their antioxidant activity. Here, the UNMET extract showed maximum inhibitory activity with an IC_{50} value of $50 \pm 2.23 \mu\text{g/mL}$ followed by UNCHL and acetone extracts with 51 ± 3.48 and $53 \pm 1.37 \mu\text{g/mL}$, respectively (Figure 3.16E). The other extracts also showed the inhibition of lipoxygenase activity in a dose-dependent manner with IC_{50} values 85 ± 6.29 and $96 \pm 3.72 \mu\text{g/mL}$ for UNPE, and water extracts, respectively. The IC_{50} value for vitamin C was $47 \pm 1.22 \mu\text{g/ml}$. UNCHL and UNACT fractions showed a similar inhibitory effect to that of Vitamin C.

Lipoxygenase enzyme activity was also inhibited by different extracts of *U.macropoda* thereby showing their antioxidant potential. UMACT extract of *U.macropoda* showed maximum inhibitory activity with an IC_{50} value of $56.01 \pm 6.22 \mu\text{g/mL}$ (Figure 3.17D). The other extracts also showed inhibition in lipoxygenase

activity in a dose-dependent manner with IC₅₀ values were >100, 74.39 ± 5.25, 63.62 ± 2.27 and >100 µg/mL for UMPE, UMCHL, UMMET, and UMAQE extracts, respectively.

3.2.6.3. Quantitative estimation of antioxidant compounds

3.3.6.3.1. Estimation of total phenol and total flavonoid content

The total phenolic content in different crude extracts was expressed in gallic acid equivalents (GAE). Total phenolic content determined in different solvent extracts of *U.narum* species is depicted in Table 3.5. The highest amount of phenolic compounds was found in the crude methanol which was 131.57 ± 1.84 mg of GAEs/g extract followed by the UNCHL (125.60 ± 3.92 mg of gallic acid equivalents/ g), UNACT extract (78.93 ± 4.66 mg of GAEs/g extract), UNPE (77.20 ± 3.67mg of GAEs/g extract), and water extract (19.44 ± 0.63 mg of GAEs/g extract) (Table 3.5). Among these extracts, the highest level of flavonoids is 66.54 ± 2.47 mg quercetin/g in acetone extract followed by 62.14 ± 1.81mg quercetin/g in methanol extract and 49.67 ± 0.92 mg quercetin/g in UNCHL extract. The total flavonoid content for UNPE extract was 14.80 ± 1.45mg quercetin/g of extract. UNAQE extract found to have a flavonoid content of 3.27 ± 0.65 mg quercetin/g of extract (Table 3.6).

CHL extract of *U.macropoda* also contained phenolics (38.82 ± 22.84 mg GAE /g extract), and flavonoids (19.3 ± 12.39 mg QTE /g extract). However, among the sample extracts the lowest concentrations of phenolics (3.77 ± 5.68 mg GAE /g extract) and flavonoids (1.12 ± 0.43 mg QTE /g extract) were observed in UMAQE extract (Table. 3.6).

Table 3.6. Total phenols and flavonoids of different extracts of *U. narum* and *U. macropoda* leaves

Extracts	Total phenol content (mg GAE /g extract)	Total flavonoid content (mg QTE /g extract)
<i>U.narum</i>		
PE	77.20 ± 3.67	14.80 ± 1.45
Chloroform	125.60 ± 3.92	49.67 ± 0.92
Acetone	78.93 ± 4.66	66.54 ± 2.47
Methanol	131.57 ± 1.84	62.14 ± 1.81
Water	19.44 ± 0.63	3.27 ± 0.65
<i>U.macropoda</i>		
PE	7.73 ± 1.24	1.93 ± 0.41
Chloroform	42.88 ± 2.79	9.14 ± 2.81
Acetone	22.95 ± 2.13	14.64 ± 2.94
Methanol	38.82 ± 22.84	19.3 ± 12.39
Water	3.77 ± 5.68	1.12 ± 0.43

Values are expressed as mean ± SD

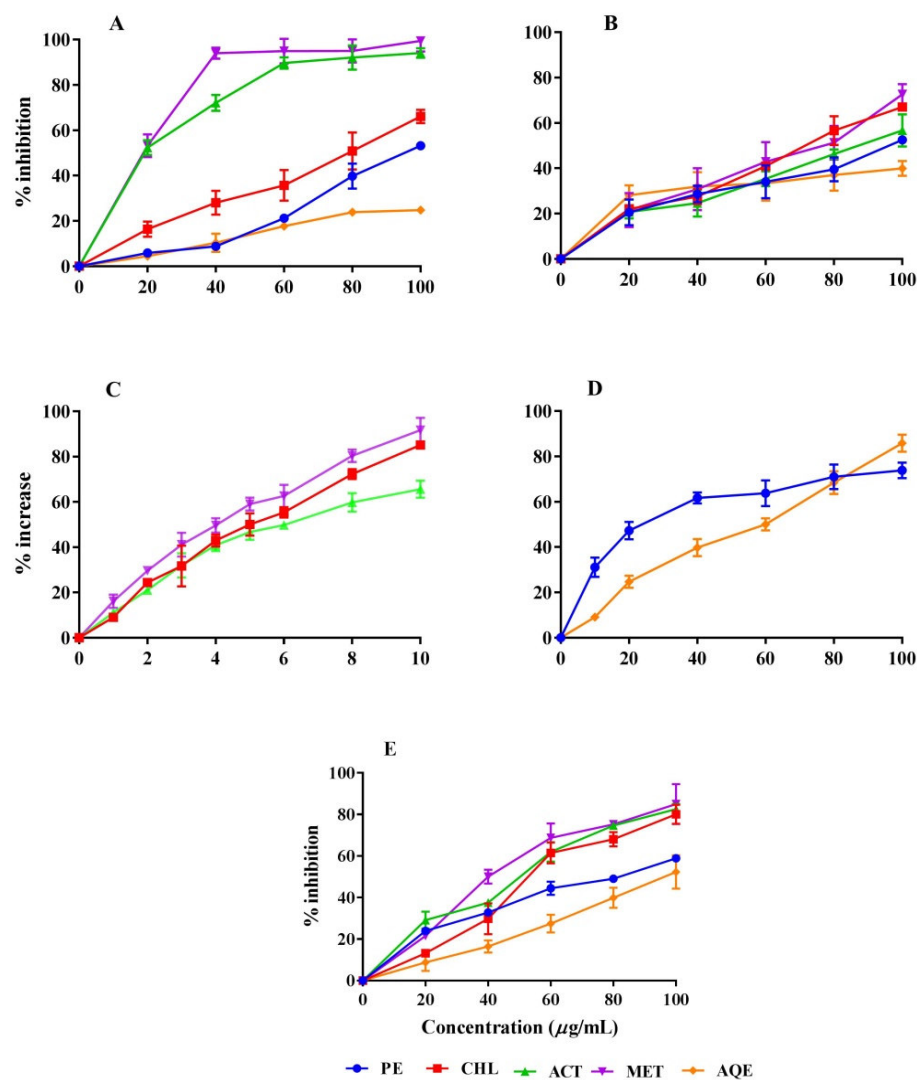


Figure 3.16. *In vitro* free radical scavenging activities of PE, chloroform, acetone, methanol and water extracts of *U. narum* leaves. A) DPPH radical scavenging assay; B) inhibition of lipid peroxidation; C) FRAP assay: chloroform, acetone and methanol extracts; D) FRAP assay: PE and water extracts and E) lipoxygenase inhibition assay. Data are the mean \pm SD.

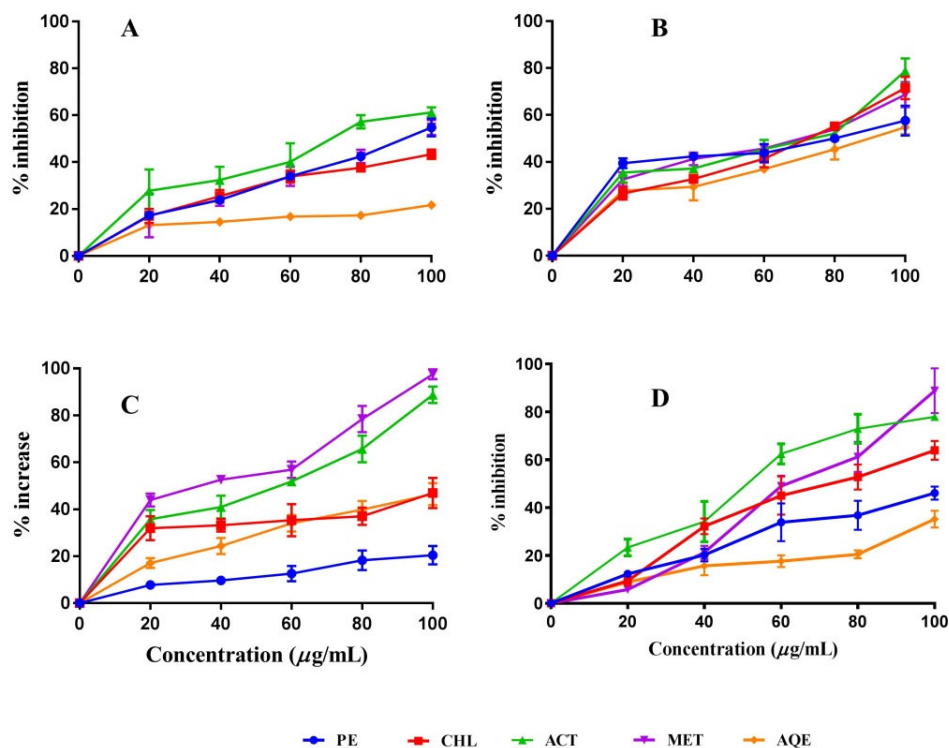


Figure 3.17. *In vitro* free radical scavenging activities of PE, chloroform (CHL), acetone, methanol (MET) and water (AQE) extracts of *U. macropoda* leaves. A) DPPH radical scavenging assay B) inhibition of lipid peroxidation and C) FRAP assay and D) lipoxygenase inhibition assay. Data are the mean \pm SD.

3.4. Discussion

In the present study, *Uvaria narum* and *Uvaria macropoda* extracts prepared based on polarity were found to possess cytotoxic/antiproliferative and antioxidant efficacies. Phytochemical identification by biochemical and TLC analysis of individual extracts of both plants reveal a more or less similar class of compounds. However, on a comparison of IC_{50} values, individual extracts employing different assay systems projects *U. narum* as a better source of cytotoxic/antiproliferative and antioxidant phytomolecules. The compounds present in each extract may vary according to their polarity due to the polarity based extraction and the concentration of phytocompounds present in each extract may vary. Synergistic action of different compounds in each

extract may vary, which could explain the differential pharmacological efficacy of the two plants.

All the extracts of *U.macropoda* (UMPE, UMCHL, UMACT, UMMET, and UMAQE) were found to be cytotoxic to DLA, EAC and spleen (normal) cells in the short term cytotoxicity assay. These extracts exhibit antiproliferative activity on all the cancer cells studied in the long-term antiproliferative assay with documented IC₅₀ values above 100 µg/ml whereas UMCHL extract is an exception, which shows cytotoxicity towards DLA cells with an IC₅₀ value of 57.60 + 1.51 µg/mL. On the other hand in both the short-term and long-term cytotoxicity assays, individual extracts of *U. narum* such as UNCHL, UNACT, and UNMET are found highly cytotoxic (IC₅₀ values below 100 µg/mL) to all cells including normal cells while the aqueous extract did not show appreciable toxicity (IC₅₀ value above 100 µg/mL). Interestingly, UNPE extract showed differential cytotoxicity, with toxicity towards cancer cell lines and less toxicity to normal spleen cells. Thus, from the screening for cytotoxicity among *U.narum* and *U.macropoda* extracts, *U. narum* petroleum ether (UNPE) extract has been found most active due to its promising differential cytotoxic/antiproliferative efficacy. This differential cytotoxicity forms the basis of the selection of PE extract for further *in vivo* antitumor studies.

Phytochemical and thin layer chromatography analysis of all extracts from both plants gave a number of compounds such as terpenoids, sterols, alkaloids, phenols, flavonoids, etc. The results obtained are similar to the results reported in earlier studies (Varghese 2013). Among these classes of compounds, terpenoids and phytosterols are detected as major phytoconstituents in UNPE extract; the most potent extract selected based on cytotoxic potential. Confirming this, these important classes of compounds could be visualized in TLC analysis of the extract. Sterols and terpenoid class of compounds are well known for their antitumor properties (Ismail *et al.* 2009; Ridley and Faulkner 2003; Menchinskaya *et al.* 2013). Vincristine and vinblastine are potent alkaloids isolated from *Vinca rosea* and taxol is a triterpene extracted from Pacific yew tree bark (*Taxus brevifolia*) (Wani *et al.* 1971). It is thus expected that the cytotoxic effect observed in the UNPE extract may be due to its terpenoid or sterol content. Many cancer chemotherapeutic drugs including vincristine, vinblastine, and taxol are reported to have side effects due to their effects

on the immune cells. The spleen is an important organ in the immune system towards which UNPE extract showed less toxicity in our studies. This indicates that the possible active components of UNPE extract (terpenoid and sterols) are likely to have fewer side effects on the body's immune system. This observation opens up avenues for further purification of the cytotoxic components in the UNPE extract to identify important chemotherapeutic drug candidates.

Moreover, the phytosterol, flavonoid and phenolic contents detected in both the *Uvaria* plant extracts may have other biological effects too. Many phytosterols are reported to elicit hormonal signaling thereby acting as important physiological regulators (Burow *et al.* 2001; Schabath *et al.* 2005; Stark and Madar 2002). Phenolics and flavonoids are important classes of antioxidant and anti-inflammatory molecules (Foti 2007; Pietta 2000). It has been found that all the extracts of both plants are effective in scavenging free radicals, inhibiting lipoxygenase mediated oxygenation and peroxidation of polyunsaturated fatty acids and enhancing ferric reducing capacity in a dose-dependent manner. In support of this, an appreciable amount of polyphenols and flavonoids are quantitatively recorded in these extracts. When their antioxidant efficacy was assessed using ferric reducing antioxidant potential (FRAP) 1, 1-diphenyl-2-picrylhydrazyl (DPPH) reducing efficacy, inhibition of lipoxygenase and lipid peroxidation, the UNMET extract showed promising antioxidant activity. However, *U. macropoda* extracts were found to be comparatively less active in the antioxidant property. The phytochemical assessment showed that UNMET is rich in polyphenols, flavonoids, terpenes, and alkaloids. The polyphenols, alkaloids, and carotenoids are reported to have antioxidant activity (Xu *et al.* 2017a; Ramesha *et al.* 2013; Tyagi *et al.* 2010; Kingston *et al.* 1977; Zlotek *et al.* 2016). The antioxidant activities of monoterpenes and diterpenes or essential oils *in vitro* have also been reported. As reported by (Lou *et al.* 2017) alkaloids from *Juglans mandshurica* markedly caused apoptosis and autophagy, in HepG2 cells. This indicates that the antioxidant properties shown by UNMET extract may be due to its terpenoid, alkaloids or polyphenolics and flavonoids contents. Chemical nature of individual phytochemicals in each extract should thoroughly be checked.

An important point to be considered in this context is that the interaction of phytoconstituents, especially combinations of hydrophilic and lipophilic antioxidant

molecules may contribute to a synergistic effect on the crude extract (Grassmann 2005). Higher amounts of phenols and flavonoids are reported in UNMET which show greater antioxidant activities. Therefore it can be concluded that *U.narum* leaf is an important source of biologically relevant cytotoxic and antioxidant phytoconstituents which require further exploration. The UNPE extract which exhibited cytotoxic potential and UNMET extract that showed high antioxidant potential are likely candidates that can be explored further to identify the bio-active components within them.

Chapter 4

*Safety evaluation of pharmacologically active extracts of *U.narum* leaves*

4.1. Introduction

Natural products and plant extracts are believed to be safer than synthetic compounds. However, there are several pharmacologically important plant extracts which are not suitable for the administration because of its toxicity. This issue has been discussed continuously for a long time. So, before the use of any plant extract or plant-derived product, it is necessary to determine the toxicity levels and safe doses for administration. The safest dose should also be calculated before the pre-clinical studies using systematic toxicity studies (OECD guidelines).

Some plants belonging to the Annonaceae have been reported to possess toxic effects on humans. For instance, studies have shown that repeated consumption of *Annona muricata* fruit could cause neuronal dysfunction and degeneration, an underlying cause of the West Indian Parkinsonian syndrome (Lannuzel *et al.* 2002). Since *U.narum* is a plant under the Annonaceae family, it is very important to evaluate the safe dose of its extract before administration in animals.

Screening of different extracts of *U.narum* and *U.macropoda* for their cytotoxicity and efficacy in preclinical studies is essential for their further advancement in drug development. Safety evaluation using acute and sub-acute toxicity models is a prerequisite for the selection of pharmacological doses of these extracts.

The present chapter describes the detailed evaluation of acute and sub-acute toxicity investigations carried out to evaluate the safety of UNPE and UNMET. The experiments followed OECD guidelines and acute toxicity studies followed for 14 days using both sexes of mice and determined the LD₅₀ value for each extract. This LD₅₀ value has been the basis for the calculation of doses for further sub-acute toxicity evaluation over a period of 28 days.

4.2. Materials and methods

4.2.1. Preparation of UNPE and UNMET extracts

UNPE and UNMET extracts were prepared by the method mentioned in Chapter 2, section 2.2.1. The extracts were prepared in 1% propylene glycol to desired concentrations for administration in mice (different doses in 0.2 mL). Propylene

glycol (1%) was administered to animals in the vehicle control group during the experimental period.

4.2.2. Animals

Male and female Swiss albino mice (25-30 g) were purchased and maintained in the animal house of Amala Cancer Research Centre for acute toxicity studies and repeated dose 28-day oral sub-acute toxicity studies as mentioned in Chapter 2, section 2.1.6. The animal experimentation protocol was approved by the Institutional Animal Ethical Committee (IAEC) (Approval No. ACR/IAEC/15/06-(I) and the care and keeping of the experimental animals were carried out as per the CPCSEA regulations.

4.2.4. Acute and sub-acute toxicity evaluation of *U.narum* extracts

4.2.4.1. Acute toxicity studies

Acute toxicity was determined as per the Organization for Economic Co-operation and Development (OECD) guideline 423(OECD 2001), where a single high dose of 2,000 mg/kg b.wt. of crude PE and MET extract of *U.narum* was administered orally to animals of both sexes three male and three female Swiss Albino mice, respectively. A single dose of each concentration of the extract such as 1000, 500, 250, 100 mg/kg b.wt. were administered orally after overnight fasting of animals. Animals in the vehicle control group (three male and three female mice) received 1% propylene glycol orally. The acute toxicity study is described in chapter 2, section 2.2.12. 1.

4.2.4.2. Repeated dose sub-acute toxicity study of UN extracts in mice

Swiss albino mice (25-30gm) were divided into 8 different groups (5 males + 5 females animals per each dose) and categorized as an untreated group; vehicle control (1% propylene glycol) treated group; 200 mg/kg b.wt. UNMET treated (LD); 300 mg/kg b.wt. UNMET (MD); 400 mg/kg b.wt. UNMET (HD), 5 mg/kg b.wt. PE extract of *U.narum* leaves treated (UNPELD); 7.5 mg/kg b.wt. UNPE extract (MD) and 10 mg/kg b.wt. UNPE extract (HD). The dosages of the extracts were determined based on acute toxicity studies. Extracts were administered daily at a specific time orally for 28 days. The animals were observed for toxic manifestation, clinical and

behavioral symptoms like diarrhea, neuromuscular problems, mortality and behavioral symptoms. The changes in body weight, food consumption, and water consumption were recorded for each group every three days during the course of the experiment. At the end of the 4th week, all the animals were sacrificed by ether anesthesia. Collection of blood was done by direct heart puncture. Blood collected in EDTA coated tubes were analyzed for hematological parameters as mentioned in chapter 2, section 2.2.12.2.1. Some amount of blood was collected in test tubes and kept for clotting, centrifuged at 3000 rpm for 10 minutes and serum was separated. This collected serum was used for determining liver function tests such as serum aspartate transaminase (SGOT/ AST), Serum glutamate pyruvate transaminase (SGPT/ALT), alkaline phosphatase (ALP) and total bilirubin, lipid profiling such as total cholesterol, triglycerides, HDL, LDL and VLDL levels and kidney function tests such as creatinine and urea. Commercial diagnostic reagent kits were used to evaluate these parameters (mentioned in chapter 2, sections 2.2.12.2.2 and 2.2.12.2.3). Electrolytes such as sodium, potassium, chloride and bicarbonate ions were determined using the collected serum (Chapter 2, section 2.2.12.2.2.1.). The weight of the brain, liver, stomach, kidney, intestine, heart, lungs, spleen and ovary/testes were excised and expressed in respect to the final day body weight and organ somatic index calculated. Sections of tissues from liver, brain, lungs, spleen, stomach, kidney, small intestine, and ovary/testes were removed, fixed in 10% neutral buffered formalin, trimmed and tissue sections of 4 μ thickness were stained with hematoxylin and eosin and histologically analyzed under light microscope (200 x) (Chapter 2, section 2.2.18.).

4.2.5. Statistical analysis

The values are expressed as a mean \pm standard error of the mean. The statistical significance was compared between control and experimental groups by one-way analysis of variance (ANOVA) followed by appropriate post-hoc Dunnet test using Graph pad in Stat software (version 3.05). Data of *U. narum* extract treated animals, as well as vehicle control groups, were compared with animals without any treatment. A value of $p < 0.05$ was considered statistically significant.

4.3. Results

4.3.1. Acute oral toxicity effect of UNPE and UNMET extracts on Swiss albino mice

Acute toxicity testing is carried out to evaluate the effect of a single dose of drug or extract on a particular animal species (Parasuraman 2011). The acute toxicity (single dose) test of *U.narum* extracts revealed that oral administration of 2000 mg/kg b.wt. of UNPE showed significant toxicity, as all animals died within the first 3 hrs. Repetition of the experiment gave the same result. The fixed dose procedure (FDP) using lower doses such as 1000, 500, 250, 100 mg/kg b.wt. were then carried out for both sexes of animals, with three animals each. The extract, when administered above 250 mg/kg b.wt. was found to have toxic effects on animals. There was a sign of sedation, lethargy, and drowsiness after the administration of plant extracts at doses above 250 mg/kg b.wt. when compared to the untreated control group. Therefore, the LD₅₀ was considered as dose >250 mg/kg b.wt. The dose lower than or equal to 1/5th and 1/10th of the lethal dose was used for further *in vivo* studies. Based on this, the safe doses for sub-acute studies were selected as 5, 7.5 and 10 mg/kg b.wt.

No treatment-related toxic symptom or mortality was observed following oral administration of a single 2000 mg/kg b.wt. of UNMET leaf extract to mice (five animals each of both sexes) during the 14 day observation period and this was confirmed in repeated tests. The general behavioral pattern of the extract treated animals and the untreated control group was monitored and found to have no drug-related changes in breathing pattern, behavior, skin, water consumption, and food intake. There was no adverse effect on the body weight of treated animals when compared to normal ones (Table 4.1). Therefore, the extract was considered to be safe at a dose level of 2000 mg/kg, and the LD₅₀ was considered to be >2000 mg/kg b.wt. So the doses selected for sub-acute toxicity studies of UNMET extract were 200, 300 and 400 mg/kg b.wt, respectively.

Table 4.1. Change in average weight (g) of mice following single dose oral administration of UNMET extract.

Groups	Days		
	1	7	14
Males			
Normal	25.425 ± 1.3	26.95 ± 0.69	28.45 ± 1.09
Vehicle control	26.01 ± 0.59	26.72 ± 1.22	27.48 ± 1.52
ME 2 g/kg b.wt.	25.55 ± 0.9	26.55 ± 1.02	27.975 ± 1.04
Female			
Normal	26.85± 0.8	27.25± 0.94	27.7± 1.04
Vehicle control	25.55 ± 0.79	25.73 ± 0.68	26.10± 0.87
ME 2 g/kg b.wt.	26.01± 0.94	26.72± 0.39	27.01± 0.51

Values are expressed as Mean ± SD for three animals

4.3.2. Sub-acute toxicity study of UN extracts in Swiss albino mice

The sub-acute toxic effects of UN extracts were evaluated as per OECD guideline 407 (OECD 2008). Repeated dose toxicity analysis is done for a minimum of 28 days (Parasuraman 2011). The animals were observed thoroughly for any changes in the general behavior, blood and biochemical parameters.

4.3.2.1. Effect on the behavioral pattern

The UNPE and UNMET extracts, when administered at sub-acute doses (mentioned in the previous section), did not show any visual symptoms of toxicity or mortality in animals compared to the control group during the entire 28 days of the experimental period. Also, no significant changes in body weight were seen in animals treated with extract when compared to the animals in the control group during treatment for 28 days. All the animals showed normal weight changes without a marked increase or decrease. The appearance of organs inside the body was also found to be normal (Figure 4.1).

General conditions and behavior of mice were not adversely affected by the administration of the UNMET extract at doses of 200, 300 and 400 mg/kg b.wt. for 28 days. There was no mortality in any of the groups. The animals were healthy and no signs of toxicity were observed during the period of study whereas animals were found to be weak and lethargic when administered a higher dose of UNPE extract (10 mg/kg b.wt.).

4.3.2.2. Body weight changes of male and female Swiss albino mice after sub-acute oral administration of UN leaf extracts

There were no significant changes ($P < 0.05$) in the body weights of the male and female mice following oral administration of UNMET as well as UNPE extracts in different doses, figures 4.2 and 4.3, respectively.

4.3.2.3. Relative organ weight of male and female Swiss albino mice upon sub-acute oral administration of UN extracts

Table 4.2 shows principal organ weights relative to body weight. Change in organ weight of liver, kidney, spleen, stomach, intestine, heart, lungs, brain and ovary/testes with respect to body mass index of UNMET administered animals were non-significant ($P > 0.05$) when compared with the untreated animals. Similarly, no significant increase in the weight of visceral organs was observed ($P > 0.05$) in UNPE administered experimental animals after 28 days when compared to the untreated normal or vehicle-treated groups. The organosomatic index for UNPE treated animals is showed in Table 4.3.

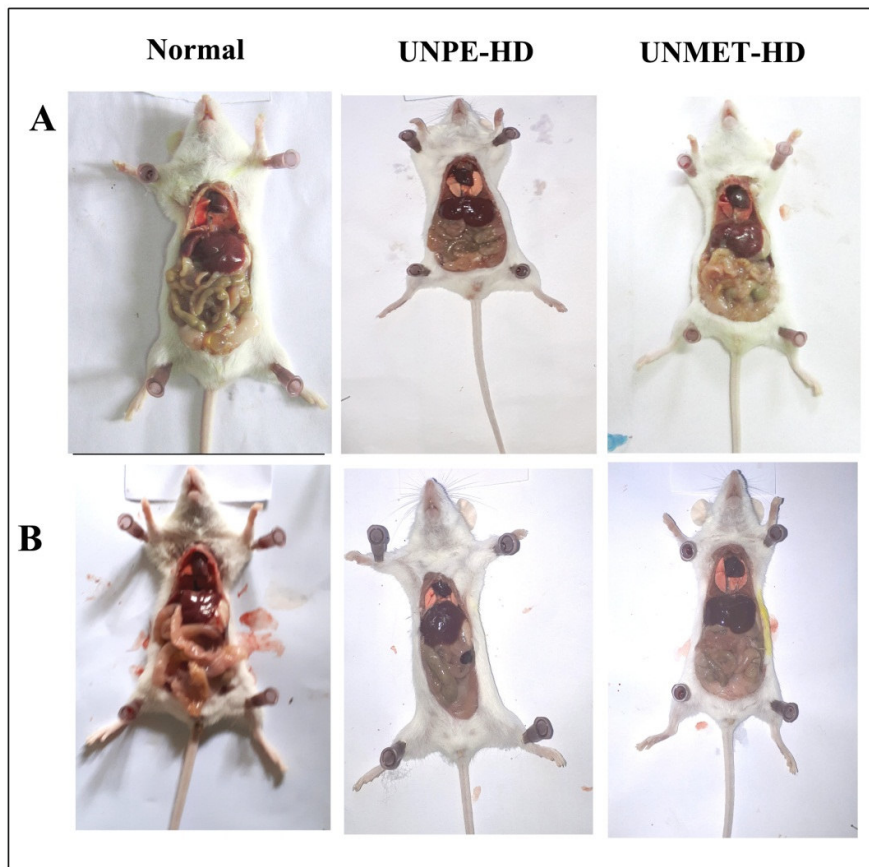


Figure 4.1. Observation of systemic organs and the body of treated animals: A) Male, B) Female Swiss albino mice were orally given UNMET-High Dose: 400 mg/b.wt and UNPE-High Dose: 10 mg/kg b.wt. of extract for 28 days. Following overnight fasting, the animals were sacrificed and dissected to monitor the organs in the thoracic and abdominal cavity.

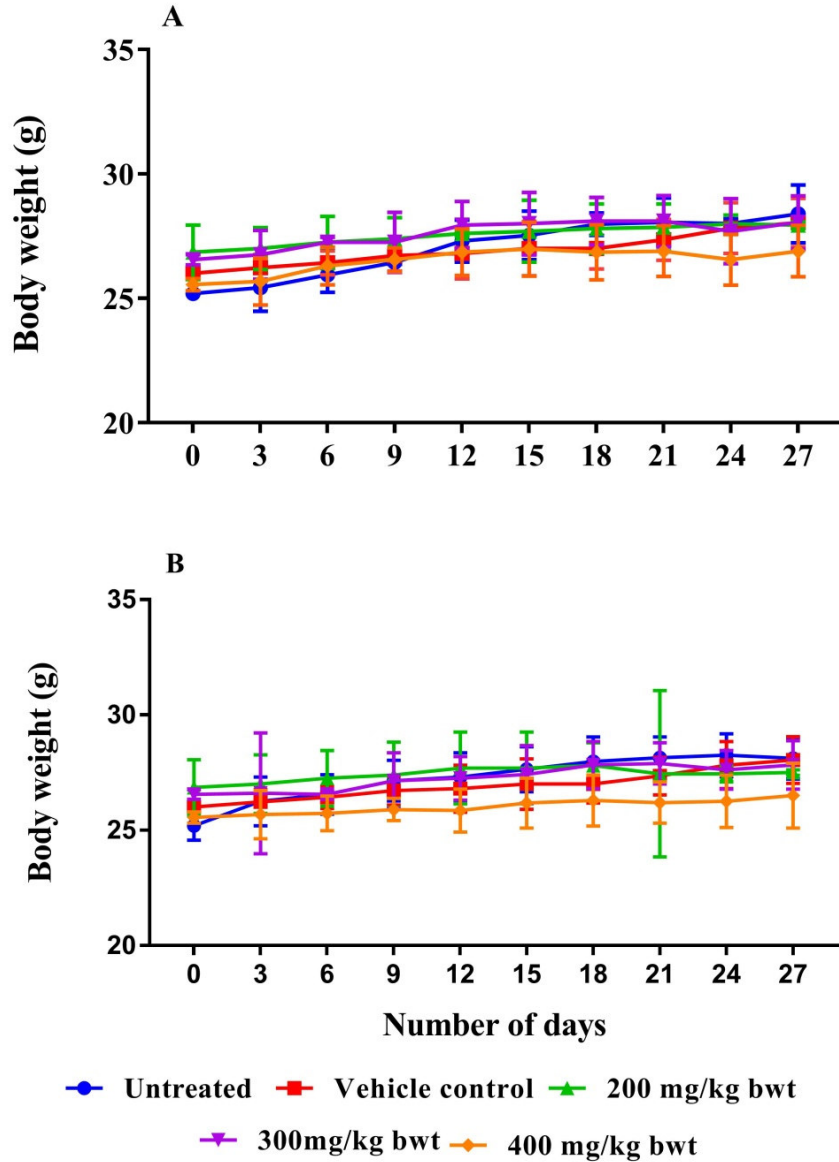


Figure 4.2. Body weights of A) male and B) female Swiss albino mice after administration of 200, 300, and 400 mg/kg b.wt. UNMET extract. Extracts were administered orally on a daily basis for 28 days. The animals were observed thoroughly and the body weights were recorded for the animals in each group every three days during the course of the experiment. Values are expressed as mean \pm SD.

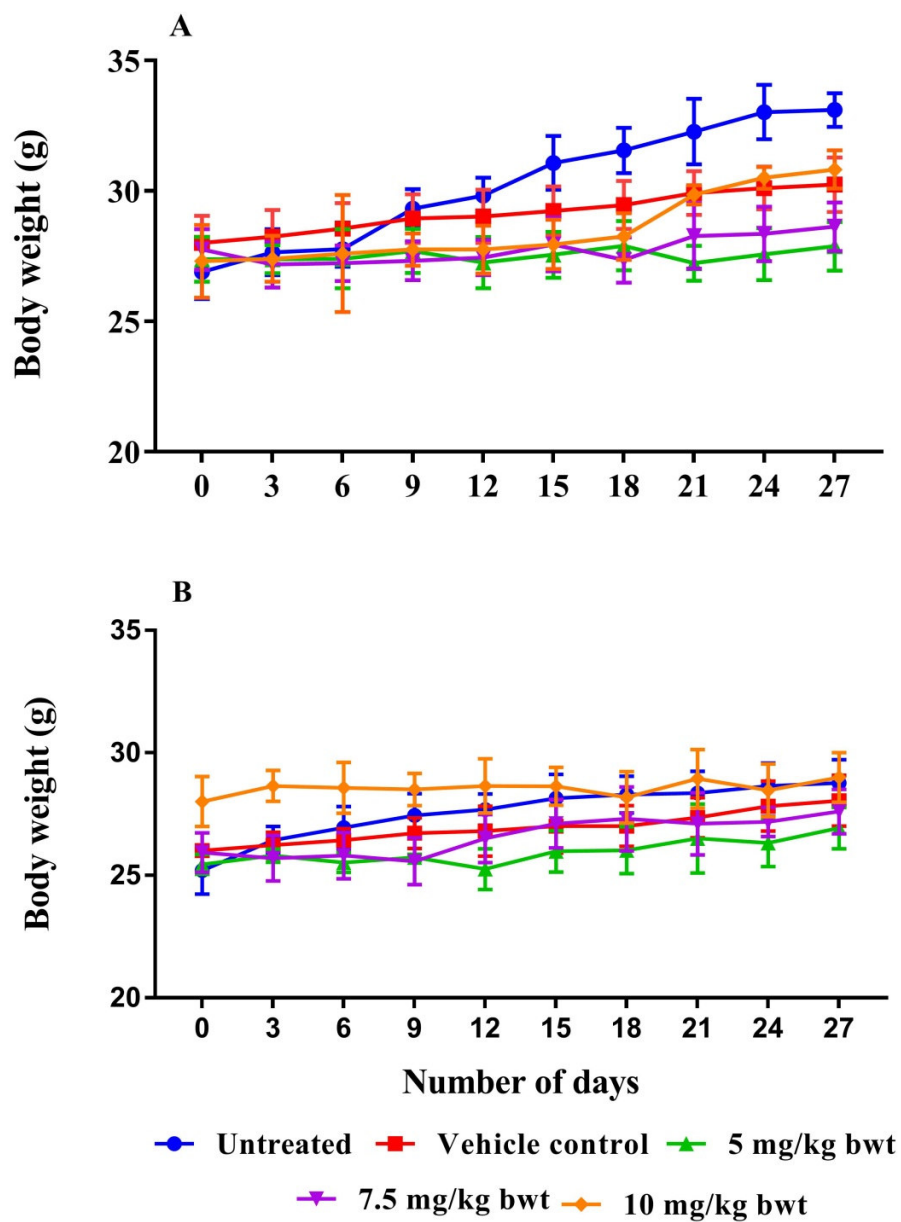


Figure 4.3. Body weight of A) male and B) female Swiss albino mice on the subacute oral administration of 5, 7.5, and 10 mg/kg b.wt. UNPE leaves. Extracts were administered orally on a daily basis for 28 days. The animals were observed thoroughly and the body weights were recorded for the animals in each group every three days during the course of the experiment. Values are expressed as mean \pm SD.

4.3.2.4. Food consumption by male and female Swiss albino mice following sub-acute oral administration of *U. narum* extracts.

Administration of UNMET extracts did not show any difference ($P > 0.05$) in the food consumption of male and female mice when compared to normal untreated groups (Figure 4.4). Similar results were found in the case of UNPE extract administered animals. In female groups, an increase in feed consumption from day 1 (4.73 g/day/ animal) to day 12 (6.12 g/day/ animal) were observed and a similar observation was noted in extract administered animals. Animals including untreated group showed a decreased food intake from day 12 to day 27 (4.86 g/day/ animal on day 27 in the untreated group) as depicted in Figure 4.5.

4.3.2.5. Changes in water consumption by male Swiss albino mice on the sub-acute oral administration of *U. narum* extracts.

Administration of UNMET extract did not produce any difference in the water intake of male and female mice when compared to normal untreated groups (Figure 4.6). The water intake was not the same throughout the experimental period. 200 mg/kg b.wt. and 400 mg/kg b.wt. administered animals showed a change in water consumption from 4.33 mL/day and 4.20 mL/ day on day 3 to 3.99 mL/ day and 4.05 mL/ day on day 27. Similar results were found in the case of UNPE extract administered animals (Figure 4.7). Female animals groups in both extract administration including untreated group showed a decreased water intake during the later stages of the experiment as depicted in Figures 4.6 and 4.7.

Table 4. 2. Relative organ weight of male and female Swiss albino mice following sub-acute administration of UNMET extract of *U.narum* leaves over 28 days.

	Liver	Kidney	Spleen	Stomach	Intestine	Heart	Lungs	brain	Ovary/Testes
Male									
Untreated	5.01 ± 0.21	1.38 ± 0.17	0.40 ± 0.01	0.93 ± 0.30	3.94 ± 0.30	0.46 ± 0.04	0.54 ± 0.08	0.97 ± 0.30	0.61 ± 0.06
Vehicle control	4.85 ± 0.02	1.19 ± 0.14	0.45 ± 0.18	0.88 ± 0.08	4.15 ± 0.11	0.40 ± 0.02	0.59 ± 0.03	1.01 ± 0.04	0.59 ± 0.04
200 mg/kg bwt	4.71 ± 0.47	1.46 ± 0.31	0.47 ± 0.05	0.85 ± 0.06	4.20 ± 0.47	0.41 ± 0.09	0.51 ± 0.04	1.16 ± 0.04	0.60 ± 0.03
300 mg/kg bwt	4.89 ± 0.21	1.37 ± 0.11	0.41 ± 0.04	0.89 ± 0.15	3.70 ± 1.70	0.48 ± 0.01	0.53 ± 0.38	1.02 ± 0.11	0.64 ± 0.03
400 mg/kg bwt	4.92 ± 0.16	1.50 ± 0.21	0.38 ± 0.08	0.86 ± 0.02	3.87 ± 1.60	0.46 ± 0.06	0.55 ± 0.04	1.01 ± 0.02	0.59 ± 0.06
Female									
Untreated	5.03 ± 0.20	1.35 ± 0.02	0.48 ± 0.27	0.80 ± .024	5.57 ± 0.40	0.52 ± 0.05	0.54 ± 0.02	1.05 ± 0.04	0.33 ± 0.11
Vehicle control	5.10 ± 0.53	1.32 ± 0.03	0.53 ± 0.12	0.84 ± 0.02	5.23 ± 0.53	0.49 ± 0.02	0.57 ± 0.03	1.12 ± 0.31	0.35 ± 0.26
200 mg/kg bwt	5.05 ± 0.30	1.28 ± 0.10	0.50 ± 0.13	0.80 ± 0.20	5.13 ± 0.22	0.42 ± 0.01	0.54 ± 0.12	1.16 ± 0.24	0.35 ± 0.07
300 mg/kg bwt	4.97 ± 1.14	1.37 ± 0.07	0.51 ± 0.05	0.81 ± 0.17	5.06 ± 1.75	0.50 ± 0.01	0.60 ± 0.17	1.11 ± 0.06	0.36 ± 0.04
400 mg/kg bwt	4.99 ± 1.14	1.39 ± 0.24	0.49 ± 0.06	0.79 ± 0.29	5.17 ± 1.68	0.48 ± 0.05	0.52 ± 0.12	1.17 ± 0.50	0.35 ± 0.07

Values are expressed as Mean ± SD for 5 animals /sex

Table 4.3. Relative organ weight of male and female Swiss albino mice upon subacute administration of UNPE extract of *U.narum* leaves over 28 days.

	Liver	Kidney	Spleen	Stomach	Intestine	Heart	Lungs	brain	Ovary/Testes
Male									
Untreated	5.01 ± 0.21	1.38 ± 0.17	0.40 ± 0.01	0.83 ± 0.30	3.94 ± 0.30	0.46 ± 0.04	0.54 ± 0.08	0.97 ± 0.30	0.61 ± 0.06
V. control	4.85 ± 0.02	1.19 ± 0.14	0.45 ± 0.18	0.88 ± 0.08	4.05 ± 0.11	0.40 ± 0.02	0.59 ± 0.03	1.01 ± 0.04	0.59 ± 0.04
5 mg/kg bwt	4.87 ± 0.36	1.42 ± 0.21	0.39 ± 0.08	0.82 ± 0.01	3.96 ± 0.60	0.46 ± 0.01	0.55 ± 0.04	0.94 ± 0.02	0.62 ± 0.06
7.5 mg/kg bwt	4.77 ± 0.70	1.43 ± 0.06	0.38 ± 0.11	0.85 ± 0.13	4.03 ± 0.90	0.43 ± 0.19	0.59 ± 0.18	0.97 ± 0.20	0.58 ± 0.08
10 mg/kg bwt	4.84 ± 0.80	1.33 ± 0.02	0.38 ± 0.04	0.85 ± 0.14	4.04 ± 0.79	0.50 ± 0.01	0.58 ± 0.06	0.95 ± 0.20	0.60 ± 0.01
Female									
Untreated	5.03 ± 0.20	1.32 ± 0.02	0.51 ± 0.27	0.80 ± 0.02	5.37 ± 0.40	0.52 ± 0.05	0.54 ± 0.02	1.05 ± 0.04	0.33 ± 0.11
V. control	5.4 ± 0.53	1.32 ± 0.03	0.53 ± 0.12	0.84 ± 0.02	5.03 ± 0.53	0.49 ± 0.01	0.57 ± 0.43	1.12 ± 0.31	0.35 ± 0.26
5 mg/kg bwt	5.1 ± 0.81	1.26 ± 0.21	0.49 ± 0.24	0.83 ± 0.30	5.30 ± 1.05	0.48 ± 0.10	0.54 ± 0.01	1.07 ± 0.20	0.36 ± 0.10
7.5 mg/kg bwt	5.3 ± 1.08	1.36 ± 0.21	0.51 ± 0.24	0.91 ± 0.31	5.25 ± 1.05	0.47 ± 0.10	0.54 ± 0.01	1.10 ± 0.20	0.36 ± 0.10
10 mg/kg bwt	5.3 ± 0.41	1.28 ± 0.01	0.52 ± 0.06	0.85 ± 0.05	5.34 ± 0.38	0.49 ± 0.04	0.54 ± 0.44	1.16 ± 0.28	0.30 ± 0.12

Values are expressed as Mean ± SD

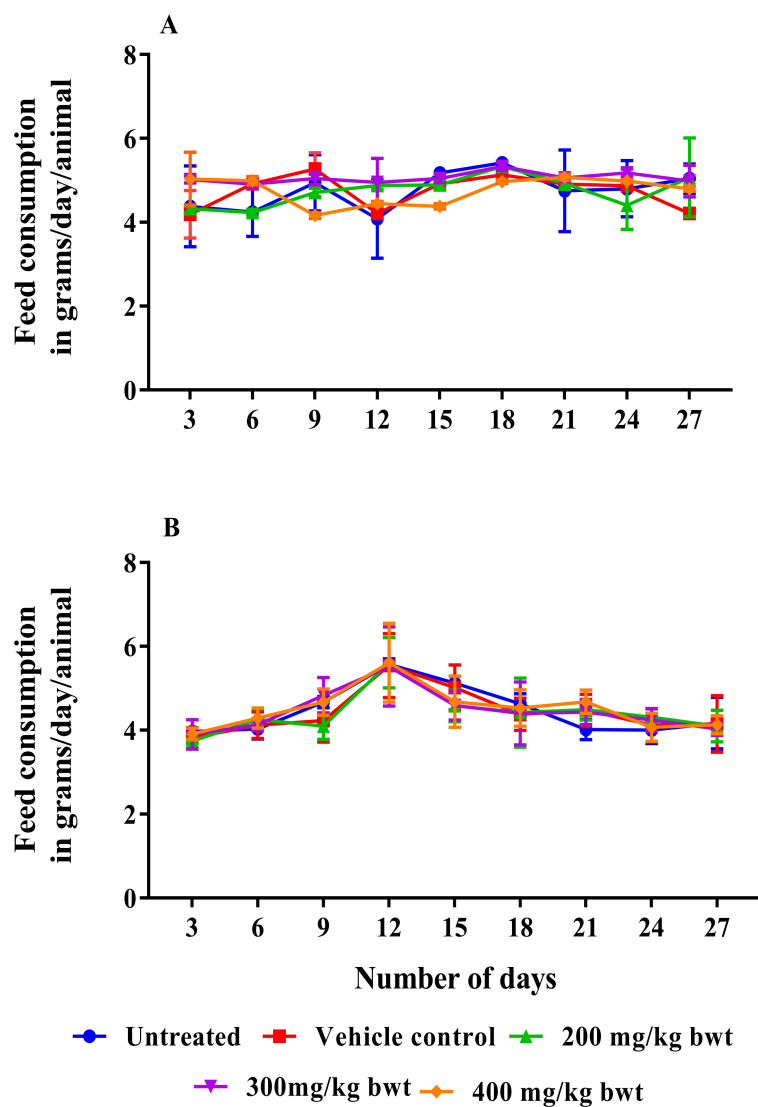


Figure 4.4. The pattern of food consumption of A) male and B) female Swiss albino mice following sub-acute administration of the UNMET extract. Methanolic extracts at different concentrations were orally administered daily for 28 days. The food consumption /gram/day per animal was recorded for each group every three days during the course of the experiment. Values are expressed as mean \pm SD.

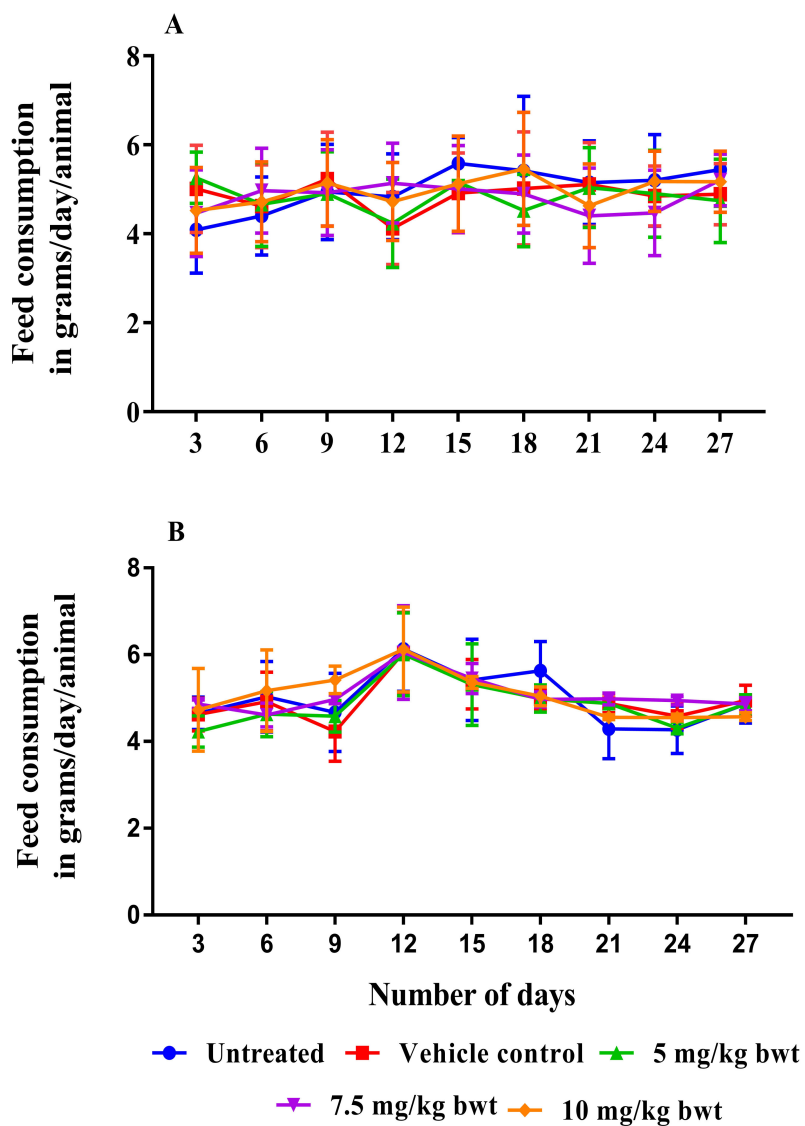


Figure 4.5. The pattern of food consumption of A) male and B) female Swiss albino mice following sub-acute administration of UNPE extract. UNPE extracts of different doses were orally administered daily for 28 days of the experimental period. The food consumption/gram/day per animal was recorded for each group every three days during the course of the experiment. Values are expressed as mean \pm SD.

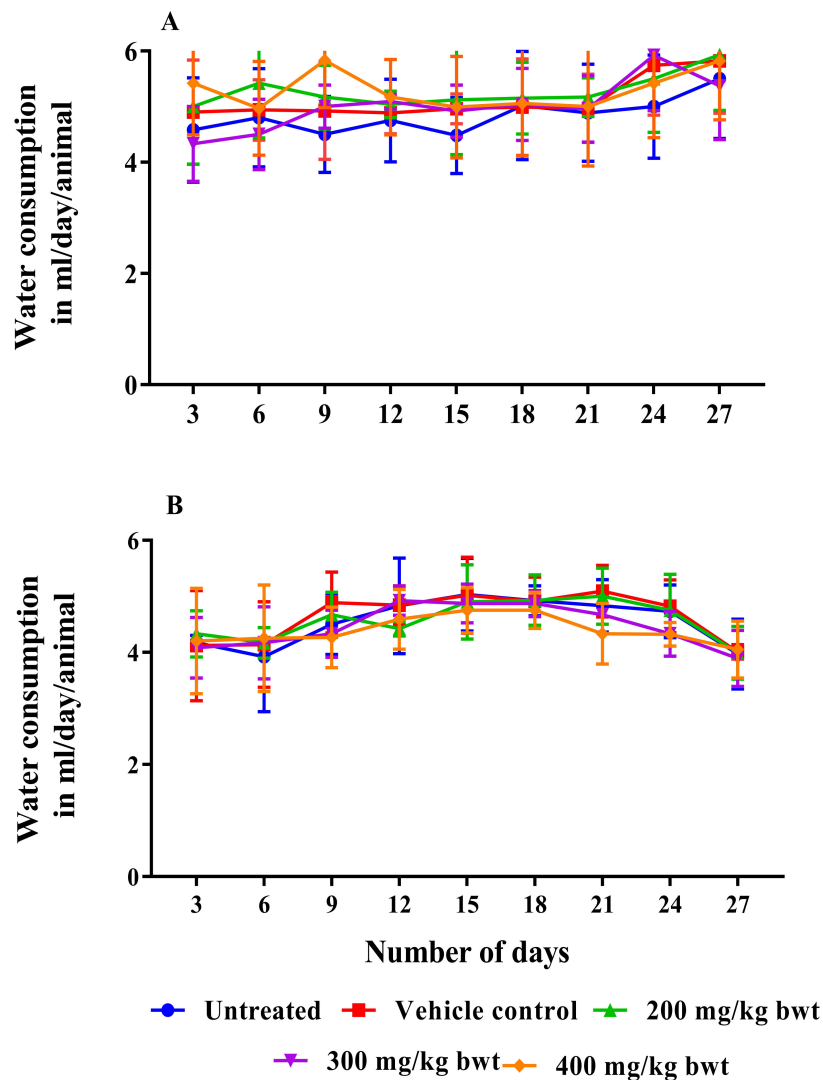


Figure 4.6. Water consumption of A) male and B) female Swiss albino mice following sub-acute administration of UNMET extract. Different doses of the extract were daily administered orally for 28 days of the experimental period. The water consumption in mL /day /animal was recorded for each group every three days during the course of the experiment. Values are expressed as mean \pm SD.

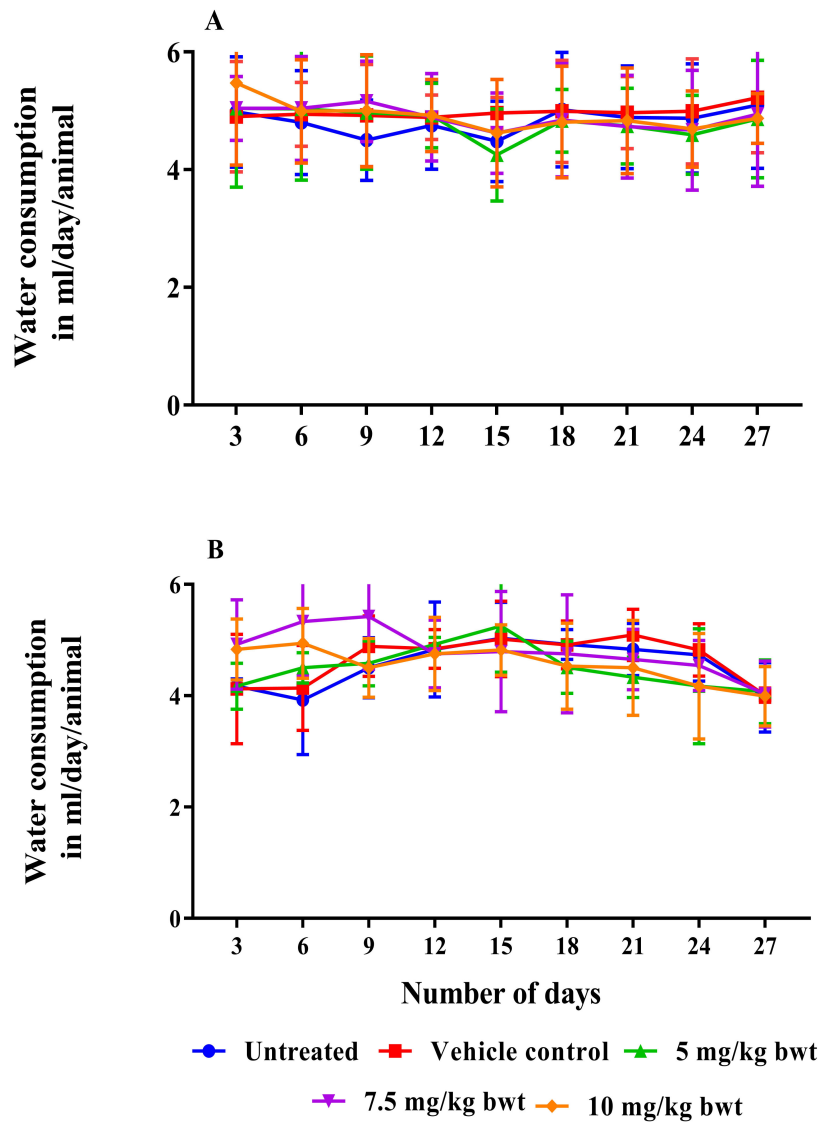


Figure 4.7. Water consumption of A) male and B) female Swiss albino mice following sub-acute oral administration of UNPE leaves extract. UNPE extract of different doses was daily administered orally for 28 days of the experimental period. The water consumption in mL/day /animal was recorded for each group every three days during the course of the experiment. Values are expressed as mean \pm SD.

4.3.2.6. Hematological parameters of male Swiss albino mice following sub-acute oral administration of *Uvaria narum* extracts.

The interaction of the phytochemicals or extract or its metabolite with cellular components may elicit significant changes in hematological parameters depending on its toxicity. RBC count represents the capacity of blood to carry oxygen for metabolic processes. The results of the study showed that the RBC values were within normal limits. The extracts did not elicit any significant toxicity to RBC and Hb levels in the blood of treated animals (Table 4.4 and 4.5). All blood parameters tested, such as Hb level, RBC, total WBC count, and differential count were found to be within normal levels in both UNMET and UNPE extract treated animals compared to the untreated normal group (Table 4.4 and 4.5). Leucocytes play a major role in the defense of the body against infection. The administration of UNMET and UNPE extracts did not change the number of leucocytes, thereby the defense functions seem to be unaltered on administering the extracts.

4.3.2.7. Serum biochemical parameters following sub-acute oral administration of *Uvaria narum* extracts

Electrolytes are essential micro components of serum along with water which maintain normal physiological function. Any disruption in the fluid and electrolyte balance in the body causes a loss in proper electrical conductivity required for cellular energy production. Thus, electrolyte imbalance adversely affects the body leading to life-threatening conditions including hypertension, kidney disorders, and cardiovascular diseases, etc. (Nnadi *et al.* 2015). Our results suggested that there was no significant change in the levels of serum electrolytes such as chloride, potassium, sodium, and bicarbonate between the *U.narum* petroleum ether (UNPE) and methanolic extract (UNME) treated female or male animals and their respective control groups. The extract treated animals maintained proper fluid and electrolyte balance as in the control group animals (Table 4.6 and 4.7).

4.3.2.8. The activity of liver function markers following sub-acute oral administration of UNMET and UNPE extracts

Uvaria narum methanolic and PE extracts at different doses did not produce any significant changes in the activity of serum marker enzymes. The elevation or alteration in the activity of liver marker enzyme levels is a sign of liver injury. Hepatic function parameters such as SGOT, SGPT, alkaline phosphatase, total bilirubin, total protein, and A/G ratio were not altered in treated animals of both sex groups (Table 4.8 and 4.9).

4.3.2.9. Changes in lipid profile of Swiss albino mice following oral administration of UNMET and UNPE extracts

The serum level of total cholesterol, HDL cholesterol, and triglycerides between normal animals and UNPE or UNMET extract did not vary significantly (Table 4.10 and 4.11). Accordingly, serum LDL, VLDL levels calculated based on estimated cholesterol and TG levels also did not vary. It was observed that all these values were within the normal limit.

4.3.2.10. Creatinine and urea levels following sub-acute oral administration of UNMET and UNPE extracts

In the present study, the renal function parameters of mice such as blood urea and serum creatinine did not show any significant variation in UNMET and UNPE extract treated animals when compared to the untreated control group. The vehicle control group also showed similar results to that of the untreated group (Table 4.12 and 4.13).

Table 4.4. Hematological parameters of male and female Swiss albino mice following sub-acute oral administration of UNMET extract.

	Hb (g/dl)	RBC (10 ⁶ /cu mm)	MCV (fl)	MCH (pg)	MCHC (g/dl)	HCT-PCV (%)	Platelet (Lakhs /cu mm)	TC (cells/cu mm)	Neutro phils (%)	Lympho cytes (%)	Eosino phils (%)
Male											
Untreated	13.6 ± 0.3	5.1 ± 0.1	88 ± 2.9	29 ± 1.0	33 ± 1.2	37 ± 0.9	2.6 ± 0.2	8900 ± 468	41 ± 4.1	56 ± 3.9	3 ± 0.10
V. control	14.9 ± 0.5	5.4 ± 1.3	87 ± 1.9	29 ± 1.3	33 ± 0.9	40 ± 1.3	2.7 ± 0.2	9100 ± 412	39 ± 1.0	58 ± 4.8	3 ± 0.05
200 mg/kg bwt	14.6 ± 0.8	5.4 ± 1.2	86 ± 1.8	28 ± 1.5	32 ± 0.9	38 ± 1.0	2.7 ± 0.1	9900 ± 764	39 ± 1.8	56 ± 2.1	3 ± 0.03
300 mg/kg bwt	13.8 ± 0.7	5.8 ± 0.5	85 ± 2.3	28 ± 1.1	33 ± 0.9	41 ± 1.3	3.1 ± 0.4	8800 ± 395	34 ± 1.6	54 ± 1.0	2 ± 0.02
400 mg/kg bwt	14.2 ± 1.9	5.9 ± 0.4	87 ± 1.5	29 ± 0.9	32 ± 0.5	43 ± 1.0	3.2 ± 0.7	9700 ± 437	42 ± 1.4	55 ± 2.0	3 ± 0.05
Female											
Untreated	13.6 ± 0.3	5.1 ± 0.4	90 ± 4.1	28 ± 1.5	31 ± 2.1	28 ± 1.1	2.6 ± 0.4	7200 ± 455	41 ± 3.7	56 ± 4.4	3 ± 0.14
V. control	12.3 ± 0.4	4.9 ± 0.6	90 ± 1.0	29 ± 1.0	32 ± 1.6	29 ± 1.3	3.4 ± 0.2	8400 ± 534	41 ± 2.9	52 ± 3.0	4 ± 0.24
200 mg/kg bwt	11.2 ± 0.5	4.8 ± 1.4	88 ± 1.9	29 ± 1.3	32 ± 0.9	30 ± 1.3	3.2 ± 0.2	7000 ± 412	40 ± 1.0	52 ± 4.8	5 ± 1.04
300 mg/kg bwt	11.2 ± 0.8	4.9 ± 0.9	89 ± 3.1	29 ± 1.1	33 ± 1.0	32 ± 1.0	3.4 ± 0.2	8300 ± 560	41 ± 2.1	54 ± 2.9	4 ± 0.10
400 mg/kg bwt	12.1 ± 1.3	4.9 ± 0.4	90 ± 3.7	30 ± 0.6	31 ± 1.6	32 ± 0.9	2.6 ± 1.4	9350 ± 572	44 ± 3.8	52 ± 2.4	4 ± 0.07

Values are expressed as Mean ± SD.

Table 4.5. Hematological parameters of male and female Swiss albino mice following sub-acute oral administration of UNPE extract.

	Hb (g/dl)	RBC (10^6 /cu mm)	MCV (fl)	MCH (pg)	MCHC (g/dl)	HCT-PCV (%)	Platelet (Lakhs /cu mm)	TC (cells/cu mm)	Neutro phils (%)	Lympho cytes (%)	Eosino phils (%)
Male											
Untreated	13.60 ± 0.2	5.20 ± 0.1	88 ± 2.9	29 ± 1.0	33 ± 1.2	37 ± 0.94	2.6 ± 0.2	8900 ± 468	41 ± 4.1	56 ± 3.9	3 ± 0.09
V. control	13.20 ± 0.4	5.00 ± 0.1	87 ± 2.0	29 ± 1.2	32 ± 2.0	39 ± 1.57	2.7 ± 0.3	7948 ± 547	41 ± 2.1	56 ± 1.3	3 ± 0.07
5 mg/kg bwt	13.40 ± 0.1	5.30 ± 0.6	88 ± 3.1	29 ± 0.9	33 ± 2.0	40 ± 1.04	2.9 ± 0.1	6800 ± 248	42 ± 3.0	55 ± 0.1	3 ± 0.08
7.5mg/kg bwt	12.60 ± 0.2	4.99 ± 0.9	86 ± 2.1	29 ± 1.2	32 ± 1.3	38 ± 0.99	2.7 ± 0.1	6900 ± 341	39 ± 2.9	56 ± 1.1	2 ± 0.04
10 mg/kg bwt	14.20 ± 0.8	5.43 ± 0.6	87 ± 1.9	29 ± 1.7	32 ± 1.3	42 ± 1.39	2.6 ± 0.0	7300 ± 421	40 ± 2.5	56 ± 0.9	3 ± 0.02
Female											
Untreated	13.60 ± 0.3	5.20 ± 0.4	90 ± 4.1	28 ± 1.5	31 ± 2.1	28 ± 1.04	2.7 ± 0.4	7200 ± 455	40 ± 3.7	56 ± 4.4	3 ± 0.14
V. control	12.40 ± 0.2	5.00 ± 1.3	89 ± 2.5	29 ± 1.0	32 ± 1.2	29 ± 1.02	2.9 ± 0.3	8100 ± 974	39 ± 1.9	57 ± 3.9	4 ± 0.07
5 mg/kg bwt	11.20 ± 0.3	4.90 ± 0.5	88 ± 3.9	29 ± 1.2	30 ± 2.8	27 ± 1.38	2.9 ± 0.3	8200 ± 578	39 ± 2.8	57 ± 2.5	3 ± 0.08
7.5mg/kg bwt	11.45 ± 0.4	4.90 ± 0.4	87 ± 2.4	28 ± 1.2	32 ± 1.3	29 ± 0.98	2.7 ± 0.1	8100 ± 341	38 ± 2.9	58 ± 1.1	4 ± 0.04
10 mg/kg bwt	12.50 ± 0.3	5.10 ± 0.7	89 ± 2.2	29 ± 1.1	32 ± 2.9	30 ± 2.89	2.9 ± 0.6	7300 ± 457	41 ± 2.1	57 ± 4.3	4 ± 0.03

Values are expressed as Mean ± SD

Table 4.6. Serum electrolytes levels of male and female Swiss albino mice following sub-acute oral administration of UNMET extract

	Sodium (mmol/L)	Potassium (mmol/L)	Chloride (mmol/L)	Bicarbonate (mmol/L)
Male				
Untreated	144 ± 2	4.8 ± 0.13	106 ± 2.7	23 ± 1.4
vehicle control	143 ± 2	4.7 ± 0.12	103 ± 2.0	24 ± 0.5
200 mg/kg bwt	142 ± 2	5.3 ± 0.14	104 ± 2.1	26 ± 1.7
300 mg/kg bwt	142 ± 2	5.6 ± 0.15	108 ± 1.9	26 ± 2.1
400 mg/kg bwt	144 ± 3	5.0 ± 0.46	107 ± 3.2	25 ± 0.1
Female				
Untreated	140 ± 2	4.9 ± 0.24	102 ± 1.2	24 ± 2.4
vehicle control	143 ± 1	5.2 ± 0.36	103 ± 1.4	23 ± 1.67
200 mg/kg bwt	148 ± 2	5.2 ± 1.14	104 ± 1.7	23 ± 2.67
300 mg/kg bwt	144 ± 2	5.4 ± 0.07	102 ± 2.8	25 ± 2.1
400 mg/kg bwt	146 ± 1	4.9 ± 0.16	103 ± 1.4	25 ± 3.4

Values are expressed as Mean ± SD

Table 4.7. Serum electrolytes levels of male and female Swiss albino mice following sub-acute oral administration of UNPE extract

	Sodium (mmol/L)	Potassium (mmol/L)	Chloride (mmol/L)	Bicarbonate (mmol/L)
Male				
Untreated	144 ± 2.01	4.8 ± 0.13	106 ± 3.2	23 ± 1.4
V. control	145 ± 1.13	4.7 ± 0.28	104 ± 4.0	24 ± 1.2
5 mg/kg bwt	143 ± 1.04	5.3 ± 0.27	105 ± 5.0	25 ± 0.20
7.5 mg/kg bwt	142 ± 1.10	5.1 ± 0.54	106 ± 4.1	25 ± 0.14
10 mg/kg bwt	141 ± 1.06	5.4 ± 0.12	104 ± 1.2	25 ± 2.04
Female				
Untreated	141 ± 1.53	4.9 ± 0.24	102 ± 2.0	24 ± 2.4
V. control	145 ± 1.24	5.1 ± 0.08	102 ± 2.7	25 ± 1.1
5 mg/kg bwt	151 ± 1.97	5.7 ± 0.27	102 ± 3.8	24 ± 3.9
7.5 mg/kg bwt	150 ± 2.64	5.6 ± 0.04	103 ± 2.8	26 ± 1.7
10 mg/kg bwt	149 ± 1.37	5.4 ± 0.12	104 ± 7.0	24 ± 2.1

Values are expressed as Mean ± SD

Table 4.8. Levels of liver function markers following sub-acute oral administration of UNMET extract

	TB	SGOT	SGPT	ALP	TP	Albumin	Globulin
Male	(mg/dl)	(U/L)	(U/L)	(U/L)	(g/dl)	(g/dl)	(g/dl)
Untreated	0.37 ± 0.03	138 ± 24	56 ± 1.24	64 ± 3.12	6.2 ± 0.73	3.2 ± 0.94	3.0 ± 0.3
V. control	0.39 ± 0.01	134 ± 27	50 ± 1.34	63 ± 2.78	6.1 ± 0.09	3.1 ± 0.91	3.0 ± 0.4
200 mg/kg bwt	0.40 ± 0.01	130 ± 38	58 ± 5.01	67 ± 3.95	6.1 ± 0.14	3.2 ± 0.14	2.9 ± 0.5
300 mg/kg bwt	0.36 ± 0.02	134 ± 27	52 ± 3.14	61 ± 4.87	6.2 ± 0.46	3.4 ± 0.57	2.8 ± 0.4
400 mg/kg bwt	0.30 ± 0.08	130 ± 13	51 ± 4.53	66 ± 4.29	6.3 ± 0.93	3.3 ± 0.97	3.0 ± 0.3
Female							
Untreated	0.31 ± 0.03	130 ± 19	45 ± 2.47	68 ± 5.37	6.8 ± 0.57	3.5 ± 0.82	3.3 ± 0.09
V. control	0.40 ± 0.02	124 ± 13	45 ± 1.86	64 ± 2.34	6.5 ± 0.57	3.4 ± 0.34	3.1 ± 0.09
200 mg/kg bwt	0.31 ± 0.01	136 ± 28	46 ± 4.28	61 ± 2.31	7.0 ± 0.24	3.7 ± 0.97	3.3 ± 0.50
300 mg/kg bwt	0.35 ± 0.08	128 ± 17	45 ± 2.67	65 ± 3.62	6.5 ± 0.31	3.4 ± 0.48	3.1 ± 0.20
400 mg/kg bwt	0.36 ± 0.07	133 ± 23	48 ± 2.11	69 ± 1.34	6.7 ± 0.59	3.8 ± 0.85	2.9 ± 0.40

TB: total bilirubin; TP: total protein. Values are expressed as Mean ± SD.

Table 4.9. Levels of liver function markers following sub-acute oral administration of UNPE extract

	TB	SGOT	SGPT	ALP	TP	Albumin	Globulin
Male	(mg/dl)	(U/L)	(U/L)	(U/L)	(g/dl)	(g/dl)	(g/dl)
Untreated	0.37 ± 0.03	138 ± 24	56 ± 1.24	64 ± 3.12	6.2 ± 0.73	3.2 ± 0.94	3.0 ± 0.3
V. control	0.39 ± 0.08	144 ± 12	52 ± 3.14	65 ± 4.20	6.3 ± 0.72	3.3 ± 0.84	3.0 ± 0.2
5 mg/kg bwt	0.36 ± 0.12	145 ± 21	65 ± 2.58	59 ± 3.40	6.1 ± 0.95	3.3 ± 0.06	2.8 ± 0.1
7.5 mg/kg bwt	0.41 ± 0.08	132 ± 16	46 ± 1.91	61 ± 2.37	6.0 ± 0.67	3.1 ± 0.84	2.9 ± 0.2
10 mg/kg bwt	0.39 ± 0.22	134 ± 34	47 ± 3.47	63 ± 4.41	6.1 ± 0.83	3.2 ± 0.90	2.9 ± 0.2
Female							
Untreated	0.31 ± 0.03	130 ± 19	45 ± 2.47	68 ± 5.37	6.8 ± 0.57	3.5 ± 0.82	3.3 ± 0.09
V. control	0.33 ± 0.02	124 ± 13	45 ± 1.86	64 ± 2.34	6.5 ± 0.57	3.4 ± 0.34	3.1 ± 0.09
5 mg/kg bwt	0.29 ± 0.02	127 ± 11	44 ± 1.29	64 ± 1.29	6.7 ± 0.68	3.5 ± 0.98	3.2 ± 0.10
7.5 mg/kg bwt	0.30 ± 0.06	130 ± 17	50 ± 1.3	65 ± 3.36	6.2 ± 0.92	3.2 ± 0.37	3.0 ± 0.30
10 mg/kg bwt	0.34 ± 0.09	127 ± 14	46 ± 2.56	64 ± 2.47	6.8 ± 0.47	3.5 ± 0.68	3.3 ± 0.20

TB: total bilirubin; TP: total protein. Values are expressed as Mean ± SD

Table 4.10. Blood lipid profile of Swiss albino mice following oral administration of UNMET extract

	Total cholesterol	Triglycerides	HDL	LDL	VLDL
Male	mg/dl	mg/dl	mg/dl	mg/dl	mg/dl
Untreated	82.0 ± 0.97	150.00 ± 9.24	32.00 ± 0.54	20.0 ± 1.4	30.0 ± 0.54
Vehicle control	80.0 ± 0.85	153.00 ± 5.12	30.00 ± 0.51	19.4 ± 1.4	30.6 ± 0.47
200 mg/kg bwt	80.0 ± 4.21	142.00 ± 6.75	35.00 ± 0.94	20.6 ± 3.12	24.4 ± 0.97
300 mg/kg bwt	79.0 ± 1.64	149.00 ± 8.83	32.00 ± 1.34	21.2 ± 1.04	25.8 ± 2.39
400 mg/kg bwt	80.0 ± 1.03	148.00 ± 3.67	30.00 ± 0.94	20.4 ± 2.94	29.6 ± 1.06
Female					
Untreated	80.0 ± 0.12	108.00 ± 5.42	34.40 ± 0.42	22.2 ± 1.75	23.4 ± 0.44
Vehicle control	74.0 ± 1.90	105.00 ± 1.30	33.00 ± 0.67	20.0 ± 1.42	21.0 ± 0.49
200 mg/kg bwt	80.0 ± 2.10	104.00 ± 4.31	34.00 ± 0.12	21.2 ± 2.14	24.8 ± 0.87
300 mg/kg bwt	77.0 ± 3.40	102.0 ± 3.36	33.00 ± 0.28	19.6 ± 2.1	20.0 ± 1.46
400 mg/kg bwt	77.0 ± 0.97	101.0 ± 4.69	34.00 ± 0.41	20.0 ± 1.97	23.0 ± 2.34

Values are expressed as Mean ± SD

Table 4.11. Blood lipid profile of Swiss albino mice following oral administration of UNPE extract

	Total cholesterol	Triglycerides	HDL	LDL	VLDL
Male	mg/dl	mg/dl	mg/dl	mg/dl	mg/dl
Untreated	82.00 ± 0.97	156.00 ± 9.24	31.00 ± 0.54	19.8 ± 1.4	31.2 ± 0.54
vehicle control	80.00 ± 0.85	153.00 ± 5.12	30.00 ± 0.51	19.4 ± 1.4	30.6 ± 0.47
5 mg/kg bwt	81.00 ± 0.17	150.00 ± 5.46	31.00 ± 0.97	20.0 ± 2.1	30.0 ± 1.06
7.5 mg/kg bwt	80.00 ± 2.45	142.00 ± 4.16	35.00 ± 0.67	19.6 ± 3.2	25.4 ± 2.34
10 mg/kg bwt	80.00 ± 2.67	147.00 ± 2.41	34.00 ± 2.47	18.6 ± 3.4	27.4 ± 3.47
Female					
Untreated	70.00 ± 0.12	99.00 ± 5.42	30.00 ± 0.42	20.2 ± 1.75	19.8 ± 0.44
vehicle control	73.00 ± 1.90	105.00 ± 1.30	32.00 ± 0.67	20.0 ± 1.42	21.0 ± 0.49
5 mg/kg bwt	70.00 ± 2.10	97.00 ± 2.58	30.00 ± 0.91	22.6 ± 3.04	17.4 ± 1.4
7.5 mg/kg bwt	73.00 ± 1.34	92.00 ± 3.64	33.00 ± 0.64	21.6 ± 3.10	18.4 ± 1.37
10 mg/kg bwt	72.00 ± 3.47	101.0 ± 4.39	34.00 ± 1.34	17.2 ± 3.47	20.8 ± 1.74

Values are expressed as Mean ± SD

Table 4.12. Creatinine and urea levels in the blood following oral administration of UNMET extract

	Serum creatinine (mg/dl)	Blood urea nitrogen (mg/dl)
Male		
Untreated	0.52 ± 0.17	20.00 ± 1.13
Vehicle control	0.49 ± 0.13	20.83 ± 1.89
200 mg/kg bwt	0.41 ± 0.08	22.08 ± 2.28
300 mg/kg bwt	0.36 ± 0.07	21.67 ± 1.36
400 mg/kg bwt	0.27 ± 0.12	21.00 ± 1.15
Female		
Untreated	0.50 ± 0.11	19.25 ± 1.64
Vehicle control	0.48 ± 0.24	21.00 ± 1.42
200 mg/kg bwt	0.40 ± 0.03	22.00 ± 0.98
300 mg/kg bwt	0.51 ± 0.01	17.75 ± 0.13
400 mg/kg bwt	0.41 ± 0.03	21.33 ± 1.68

Values are expressed as Mean ± SD

Table 4.13. Creatinine and urea levels in the blood following oral administration of UNPE extract

	Serum creatinine (mg/dl)	Blood nitrogen (mg/dl)	urea
Male			
Untreated	0.52 ± 0.17	20.00 ± 1.13	
vehicle control	0.39 ± 0.13	20.83 ± 1.89	
5 mg/kg bwt	0.35 ± 0.06	22.33 ± 1.15	
7.5 mg/kg bwt	0.37 ± 0.11	22.83 ± 1.37	
10 mg/kg bwt	0.36 ± 0.10	21.00 ± 1.37	
Female			
Untreated	0.50 ± 0.11	19.25 ± 1.64	
vehicle control	0.49 ± 0.24	20.0 ± 1.78	
5 mg/kg bwt	0.42 ± 0.04	17.08 ± 1.60	
7.5 mg/kg bwt	0.33 ± 0.09	21.67 ± 2.69	
10 mg/kg bwt	0.34 ± 0.08	19.00 ± 2.46	

Values are expressed as Mean ± SD

4.3.2.11. Effect of oral administration of UNMET and UNPE extracts on the architecture of different tissues.

Macroscopic examination of the main organs of the animals administered *U.narum* extracts was performed at the end of sub-acute study (after 28 days). Figures 4.8 and 4.9, respectively show the representative photographs of histological details of organs of untreated normal, as well as *U.narum* extracts, treated mice. The study revealed no characteristic changes in the liver, kidney, brain, spleen, stomach, intestine, lungs and heart from both control and treated mice on histopathology analysis. Liver sections showed normal hepatic architecture and hepatocytes. Histological sections of lungs showed normal appearance with normal alveolus and bronchioles. Histological examination of the kidney of treated groups showed normal renal architecture with a normal appearance of glomerulus and tubules as in the normal group. Normal myocardial appearances are in the heart sections of treated animals when compared to a normal heart. The spleen and brain sections displayed normal architecture whereas infiltration of leucocytes was seen in the sections of the stomach from both methanolic extract and UNPE treated animals, infiltration was more in UNPE extract treated animals. The testis section showed detached lobuli of the testis with slightly defective spermatogenesis in the *U.narum* treated animal groups. Ovarian sections of the treated animals do not seem to show any gross morphological alterations. So the histopathological examination showed that treatment with *U.narum* produced no significant pathological organ lesions for main organs except testis during the period of 28 days of subacute toxicity analysis (Figure 4.8 and 4.9).

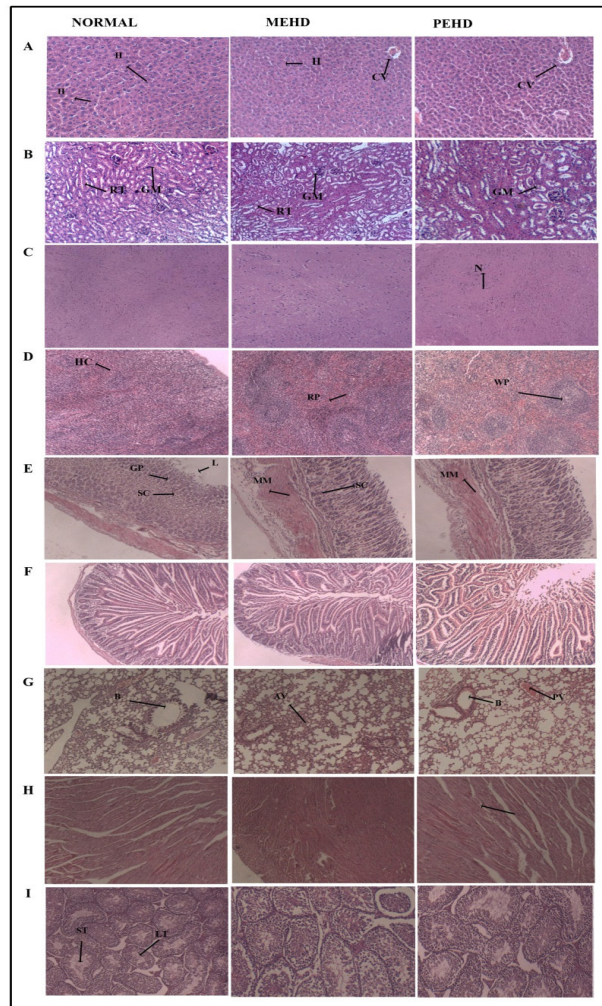


Figure 4.8. Histological examinations of the main organs in male Swiss albino mice following sub-acute toxicity study. Representative photomicrographs of (A) liver; (B) kidney; (C) brain; (D) spleen; (E) stomach; (F) intestine (G) lungs (H) heart (I) testis sections stained with hematoxylin and eosin, respective groups normal untreated; UNMET, 400 mg/kg b.wt. and UNPE, 10 mg/kg b.wt. treated groups. At the end of the experiment, all animals were sacrificed and tissues from the liver, brain, lungs, spleen, stomach, kidney, small intestine, and ovary/testes were histologically analyzed (200 X). H: hepatocytes; CV: central vein; BV: blood vessel; GM: glomerulus; RT: renal tubule; N: nerve cells; HC: haemopoietic cells; WP: white pulp; RP: red pulp; GP: glandular pit; SC: surface mucosa cells; L: lumen; ST: seminiferous tubules; LT: lobuli testes; IC: intestinal crypts; MC: myocardium; MM: muscular mucosa; AV: alveoli; B: bronchiole.

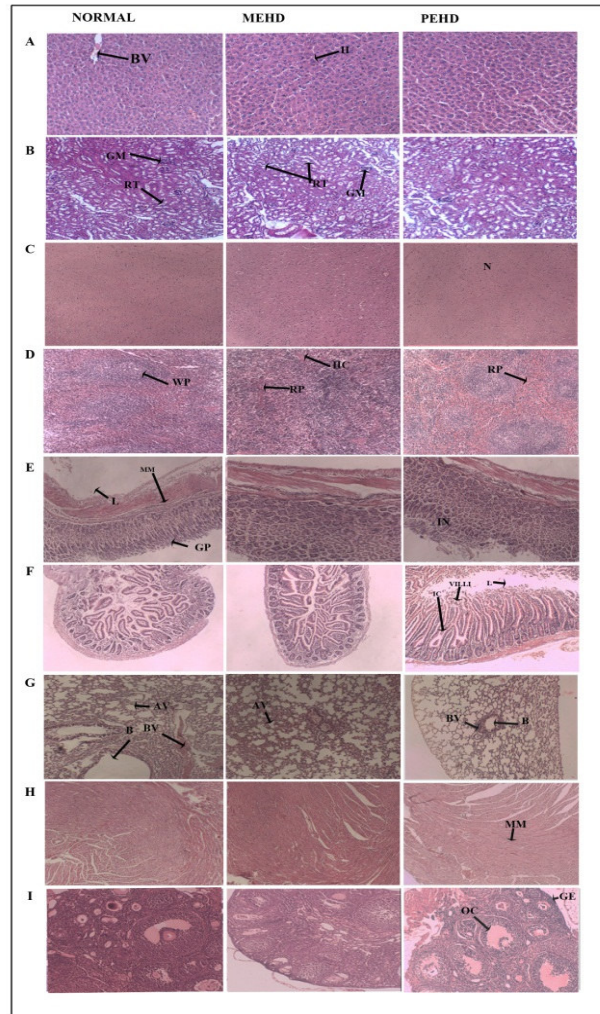


Figure 4.9. Histological examinations of the main organs in female Swiss albino mice following 28 days of repeated dose sub-acute toxicity study. Representative photomicrographs from (A) liver; (B) kidney; (C) brain; (D) spleen; (E) stomach; (F) intestine (G) lungs (H) heart (I) ovary sections stained with hematoxylin and eosin (200x), respective groups normal untreated; UNME-HD, 400 mg/kg b.wt. and UNPE-HD, 10mg/kg b.wt. treated groups. At the end of the experiment, all animals were sacrificed and tissues excised and analyzed histologically (200 X). H: hepatocytes; CV: central vein; BV: blood vessel; GM: glomerulus; RT: renal tubule; N: nerve cells; HC: haemopoietic cells; WP: white pulp; RP: red pulp; GP: glandular pit; L: lumen; IC: intestinal crypts; IN: infiltration of leucocytes. MC: myocardium; MM: muscular mucosa; AV: alveoli; B: bronchiole; GE: germinal epithelium; OC: oocyte.

4.4. Discussion

In the present study, the evaluation of the safety of UNMET and UNPE extracts in animals was carried out. Acute toxicity study (as per OECD guidelines 423) using UNMET extract did not cause mortality for up to 14 days when tested at a dose of 2000 mg/kg b.wt. Also, there was no observed hair loss, behavioral changes or significant changes in the body weight and water consumption, throughout the experimental period. This suggests that the animals tolerated UNMET extract upto a dose of 2000 mg/kg body wt and that the LD₅₀ value of UNMET extract may be above this dose. For further sub-acute toxicity studies, doses between 1/10th (200 mg/kg b.wt.) and 1/5th (400mg/kg b.wt.) of the maximum tolerable dose observed was selected. The sub-acute oral administration of the different doses of UNMET extracts did not elicit any changes in body weight, the pattern of food consumption, water consumption, and organ somatic index, etc. The blood parameters such as serum electrolytes, lipid profile, liver function, and levels of urea and creatinine remained unaltered following administration with UNMET extract for 28 days. Histology of important organs revealed no structural changes between normal and treated animals. This indicates the safety of UNMET extract upon administration. The hepatoprotective effect of a methanolic extract of *U. narum* reported by Patel *et al.* 2013 supports the findings in this study. Hence, the methanolic extract at a dose range of 200 to 400 mg/kg b.wt. could be utilized safely for further animal experiments.

On the other hand, the acute oral administration of UNPE extract above 250 mg/kg b.wt., was found to be highly toxic to animals. At the higher concentration tested (2000, 1000, 500 and 300 mg/kg b.wt.) significant toxicity was observed that led to the death of animals. However, 250 mg/kg was the limit dose of the extract that did not have adverse effects on the animals. As per the LD₅₀ value, the doses of UNPE extract selected for repeated dose studies for a period of 28 days were 5, 7.5 and 10 mg/kg b.wt. The sub-acute toxicity study indicated that repeated intake of extract in high, medium and low doses (10, 7.5 and 5 mg/kg b.wt.) for 28 days didn't elicit changes in lipid profile and hemogram of the mice. Liver function markers such as aspartate aminotransferase and alanine aminotransferase activities were within normal levels. Alteration or elevation of marker enzyme activities denotes the liver injury (Johnston 1999). Therefore, it is assumed that the UNPE extract at the tested dose

produced no injury. In corroboration, histological examination of major tissues did not show any toxic alteration among treated group animals compared to normal animals. However, the testes of animals exposed to 10 mg/kg b.wt. UNPE extract experienced marginal toxicity. The possible reproductive toxicity of UNPE extract has to be evaluated systematically. The UNMET extract also showed similar toxicity towards testes. It may be due to a phthalic acid mono-2-Ethylhexyl ester, detected in the UNMET extract by LC-MS analysis. The compound is a known germ cell toxic chemical and studies has revealed the apoptosis-inducing effect of phthalic acid mono-2-Ethylhexyl ester in germ cells (through Fas (Apo-1, CD95) death receptor pathway) altering spermatogenesis (Giammona *et al.* 2002; Richburg and Boekelheide 1996). However, this observation raises two important possibilities; UN extract could be specifically used in reproductive cancer studies which need to be tested using cell culture and animal model. Another possibility is that reproductive organ-specific toxic chemical can be purified from this extract which can be used in pre-clinical researches as a targeting agent. However, the serum level of urea and creatine were also unaltered among normal and treated group animals. This indicated that the extract did not induce any renal toxicity in mice. Hence, these results suggested that the UNPE extract is safe for the use in animal experiments between 5 and 10 mg/kg b.wt.

Chapter 5

*Cytotoxic and antitumor principles of UNPE extract and
their mechanism of action*

5.1. Introduction

Great breakthroughs have been made in the development of promising therapeutics for cancer treatment. Among the emerging chemotherapeutic agents, only about five percent of cancer drugs are approved on efficacy level (Day *et al.* 2015). This has led to the importance of preclinical evaluation and use of animal models for the early stage drug development. The simple and refined methods with the increased availability of mouse models of cancer have made it an important avenue for the preclinical testing of chemotherapeutic agents (Kim and Sharpless 2012).

In a detailed screening for cytotoxic and antioxidant properties of *U. narum* and *U. macropoda* leaves, *U.narum* has been found as the most potent one. The petroleum ether extract of *U.narum* (UNPE extract) being differentially cytotoxic (Chapter 3) warranted further study to assess for its cytotoxicity and antitumor efficacy using model cell lines and *in vivo* animal models. Attempts were also made to purify the active component to the maximum extent possible and explore the mechanistic basis of its action.

One of the commonest forms of experimental tumor models in mice is Ehrlich ascites carcinoma (EAC). It appeared as a spontaneous breast carcinoma in a female mouse and it is being used as an experimental model for inducing tumor by injecting subcutaneously (Ozaslan *et al.* 2011). Many preclinical studies have exploited EAC induced tumor models for checking the efficacy of chemotherapeutic agents. Several studies have reported antitumor efficacy using EAC induced ascites tumor models (Agrawal *et al.* 2011a, b; Patel *et al.* 2014; Pandya *et al.* 2013; Dhamija *et al.* 2013).

Another model for the identification of a chemotherapeutic drug is solid tumors. Dalton's lymphoma is a transplantable T-cell lymphoma of spontaneous origin in the thymus of murine host and it is exploited as an important model in cancer research (Rajkumar 2016). Dalton's lymphoma has been found very useful in a preclinical system for evaluating emerging drugs in the treatment of various cancers (Rajkumar 2016). In the current study, we selected DLA and EAC induced solid and ascites tumor models to demonstrate the cytotoxic effect of the UNPE extract.

Based on the acute and sub toxicity of UNPE extract in mice, sub-lethal doses suitable for further studies have been calculated (Chapter 4). In this chapter, our aim was to study the antitumor activity of the UNPE extract using Dalton's lymphoma ascites (DLA) and Ehrlich ascites carcinoma (EAC) cell induced mice, tumor models. The bioassay-guided partial purification of PE extracts and further, possible mechanism of action of the active fraction was also investigated.

5.2. Materials and methods

5.2.1. Collection and preparation of the plant extract

The collection of *Uvaria narum* leaves and the preparation of petroleum ether extract (PE) was performed as described in Chapter 2, section 2.1.5.3. and 2.2.1.

5.2.2. Animals

Female Swiss Albino mice (25 - 30 g) were purchased and maintained as described in Chapter 2, section 2.1.6.

5.2.3. Cell lines

The Daltons lymphoma ascites (DLA) and Ehrlich Ascites Carcinoma (EAC) cells were used for short-term *in vitro* cytotoxicity and antitumor study. The maintenance of DLA and EAC cells are mentioned in Chapter 2, section 1.7. Normal spleen cells for *in vitro* cytotoxicity studies were isolated as per the procedure mentioned in Chapter 2, section.2.1.7.

5.2.4. Analysis of the antitumor effect of UNPE extracts using *in vivo* mice model

5.2.4.1. Determination of the antitumor activity of UNPE extract using DLA cell induced solid tumor model

Swiss Albino female mice (20-25 g) were divided into 8 groups comprising 6 animals each. Group I: Control - Untreated; Group II: Prevehicle control (1% propylene glycol (p.o.) treated animals 5 days before tumor induction); Group III: vehicle control (1% propylene glycol treated animals simultaneously with tumor induction); Group IV: Standard (cyclophosphamide, 15 mg/kg b.wt.,p.o.); Group V & VI: 5 and 10 mg/kg

UNPE extract orally 5 days before tumor induction; Group VII & VIII: 5 and 10 mg/kg UNPE extracts simultaneously with tumor induction. All treatments were done orally (p.o.). DLA cells were aspirated from the peritoneal cavity of tumor-bearing mice washed thrice and suspended in PBS to a known volume. Cell suspension, 0.1 mL containing 1×10^6 cells was injected intramuscularly into the right hind limb of Swiss albino mice. Drug administration (propylene glycol, cyclophosphamide, and extracts) were continued for 10 consecutive days after tumor induction and diameter of the tumor was measured using a Vernier caliper in two perpendicular planes at every three days intervals following tumor induction. The tumor volume was calculated using the formula, $V = 4/3\pi \cdot a^2 \cdot b/2$ where 'a' is the minor diameter and 'b' is the major diameter (Ma *et al.* 1991). The weight of the animal, as well as the total WBC count, was determined every third day following tumor induction for 4 weeks. Percentage inhibition was calculated by the formula, Percentage inhibition = $C - T/C \cdot 100$, where 'T' and 'C' represent the number of days that treated and control animals survived.

5.2.4.2. Determination of the effect of UNPE extract on the survival of ascites tumor-bearing animals

Swiss Albino female mice (20-25 g) were divided into 8 groups comprising 6 animals each. Group I: Control - Untreated; Group II: Prevehicle control (1% propylene glycol (p.o.) treated animals 5 days before tumor induction); Group III: vehicle control (1% propylene glycol treated (p.o.) animals simultaneously with tumor induction); Group IV: Standard (cyclophosphamide, 15 mg/kg b.wt., p.o.); Group V and VI: 5 and 10 mg/kg *U. narum* leaf PE extract (p.o.) respectively, 5 days before tumor induction; Group VII and VIII: 5 and 10 mg/kg *U. narum* leaf PE extract (p.o.) respectively, simultaneously with tumor induction. Animals in all the groups were injected 1×10^6 EAC cells/animal intraperitoneally (0.1 mL) to induce ascites tumor. The treatments were done for 10 consecutive days. The death pattern of animals due to tumor burden was noted and the percentage of increase in lifespan was calculated using the formula, % increase in lifespan = $(T - C)/C \cdot 100$ where, 'T' and 'C' represent the number of days that treated and control animals survived, respectively.

5.2.5. GC-MS analysis of PE extract of *U. narum* leaves

The crude petroleum ether extract was subjected to GC-MS analysis, using a QP2010S Shimadzu system, Rxi-5Sil MS column with 30-meter length, 0.25 mm ID and 0.25 μm thickness and an MS Shimadzu detector. A carrier gas, Helium (99.99%) was used at a constant flow of 3 mL/minute in a splitless mode at 173 Pascal inlet pressure. An aliquot of 1.0 μL ethanol solution of the sample was injected to the MS column with an injection temperature of 260 °C; the GC column oven temperature was 80 °C, with an increment rate of 5 °C/min. The detector was set at a temperature of 280 °C. Ion source temperature was maintained at 200 °C. The mass spectrum of compounds in the UNPE extract was obtained by electron ionization at 70 eV. The detector was working in the scan mode range of 50-500 amu. The total running time was 45 min. The peaks were obtained using software, GC-MS solutions and confirmed with libraries of National Institute Standard and Technology, NIST 11 and WILEY 8 mass spectral databases.

5.2.6. Bioassay-guided partial purification of fractions from *U.narum* PE extracts using column chromatography

UNPE extract was further subjected to bioassay-guided fractionation using column chromatography. The crude extract of UNPE was subjected to column chromatography in a glass column of dimensions 300 x 10 mm for the purification of the active fraction. Silica gel (from Merck) of mesh size 60–120 was used as the stationary phase and the solvents used were hexane and methanol for separation of different phytochemical fractions. Approximately 100 mg of the UNPE extract was dissolved in a minimum volume of methanol and then mixed with 500 mg of silica gel for column chromatography, kept at 40 °C for evaporation to dryness and made to a fine powder using mortar and pestle. The column bed was made with silica gel (8 g) and the solvent methanol used to run before the sample loading. The dried powdered extract was loaded over the column bed and chromatographic separation was carried out by using methanol followed by hexane as mobile phase. The column eluate was collected as 100 mL fractions up to a total volume of 200 mL yielding methanol and hexane fractions. Each fraction was evaporated to remove solvents, washed and dried. The fractions were subjected to *in vitro* short-term cytotoxicity assay as mentioned in Chapter 2, section.2.2.5 and evaluated for the antiproliferative effect using different cell lines (Chapter 2, section 2.6) in long-term culture.

The most cytotoxically active fraction was sub-fractionated into ethyl acetate and toluene fractions using column chromatography. The dried fraction obtained from the first fractionation was subjected to silica gel column chromatography using ethyl acetate and toluene as mobile phases as per the method described as above. The ethyl acetate and toluene fractions were collected, dried and the resulting fractions were subjected to cytotoxicity and antiproliferative assay as described in Chapter 2, section 2.2.5 using different cell lines.

5.2.6.1. High-performance thin layer chromatography analysis

The methanolic and hexane fractions obtained from UNPE extract were subjected to qualitative high HPTLC as the method mentioned in section 2.2.4, Chapter 2. The number of bands and derivatized bands was determined for both fractions and Rf values were calculated.

5.2.7. Cell morphological analysis of the apoptosis-promoting efficacy of active ethyl acetate fraction

5.2.7.1. Acridine orange/Ethidium bromide (AO/EtBr) dual staining

HeLa cells were seeded on a 6 well plate at a density of 2×10^5 cells/mL in DMEM and allowed to grow till 80% confluence. The cells were then exposed to different concentrations (10-100 $\mu\text{g/mL}$) of the extract in fresh DMEM and incubated further for 24 hrs. Then, the media was removed and the cells were washed with ice-cold PBS. The cells were collected after trypsinization and by centrifuging at 2000 rpm for 10 minutes. The cell pellet was then re-suspended in PBS (50 μL). Resuspended cells were mixed with equal volume AO/EtBr dual staining solution (Preparation of AO/EtBr stain is described in chapter 2 section 2.4.1. The cell morphology was analyzed under a Leica fluorescent microscope with red and green filters and photographed.

5.2.7.2. Annexin V-FITC staining

HeLa cells were plated in 6 well plates at a density of 2×10^5 and incubated for 12 hrs following which 50 and 100 $\mu\text{g/mL}$ of ethyl acetate fraction was added and incubated for further 24 hrs. Then, the cells were trypsinized and washed with pre-chilled PBS

and resuspended with 100 μ l of 1X binding buffer containing 5 μ L of Annexin V-FITC and 5 μ L of propidium iodide for 15 min in dark. Then 400 μ L of binding buffer was added and cells were filtered through a cell strainer and analyzed using FACS Aria, BD, USA scanner.

5.2.7.3. Cell cycle analysis by flow cytometry

HeLa cells were cultured in DMEM with 10% FBS in 5% CO₂ humidified incubator. After reaching 70% confluence of cells, media was removed and washed with 3 mL PBS. Cells were trypsinized with 1X trypsin and FBS containing media was added to inhibit the trypsin action after detachment of cells. The cell suspension was centrifuged and pelleted at 1500 rpm for 2 min and the cell pellet was washed twice with PBS to remove any cell debris. The cells were resuspended in 1 mL DMEM and counted using hemocytometer. Cells having density 2×10^5 were seeded in 6-well plate and incubated for 24hrs for cell attachment and to attain their morphology. After 24 hrs cells were washed with PBS and exposed with ethyl acetate fraction at 50 and 100 μ g/mL concentration for 24 hrs and cells were then washed ones with PBS, trypsinized and washed again with ice-cold PBS. The pellets were resuspended and fixed with ice-cold 70% ethanol and incubated in ice for 45 min. The fixed cells were again washed with ice-cold PBS twice and resuspended in 100 μ L of PBS containing RNase A (1 mg / mL) for 30 min at 37 °C. A 10 μ L of propidium iodide (1 mg/mL) was added followed by an incubation of 15 min in dark and analysis was carried out by BD FACS Aria system.

5.2.8. GC-MS analysis of ethyl acetate column fraction of UNPE extract

The GC-MS analysis of the ethyl acetate fraction was carried out using the previously mentioned method (section 5.2.5.).

5.2.9. Statistical analysis

All *in vitro* assays were performed in triplicate and values represented are mean \pm SD. Statistical evaluation of the data was done by one way ANOVA using graph pad software (Instat 9.0). Results were considered statistically significant when $p < 0.05$.

5.3. Results

5.3.1. Antitumor effect of UNPE extract

Oral administration of sublethal concentrations (5 and 10 mg/kg b.wt.) of the extract was found to inhibit DLA induced solid tumor development in mice. Compared to cyclophosphamide (CTX), a known antitumor agent, the tumor reducing the efficacy of UNPE extract was less, however, the extract showed a considerable reduction in CTX induced myelosuppression.

5.3.1.1. Effect of UNPE extracts on solid tumor mice models

Oral administration of UNPE extract at 5 and 10 mg/kg body weight in animals for 5 days prior to tumor induction and in animals that received the same dose simultaneously with tumor induction was found to limit tumor development. There was a steady increase in tumor volume from an initial value of 0.24 ± 0.03 to $7.86 \pm 0.85 \text{ cm}^3$ following tumor injections over a period of 30 days in control group animals (Figure 5.1). Animals fed cyclophosphamide at 15 mg/kg b.wt. simultaneous with tumor induction orally, offered significant ($P < 0.01$) protection and showed a decrease in tumor volume from the initial 0.24 ± 0.13 to $0.98 \pm 0.16 \text{ cm}^3$ on day 15 and thereafter tumor growth reduced to $0.85 \pm 0.37 \text{ cm}^3$ (Figure 5.1). In the animals given pre-dose of *U.narum*, 5 mg/kg b.wt. there was a steady increase in tumor volume from an initial volume of 0.23 ± 0.04 to 3.82 ± 0.99 on day 15 and thereafter, it reduced to $3.46 \pm 0.54 \text{ cm}^3$. A similar pattern was observed with pre-dosing of *U. narum* at 10 mg/kg body weight orally with an initial tumor volume of $0.23 \pm 0.01 \text{ cm}^3$ growing to $3.59 \pm 0.54 \text{ cm}^3$ on day 15 and thereafter reducing to $3.31 \pm 0.723 \text{ cm}^3$ (Figure 5.1, Table 5.1). Simultaneous oral administration of UNPE extracts at 5 and 10 mg/kg b.wt. significantly inhibited the increase in tumor burden. In 10 mg/kg b.wt. group of animals, the initial tumor volume was found to be $0.23 \pm 0.032 \text{ cm}^3$ which increased only up to $3.08 \pm 0.73 \text{ cm}^3$ (Figure 5.1, Table 5.1). The percentage of inhibition in tumor growth at a concentration of 5 and 10 mg/kg b.wt. was found to be 57.30 and 61.07%, when compared to that of control. Percentage inhibition in tumor growth on the 30th day following cyclophosphamide treatment was 89.21 % (Table 5.1). In all the treatment groups, the inhibition of tumor growth was significant ($P < 0.01$) when compared to untreated control and vehicle control.

Figure 5.2.II depicts the WBC counts in the experimental groups from days 1 to 28. In the control group animals, the total WBC count remained the same throughout. In the cyclophosphamide-treated group, there was a significant decrease in WBC count from an initial value of 15050 ± 2140 to 4525 ± 388 on day 28. In animals that received a pre-dose of UNPE extract, there was no significant change in WBC count during the experimental period. In animals receiving 5 mg/kg b.wt. pre-dose, the count on day 28 was 11525 ± 2156.68 which was less than the day 1 count (13574 ± 975). On the 28th day, WBC count of 5 mg/kg b.wt. UNPE extract treated animals increased up to 11550 ± 1343.51 . Similarly, in pre-dose 10 mg/kg body weight animals, the initial count of 14782 ± 845 was reduced to 13100 ± 2757 on day 28 but the count was within normal limits. Simultaneous addition of 10 mg/kg b.wt. dose, however, maintained the body weight without any drastic change, throughout the period. But a slight reduction in WBC count was observed in 10 mg/kg body weight of UNPE extract, that was administered simultaneously but the numbers increased on day 28. The body weight of tumor-bearing animals over a period of 30 days did not show much change in any of the treated groups (Figure 5.2(I)).

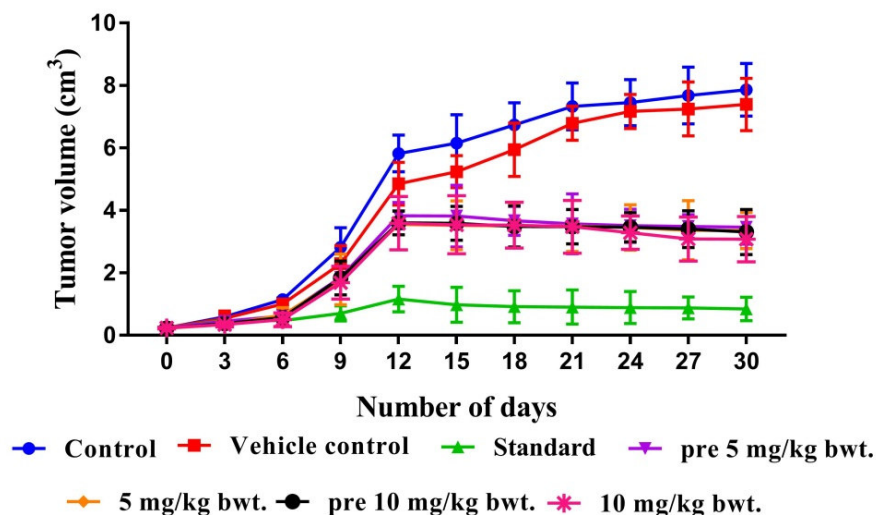


Figure 5.1. Solid tumor volume (cm³) in Swiss Albino mice after UNPE administration. Animals were inoculated with 1x10⁶ cells in the right hind limb. Test materials were given orally 5 days prior or simultaneously to inoculation. Tumor volume was measured using vernier calipers every 3rd day upto 28 days. P < 0.01 for all treated groups except vehicle control which is not significant when compared to control.

Table 5.1. The inhibitory effect of *U.narum* PE leaf extract on DLA induced solid tumor

Group	Tumor volume on the 30 th day	% tumor inhibition
Control	7.86 ± 0.85	--
Vehicle control	7.39 ± 0.84 ^{ns}	5.94
Standard	0.85 ± 0.37**	89.21
Pre 5 mg/kg bwt.	3.46 ± 0.54**	54.83
Pre 10 mg/kg bwt.	3.31 ± 0.72**	57.88
5 mg/kg bwt.	3.36 ± 0.58**	57.30
10 mg/kg bwt.	3.08 ± 0.78**	61.07

Values are expressed as mean \pm SD for 6 animals. **p < 0.01, when compared to control, ns: non-significant, p>0.05, (Vehicle control compared to control).

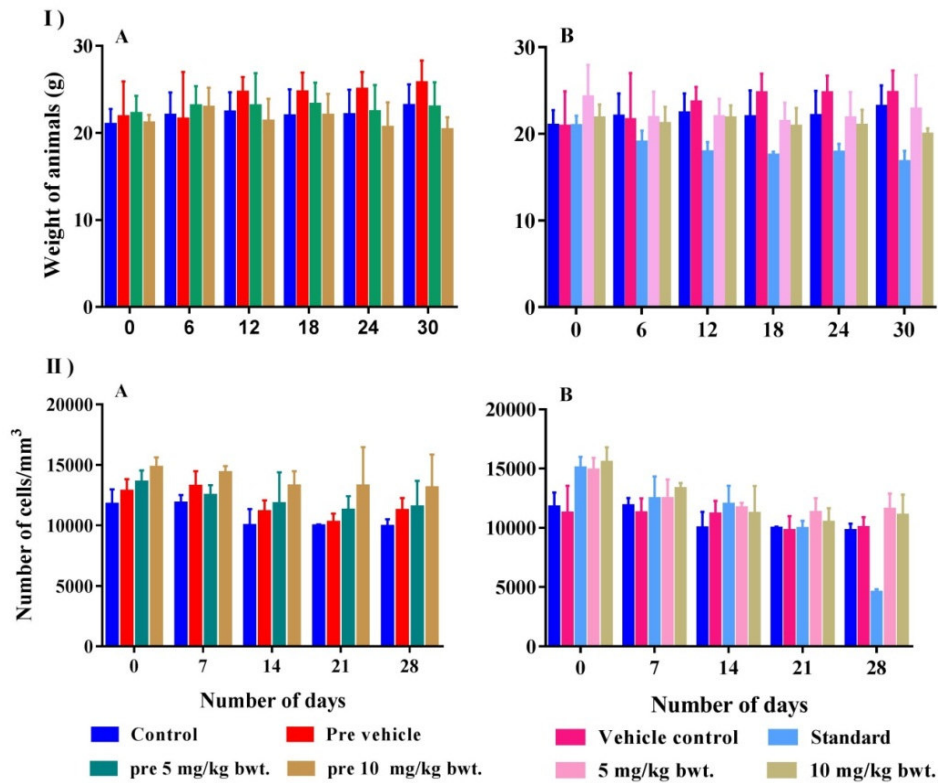


Figure 5.2. I(A): Body weight of UNPE pretreated animals; I(B): body weight of animals that got simultaneous treatment of UNPE along with tumor induction; II(A): total WBC count of DLA induced solid tumor-bearing mice that received pretreatment of different doses of extract; II(B): total WBC count of DLA induced solid tumor-bearing mice that received simultaneous extract treatment with tumor induction. Animals were inoculated with 1×10^6 cells in the right hind limb. Test materials were given orally 5 days prior or simultaneously to inoculation. Blood was collected every third day by tail vein puncture and WBC was counted. Body weight was measured weekly upto 28 days. Values are expressed as mean \pm SD for 6 animals.

5.3.1.2. Effect of UNPE extracts on ascites tumor development in mice model

The lifespan of ascites tumor-bearing mice treated with UNPE extract was found to be significantly increased compared to the control group animals (19 ± 0.71) days. UNPE extract treated animals with 5 and 10 mg/kg b.wt. survived 28.00 ± 1.52 and 29.75 ± 2.27 days with an increase in lifespan of 47.36 ± 2.07 and 56.58 ± 4.15 %, respectively (Figure 5.3 and 5.4, Table 5.2). UNPE extract pretreatment was found to have a similar effect, showing an increase in lifespan of 40.35 ± 2.46 % for 5 mg/kg b.wt (26.67 ± 2.12 days) and 52.63 ± 3.61 % for 10 mg/kg b.wt (29.00 ± 2.89 days) in comparison to the control group (19.00 ± 0.71 days). Mean survival time (MST) values showed no significant variation among the individuals of prevehicle and vehicle groups (19.60 ± 2.94 and 19.50 ± 1.73 days, respectively) with that of control animals (Figure 5.3 & 5.4, Table 5.2). However, there was a marked increase in cyclophosphamide-treated animals (31.75 ± 1.13 days) and UNPE treated animals. The predoses treated animals had MST values of 26.67 ± 2.12 and 29.00 ± 2.89 days in 5 and 10 mg/kg b.wt. treated animals, respectively. With respect to the control group animals, Cyclophosphamide group had 67.10 ± 3.27 % increases in lifespan (Figure 5.3 and 5.4, Table 5.2).

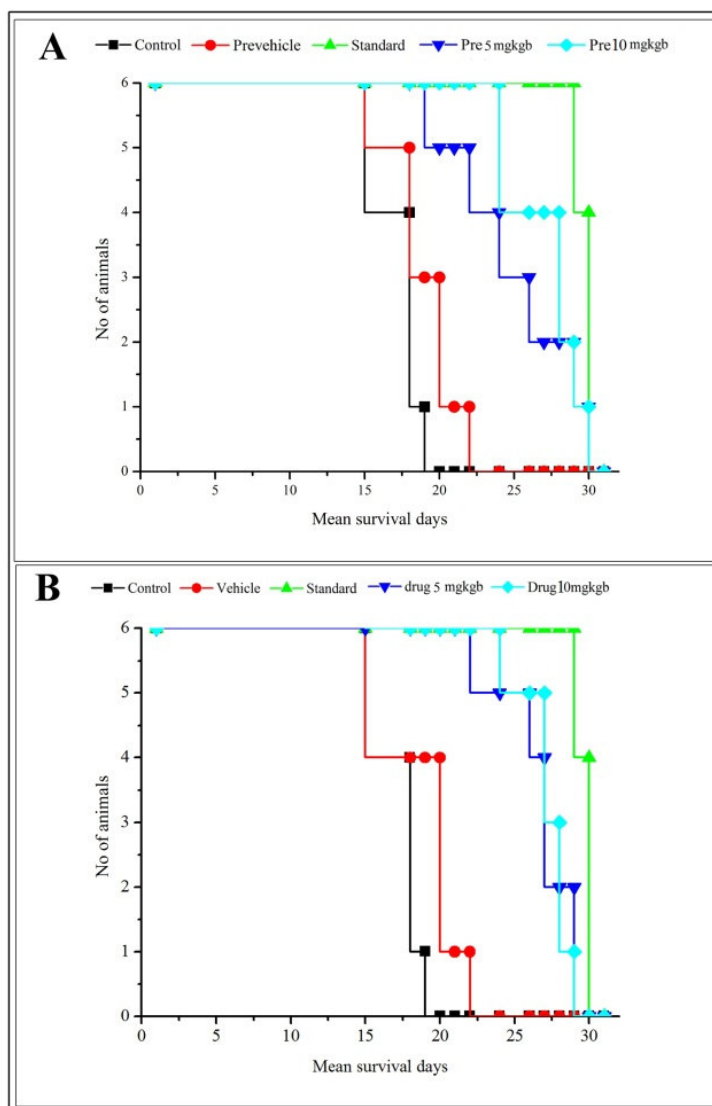


Figure 5.3. The survival rate of ascites tumor bearing mice following UNPE extract administration. Animals were inoculated with 1×10^6 cells into the peritoneal cavity. Test materials were given orally 5 days prior or simultaneously to inoculation. A: Groups with treatment scheduled for 5 days before tumor induction. B: Groups with treatment scheduled simultaneously with tumor induction. Values are expressed as mean \pm SD for 6 animals.

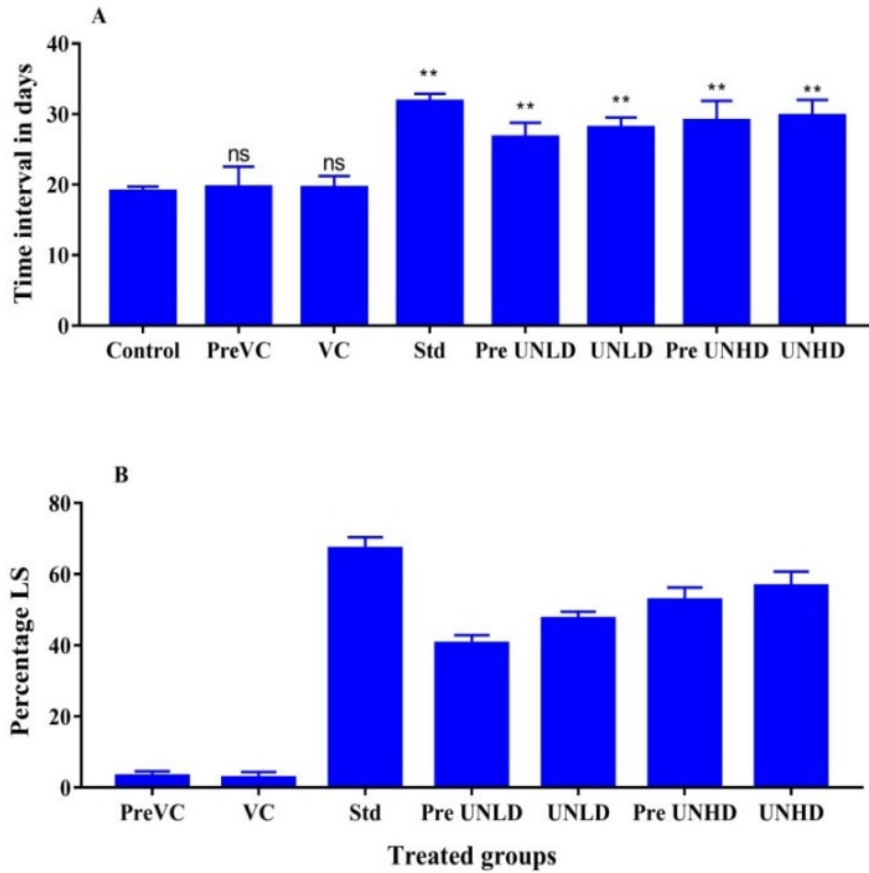


Figure 5.4. Survival and extent of lifespan in tumor-bearing animals following UNPE extract administration. Animals were inoculated with 1×10^6 cells into the peritoneal cavity. Test materials were given orally 5 days prior or simultaneously to inoculation. A: survival rate and B: % increase in lifespan of ascites tumor-bearing mice. ('Pre' denotes the treatment started before 5 days of tumor induction; UNLD: 5 mg/kg b.wt.; UNHD: 10 mg/kg b.wt.; Std: cyclophosphamide 15 mg/kg b.wt.). Values are expressed as mean \pm SD of 6 animals per group. $P < 0.01$ for all treated groups except vehicle control which is not significant when compared to control.

Table 5.2. Mean survival days and % increase in lifespan of ascites tumor-bearing mice treated with UNPE extract.

Group	Mean survival days	% increase in lifespan
Control	19.00 ± 0.71	----
Pre vehicle	19.60 ± 2.94 ^{ns}	3.16 ± 1.42
Vehicle control	19.50 ± 1.73 ^{ns}	2.63 ± 1.74
Standard	31.75 ± 1.13**	67.10 ± 3.27
Pre 5 mg/kg bwt.	26.67 ± 2.12**	40.35 ± 2.46
Pre 10 mg/kg bwt.	29.00 ± 2.89**	52.63 ± 3.61
5 mg/kg bwt.	28.00 ± 1.52**	47.36 ± 2.07
10 mg/kg bwt.	29.75 ± 2.27**	56.58 ± 4.15

Values are expressed as mean ± SD for 6 animals. **p < 0.01, when compared to control, ns: non-significant, p>0.05, (Pre vehicle and Vehicle control compared to control)

5.3.2. GC- MS analysis of UNPE extract

The results obtained from GC-MS analysis of UNPE extract lead to the identification of a number of compounds. Compounds were determined by comparing the ion chromatogram spectra with the spectra of available compounds in the database of the library of National Institute of Standard and Technology (NIST Library) and WILEY 8 on the basis of retention time (RT), m/z ratio & and peak area.

The representative GC-MS chromatogram of UNPE extract is provided in Figure 5.5 and a list of compounds identified is given in table 5.3. UNPE was found to contain 53 compounds in the GC-MS analysis (Figure 5.5 and Table 5.3). The total ion chromatogram of UNPE extract has shown that the retention time of different phytocompounds ranged from 12.72 to 44.38 minutes. Tetratetracontane, 2(1h)-pyridone, 6-(Acetoxymethyl)-5-(benzyloxy)-5,6-dihydro- and Guaiacol benzoate are the compounds found with higher abundance (high peak area percent) in the extract (figure 5.5 and table 5.3). Table 5.4 shows various compounds of pharmaceutical importance identified in UNPE extract by GC-MS analysis.

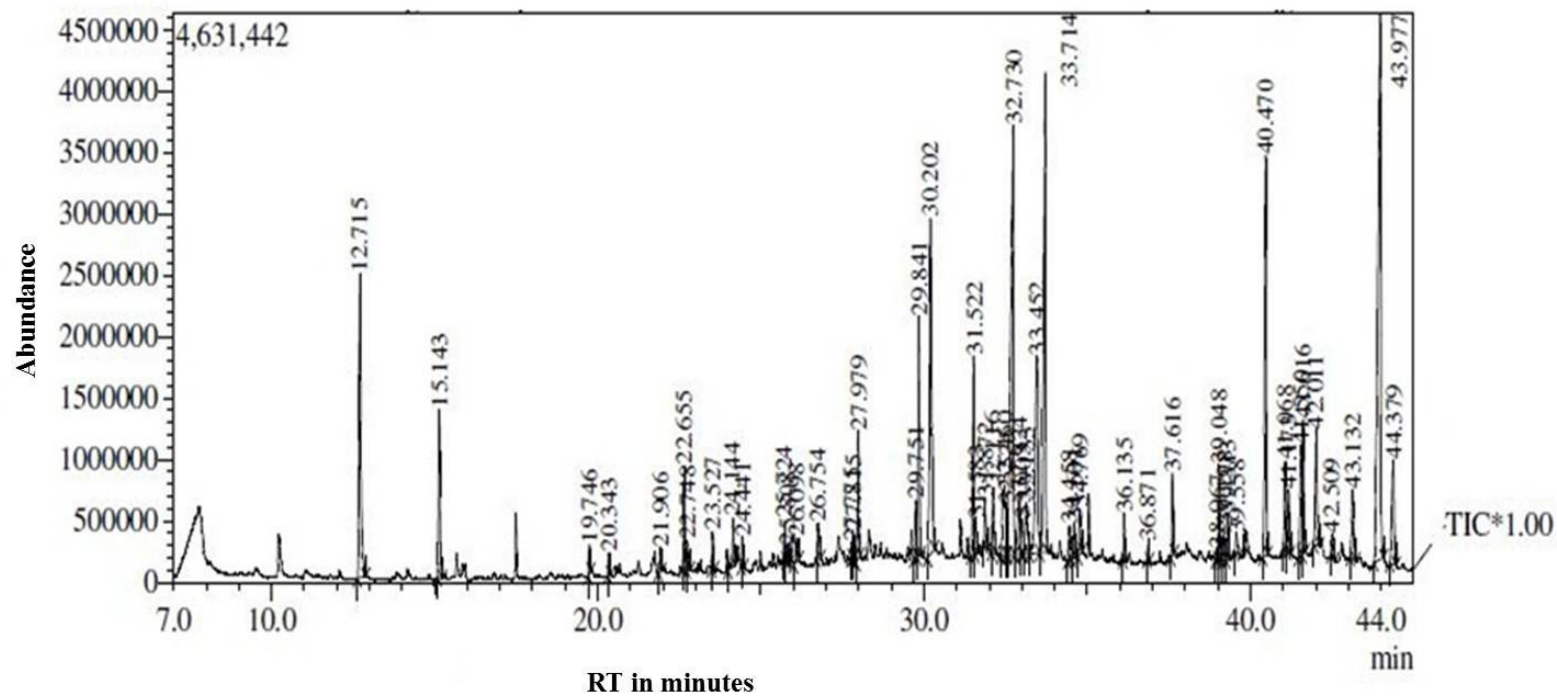


Figure 5.5. Total ion chromatogram (TIC) of UNPE extract of *U.narum* leaves when subjected to GC MS analysis.

Table 5.3. Total compounds identified in the of UNPE extract of *U.narum* leaves by GC-MS analysis.

Peak	R.Time	Area%	Name	Molecular Formula	Molecular weight(KDa)
1	12.715	5.44	Hexadecane	C ₁₆ H ₃₄	226.448
2	15.143	2.30	Pentadecane	C ₁₅ H ₃₂	212.421
3	19.746	0.31	Heptadecane	C ₁₇ H ₃₆	420.725
4	20.343	0.24	Hexahydrofarnesol	C ₁₅ H ₃₂ O	228.42
5	21.906	0.28	Nonadecane	C ₁₉ H ₄₀	268.529
6	22.655	1.18	Neophytadiene	C ₂₀ H ₃₈	278.524
7	22.748	0.44	Hexahydrofarnesyl Acetone	C ₁₈ H ₃₆ O	268.485
8	23.527	0.41	9-Eicosyne	C ₂₀ H ₃₈	278.524
9	24.144	0.70	Farnesyl Acetone C	C ₁₈ H ₃₀ O	262.437
10	24.441	0.27	Hexadecanoic Acid, Methyl Ester	C ₁₇ H ₃₄ O ₂	270.457
11	25.724	0.45	6,10-Dodecadien-1-Yn-3-Ol, 3,7,11-Trimethyl-	C ₁₅ H ₂₄ O	220.3505
12	25.808	0.21	1-Hexacosene	C ₂₆ H ₅₂	364.702
13	26.098	0.37	1-Phenyl-4-Penten-1-One	C ₁₁ H ₁₂ O	160.216
14	26.754	0.56	3-(Benzoylsulfanyl)-2-Methylpropanoic Acid	C ₁₁ H ₁₂ O ₃ S	224.276

15	27.775	0.15	9-Octadecenoic Acid (Z)-, Methyl Ester	C ₁₉ H ₃₆ O ₂	296.4879
16	27.815	0.26	Iron, Tricarbonyl[N-(Phenyl-2-Pyridinylmethylene) Benzenamine-N,N']	C ₂₁ H ₁₄ FeN ₂ O ₃	398.193
17	27.979	1.67	Phytol	C ₂₀ H ₄₀ O	296.531
18	29.751	0.78	Carbonic Acid, Hexadecyl Propyl Ester	C ₂₀ H ₄₀ O ₃	328.530
19	29.841	2.95	Phytol, Acetate	C ₂₂ H ₄₂ O ₂	338.568
20	30.202	6.56	2-Cyclohexene-1,4-Dione, 5,6-Dibromo-2,6-Dimethyl-, 1-Oxime, O-Benzoyl-	C ₉ H ₁₂ O ₂	152.190
21	31.522	2.79	9-Borabicyclo[3.3.1]Nonane, 9-(Benzoyloxy)-	C ₁₅ H ₁₉ BO ₂	242.121
22	31.583	0.45	Benzoic Acid, 1,3,3-Trimethyl-2-Oxa-Bicyclo[2.2.2]Oct-6-Yl Ester	C ₁₇ H ₂₂ O ₃	274.355
23	31.872	0.66	5'-O-(4-Oxopentanoyl)-2'-O-Uridine	C ₂₁ H ₂₂ N ₂ O ₉	446.412
24	32.116	0.88	4,8,12,16-Tetramethylheptadecan-4-Olide	C ₂₁ H ₄₀ O ₂	324.549
25	32.460	2.06	Benzo[B]Thiophene-3-Carboxylic Acid, 4,5,6,7-Tetrahydro-2-Benzoylamino-7-Oxo-, Ethyl Ester	C ₂₂ H ₂₈ N ₂ O ₃ S	400.54
26	32.525	0.58	Hexadeca-2,6,10,14-Tetraen-1-ol, 3,7,11,16-Tetramethyl-	C ₂₀ H ₃₄ O	332.52
27	32.730	11.24	Benzoic Acid, (5,5-Dimethyl-4-Oxo-2-Cyclohexenyl) Ester	C ₁₅ H ₁₆ O ₃	244.27 g/mol
28	32.924	1.51	2(1H)-Pyridone, 6-(Acetoxymethyl)-5-(Benzoyloxy)-5,6-Dihydro-	C ₁₅ H ₁₅ NO ₅	289.28
29	33.003	0.49	Eicosane	C ₂₀ H ₄₂	282.556
30	33.133	1.30	Benzoic Acid, Dec-2-yl Ester	C ₁₇ H ₂₆ O ₂	262.3871
31	33.452	5.18	Benzoic Acid 3-Carbamoyl-2,2,5,5-Tetramethyl-2,5-Dihydro-Pyrrol-1-Yl Ester	C ₁₆ H ₂₀ N ₂ O ₃	288.342

32	33.714	9.00	Guaiacol Benzoate	$C_{14}H_{12}O_3$	228.2433
33	34.458	0.56	2-Ethylbutyric Acid, Eicosyl Ester	$C_{26}H_{52}O_2$	396.6899
34	34.601	0.49	Pentacosane	$C_{25}H_{52}$	352.6804
35	34.769	0.68	Palmitic acid .beta.-monoglyceride	$C_{19}H_{38}$	330.503
36	36.135	0.50	Tricosane	$C_{23}H_{48}$	324.6272
37	36.871	0.18	4,4'-((P-Phenylene)Diisopropylidene)Diphenol	$C_{24}H_{26}$	346.462
38	37.616	0.87	Celidoniol, Deoxy-	$C_{29}H_{60}$	408.787
39	38.967	0.15	Decanedioic Acid, Bis(2-Ethylhexyl) Ester	$C_{26}H_{50}$	426.673
40	39.048	1.11	Heptacosane	$C_{27}H_{56}$	380.733
41	39.198	0.41	Squalene	$C_{30}H_{50}$	410.718
42	39.313	0.54	Phenacyl 11-Octadecenoate	$C_{26}H_{40}O_3$	400.594
43	39.558	0.32	14-.Beta.-H-Pregna	$C_{21}H_{36}$	288.510
44	40.470	5.91	Pentatriacontane	$C_{35}H_{72}$	492.946
45	41.068	1.33	9-(Benzoyloxy)-9-Borabicyclo[3.3.1]Nonane #	$C_{15}H_{19} BO_2$	242.121
46	41.173	0.84	Benzoic acid , (6-[benzoyloxy] methyl-2,8-dioxabicyclo ,3,2,1 Oct-7-yl) ester	$C_{21}H_{20}O_6$	368.380
47	41.550	2.00	Methyl-2-deoxyribofuranose 3,5-dibenzoate	$C_{20}H_{20}O_6$	356.369
48	41.616	1.80	2-Oxobicyclo(3.2.2)nona-3,6-dien-1-yl benzoate	$C_{16}H_{14}$	254.281
49	42.011	1.96	2-methyloctacosane	$C_{29}H_{60}$	408.787

50	42.509	0.36	Benzene acetic acid, .alpha.-oxo-, ethyl ester	$C_{10}H_{10}O_3$	178.187
51	43.132	1.28	2-Cyclohexene-1,4-dione, 5,6-dibromo-2,6-dimethyl-, 1-oxime, o-benzoyl-	$C_{15}H_{13}Br_2NO_3$	415.077
52	43.977	14.87	Tetratetracontane	$C_{44}H_{90}$	619.185
53	44.379	2.21	Vitamin E	$C_{29}H_{50}O_2$	430.7061

Table 5.4. Compounds in PE extract of *U.narum* leaves identified by GC-MS analysis and their reported biological activity

Sl. No	Compound name	Biological activities
1.	Phytol	Antibacterial, antiradical (Pejin <i>et al.</i> 2014b), cytotoxic (Pejin <i>et al.</i> 2014a), antitumor (Kim <i>et al.</i> 2015), anti-inflammatory (Silva <i>et al.</i> 2014)
2.	Squalene	Antioxidant (Gunes 2013), chemopreventive, antitumor, decreasing LDL cholesterol, additive (Spanova and Daum 2011).
3.	Hexadecane	Antimicrobial, antioxidant, cholesterol reducing, hemolytic, nematocidal (Candra <i>et al.</i> 2018).
4.	Pentadecane	Antimicrobial (Bruno <i>et al.</i> 2015)
5.	Heptadecane	Anti-inflammatory (Kim <i>et al.</i> 2013).
6.	Hexadecanoic acid, methyl ester	Antimicrobial, antioxidant, cholesterol reducing, hemolytic, and nematocidal (Candra <i>et al.</i> 2018).
7.	Eicosane	Antioxidant, cytotoxic, antibacterial and antitumor properties (Phillips <i>et al.</i> 2015; Akpuaka <i>et al.</i> 2013; Yu <i>et al.</i> 2005).
8.	Vitamin E	Hepatoprotective and neuroprotective (Seo <i>et al.</i> 2015), Free radical scavenging (Rastogi <i>et al.</i> 2014), antioxidant (Jayasinghe <i>et al.</i> 2013).

5.3.4. Bioassay-guided partial purification of UNPE extract and selection of active fraction

Partial purification of UNPE extract using column chromatography yielded partially polar (methanolic soluble) and highly polar (hexane soluble) fractions. The cytotoxically active fraction was found to be in the partially polar methanolic fraction which was further subfractionated into toluene and ethyl acetate used as mobile solvents.

5.3.4.1. *In vitro* short-term cytotoxicity assay of methanolic and hexane fractions

The methanol fraction of UNPE was found to be more cytotoxic towards DLA and EAC cells compared to hexane fraction. IC₅₀ values of methanol extract on DLA, EAC, and spleen cells are found to be 27.82 ± 1.02 , 47.27 ± 2.13 and $>100 \mu\text{g/mL}$, respectively. Hexane extract showed less cytotoxicity, with IC₅₀ values $>100 \mu\text{g/mL}$ for all these three cell lines. So the methanolic extract was found to be a more cytotoxic fraction (Figure.5.6).

5.3.4.2. Antiproliferative activities of methanolic and hexane fractions of *U.narum* PE extract

Antiproliferative assay by MTT was also done to determine the most active cytotoxic fraction from UNPE extract. The methanolic extract showed more antiproliferative activity towards HeLa, Vero, HepG2 and HCT-15 cells with documented IC₅₀ values of 23.09 ± 2.54 , 98.53 ± 5.47 , 26.05 ± 1.24 and $42.21 \pm 2.08 \mu\text{g/mL}$, respectively. Hexane fraction, however, showed dose-dependent cytotoxicity with IC₅₀ values 78.16 ± 1.91 , 99.94 ± 2.75 , 67.58 ± 2.07 and $>100 \mu\text{g/mL}$ for HeLa, Vero, HepG2 and HCT-15 cell lines, respectively. Methanolic fraction possessed significant antiproliferative activity than hexane extract (Figure.5.7).

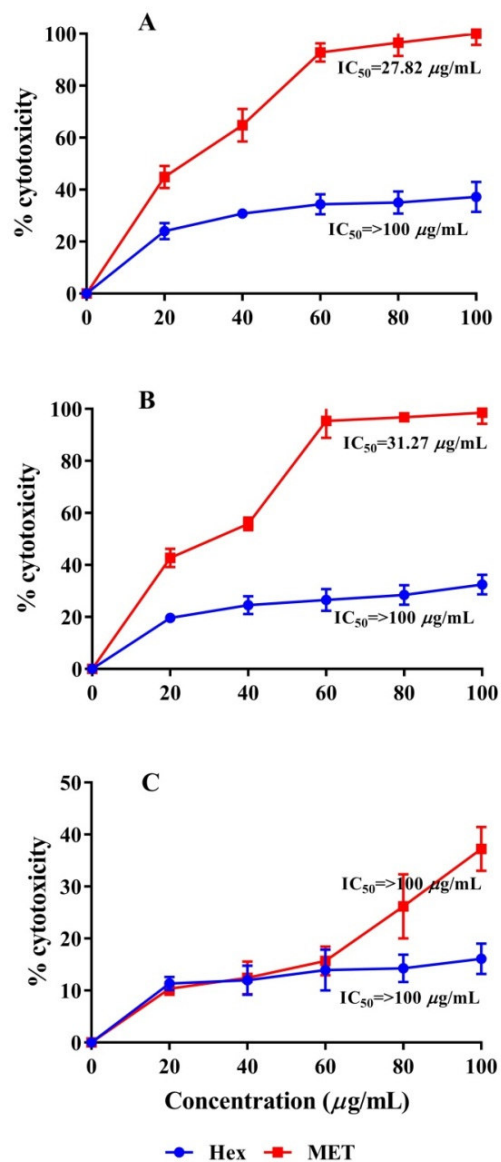


Figure 5.6. Cytotoxic effect of fractions of UNPE extract: Methanolic and hexane fractions were added to A: DLA, B: EAC and C: spleen cells in test tubes at a varying concentration as indicated and incubated at 37 °C for 3 hrs following which cell death in the cell population was detected using Trypan blue method. Percentage change in cell death (% cytotoxicity) was calculated in the treated tubes compared to untreated cells. Values are mean \pm SD of three independent experiments.

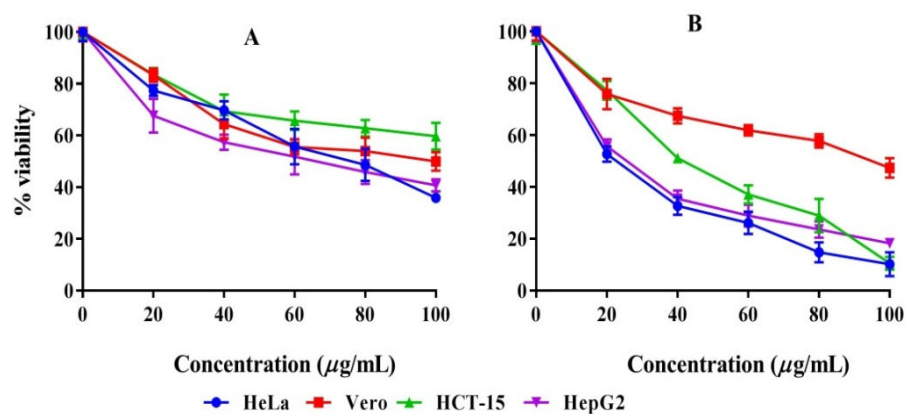


Figure.5.7. Antiproliferative activity of fractions of UNPE extract: Hexane (A) and methanol (B) fractions at varying concentrations (indicated in the figure) were added to HeLa, Vero, HCT-15, and HepG2 in the culture at 70-80% confluence and allowed to incubate for 48 hrs. Cell viability was determined by MTT assay. Percentage changes in the viability of treated cells against untreated control groups were calculated. Values represent means \pm SD of at least 3 replica cultures.

5.3.4.3. HPTLC analysis

Comparison of methanolic and hexane fractions as well as crude UNPE extract based on HPTLC separation followed by detection at 254 and 366 nm and also derivatization using anisaldehyde spray reagent (550 nm) confirmed the presence of terpenoids and sterol compounds in the extract. There were 4 bands visualized in UV at 254 nm, 366 nm and after derivatization with anisaldehyde sulphuric acid reagent (550 nm). All the 6 bands separated in hexane fraction lies in the upper portion of the chromatogram with Rf values ranging from 0.58 and 0.86 and among them, compounds with Rf values 0.69 and 0.80 were found to be terpenoid upon reagent spray. Methanolic fraction contained 8 bands that separated in the lower part of chromatogram Rf values ranging from 0.21 to 0.56. And these bands were found to be sterol or terpenoid. Two terpenoid bands with Rf values 0.69 and 0.80 were found in the upper half of chromatogram. The Rf values are given in figure 5.8.

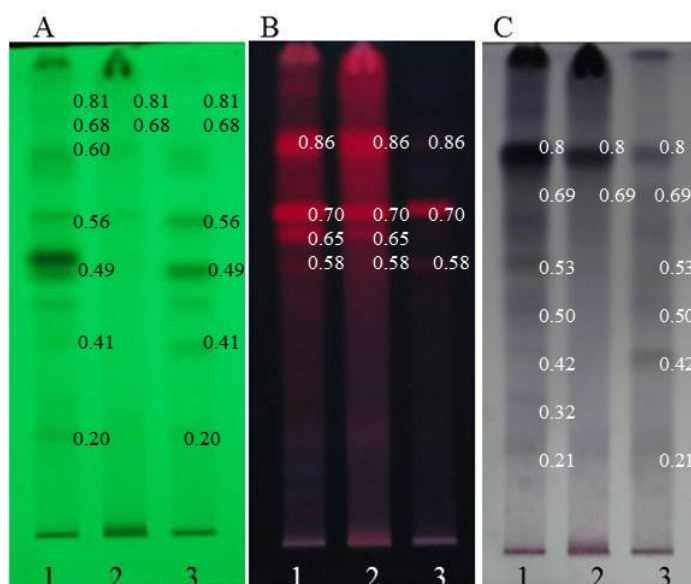


Figure 5.8. HPTLC analysis of 1) UNPE extract, 2) Hexane and 3) methanol fractions visualized under A) UV 254 nm B) 366 nm and C) derivatization using spraying reagent anisaldehyde sulphuric acid using solvent system of toluene: ethyl acetate: formic acid (8:2:0.2 v/v/v). 10 μ L of the sample was loaded on pre-coated silica gel TLC plates using spotting device. The developed plates were scanned at 366 and 254 nm with a CAMAG TLC Scanner. Derivatization of the plate was done using anisaldehyde - sulphuric acid spraying reagent and visualized after 10 min at 115° C in a hot air oven. The obtained data were analyzed using CAMAG WinCat software.

5.3.4.4. Short term cytotoxicity assay of ethyl acetate and toluene sub-fractions

The cytotoxic potential of methanol fraction of UNPE extract upon further fractionation yielded toluene and ethyl acetate sub-fractions. The *in vitro* cytotoxicity analysis of ethyl acetate sub-fraction showed higher cytotoxicity towards DLA cells with IC_{50} value $49.83 \pm 1.48 \mu\text{g/mL}$ and less toxic to EAC and normal spleen cells with an IC_{50} value greater than $100 \mu\text{g/mL}$ (Figure.5.9). Toluene extract, on the other hand, was found to be less toxic to three of these cells with IC_{50} value $>100 \mu\text{g/mL}$.

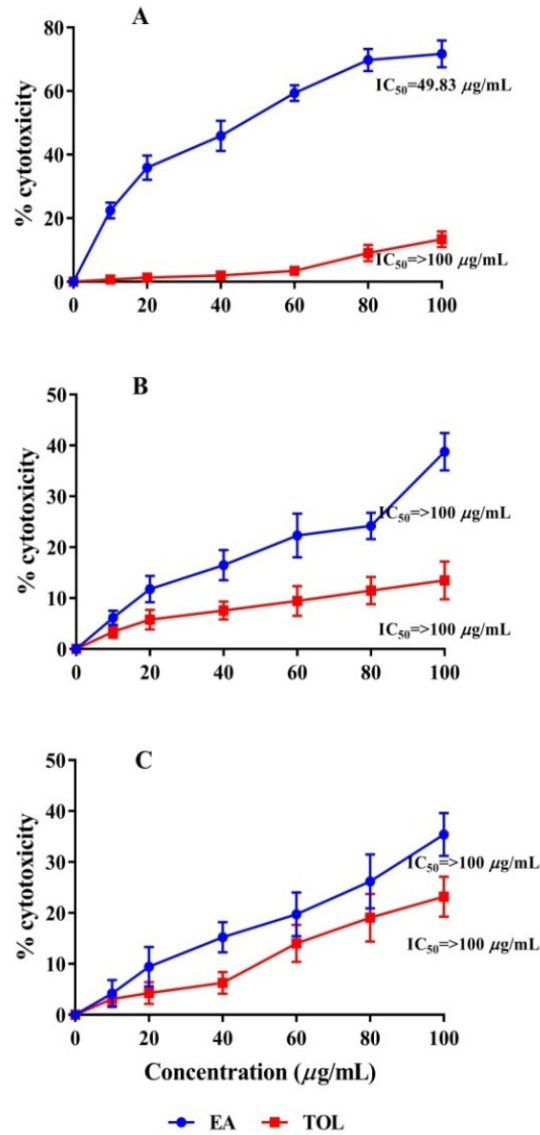


Figure 5.9. Cytotoxic effect of sub-fractions of UNPE extract MET fraction: ethyl acetate and toluene sub-fractions at varying concentration (as indicated) were added to A: DLA, B: EAC and C: spleen cells in test tubes and incubated at 37 °C for 3hrs following which cell death in the cell population was detected using Trypan blue method. Percentage change in cell death (% cytotoxicity) was calculated in the treated cells compared to untreated ones. Values are mean \pm SD of three independent experiments.

5.3.4.5. Antiproliferative activities of ethyl acetate and toluene fractions

Among the sub-fractions of UNPE extract under preliminary screening with the antiproliferative assay, ethyl acetate sub-fraction showed cytotoxicity in a dose-dependent manner. Ethyl acetate sub-fraction showed toxicity towards various cell lines such as HeLa, Vero, HCT-15 and HepG2 cells with IC_{50} values of 97.42 ± 2.41 , 90.92 ± 1.45 , 47.92 ± 1.03 and $94.47 \pm 1.09 \mu\text{g/mL}$, respectively (Figure 5.10). Toluene sub-fraction was found to be non-toxic with IC_{50} values more than $>100 \mu\text{g/mL}$ in all cell lines tested, except HCT-15 (IC_{50} of $61.38 \pm 2.14 \mu\text{g/mL}$). HCT-15 cells are most susceptible to the treatment of both ethyl acetate and toluene fractions. (Figure 5.10).

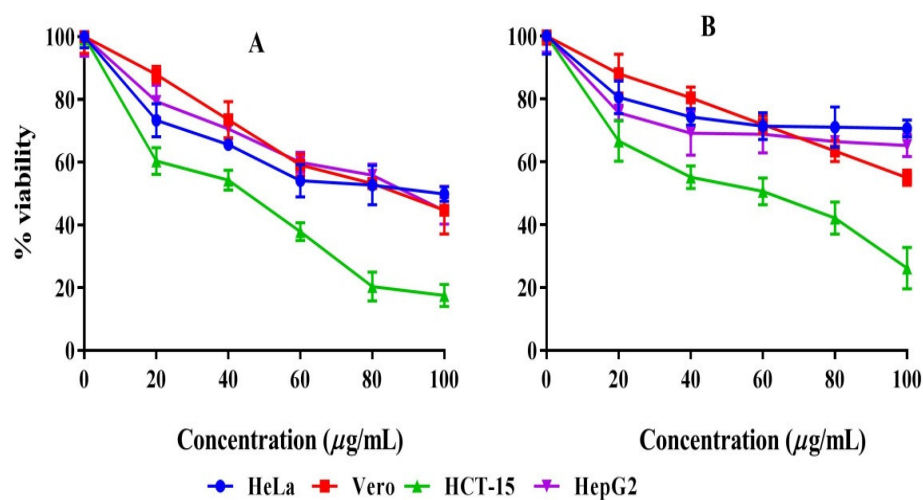


Figure 5.10. Antiproliferative activities of sub-fractions of UNPE extract MF: Ethyl acetate (A) and toluene (B) sub-fractions at varying concentrations (indicated in the figure) were added to HeLa, Vero, HCT-15 and HepG2 in the culture at 70-80% confluence and allowed to incubate for 48 hrs. Cell viability was determined by MTT assay. Percentage change in the viability of treated cells against untreated control groups was calculated. Values represent means \pm SD of at least 3 replica cultures. The concentration of 50% viability was calculated.

5.3.5. Cell morphological analysis of apoptosis-inducing efficacy of UNMET extract and active sub-fractions

5.3.5.1. Acridine orange/Ethidium bromide (AO/EtBr) dual staining

The cells exposed to UNPE extract at $15\mu\text{g/mL}$ resulted in late apoptosis with orange coloration and denaturation of DNA (5.11.B). The methanol fraction of UNPE extract ($20\mu\text{g/mL}$) (UNPE MF) exposure showed similar results and ethyl acetate sub-fraction in the concentration of $90\mu\text{g/mL}$ treated resulted in nuclear chromatin condensation and blebbing in HeLa cells (Figure 5.11.C) as the sign of early or moderate apoptosis (Figure 5.11D). The untreated viable cells are in bright green coloration (5.11.A).

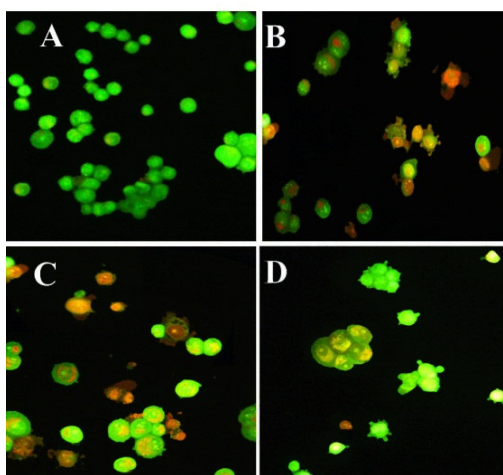


Figure 5.11. Induction of apoptosis in HeLa cells by UNPE extracts and their sub-fractions. HeLa cells in culture was incubated with UNPE extract at $15\mu\text{g/mL}$ (B) UNPE MF at $20\mu\text{g/mL}$ (C) and Ethyl acetate sub-fraction at $90\mu\text{g/mL}$ (D) for 48hrs. The cells were harvested and suspended in AO/ EtBr in $100\mu\text{L}$ PBS buffer for 3 min. Cells in $10\mu\text{L}$ suspension were then spread on a glass slide and observed using a fluorescent microscope under green and red filters. Photographs (20X magnification) were taken and merged. Cellular morphology in comparison to Untreated HeLa cells (A) was assessed.

5.3.5.2. Annexin V-FITC staining and detection of cell cycle flow cytometry analysis

Annexin-V-FITC (fluorescein isothiocyanate (FITC) conjugated form of Annexin V) staining was performed in order to determine the cells in the apoptotic stage to distinguish different stages of apoptosis. Here, Annexin V-FITC staining was done for untreated control cells and ethyl acetate sub-fraction 50 and 100 $\mu\text{g}/\text{ml}$ treated cells and analyzed using FACS Aria, BD, USA scanner. HeLa cells were treated with ethyl acetate sub-fraction 50 and 100 $\mu\text{g}/\text{ml}$ treated cells to demonstrate the dose-dependent effect on apoptosis. Figure 5.12 and Table 5.5 show- data from the HeLa cells dose response experiment as Annexin V FITC-A vs PI-A contour plots with quadrant gates showing four populations. The results showed that the majority of untreated cells were non-apoptotic and seen in the Quadrant Q3. There was an increase in the accumulation of cells in Q2 quadrant which represented cells in the late apoptotic phase and showed a dose-dependent increase in the Annexin V-FITC + PI population when compared to the untreated control. 100 $\mu\text{g}/\text{mL}$ EA sub-fraction induced apoptosis in HeLa cells at 24 hrs incubation (Figure 5.12 and Table 5.5). The results showed that apoptotic cells are more in treated cells especially in 100 $\mu\text{g}/\text{ml}$ of treated cells compared to the untreated one.

Cell cycle analysis of HeLa cells by flow cytometry revealed that cells treated with ethyl acetate sub-fraction (EA Fr) were arrested predominantly in sub G1 phase. The percentage of cells in the sub-G1 phase of the cell cycle for the untreated control was 2.35 and for the treated cells with EA sub-fraction at 50 and 100 $\mu\text{g}/\text{ml}$ concentrations were to 4.3 and 9.3%, respectively. In the G1 phase, the relative percentage of cells were 77.6, 77.6 and 73.6% for control, 50 and 100 $\mu\text{g}/\text{mL}$ EA Fr treated cells, respectively. The relative percentage of cells in the S phase of the cell cycle was reduced in the EA sub-fraction treated cells. No significant changes were observed in other phases. A representative diagram of cell cycle analysis by flow cytometry of control as well as EA sub-fraction treated groups are depicted in figures 5.13 and 5.14 and table 5.6. These results demonstrated the apoptotic effects of EA sub-fraction in tumor cells.

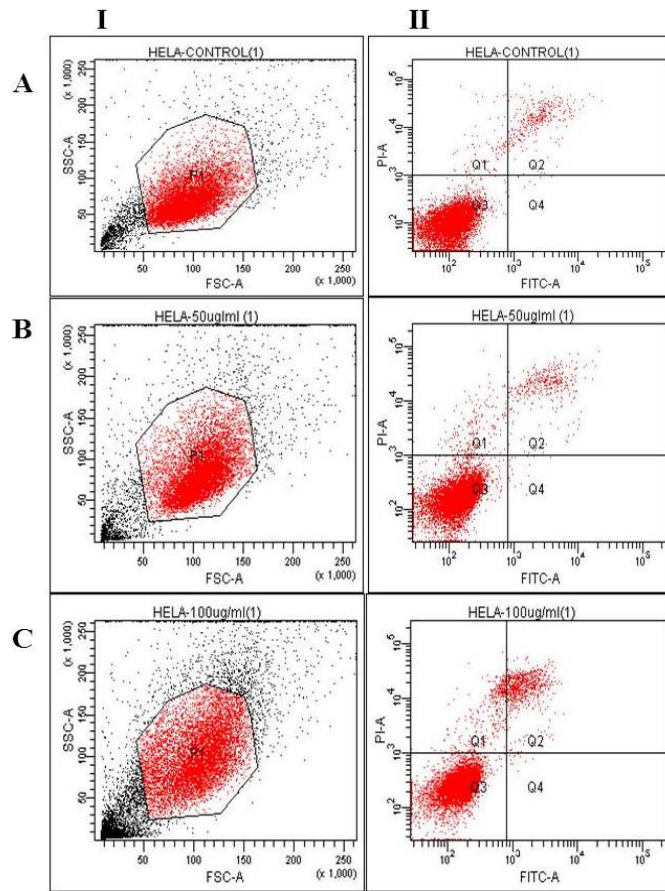


Figure 5.12. showing the apoptotic pattern of A) untreated control HeLa cells and ethyl acetate sub-fraction B) 50 and C) 100 $\mu\text{g}/\text{ml}$ treated cells. HeLa cells of density 2×10^5 were plated incubated for 12 hrs and following incubated for further 24 hrs with 50 and 100 $\mu\text{g}/\text{mL}$ of ethyl acetate fraction. Trypsinized cells were washed with pre-chilled PBS and resuspended with 100 μL of 1x binding buffer containing 5 μL of Annexin V-FITC and 5 μL of propidium iodide for 15 minutes in dark. 400 μL of binding buffer added cells were filtered through a cell strainer and analyzed using FACS Aria, BD, USA scanner. Cell counts in I) Dot plot I represent: Forward scatter Annexin (FSC-A) vs. Side scatter Annexin (SSC-A) with a gate for cells II) Dot plot II: -FITC-A (Annexin V-FITC) vs. PI (Propidium iodide). Untreated control cells are in the Q3 quadrant with non-apoptotic cells and 100 $\mu\text{g}/\text{mL}$ treated cells were in Q2 with cells of late apoptotic phase.

Table 5.5. Percentage of the cell population in control and ethyl acetate sub-fraction treatment after staining with Annexin V-FITC staining and analyzed using FACS scanner

	Q1	Q2	Q3	Q4
Control	0.9	3.5	95.4	0.1
50 $\mu\text{g/mL}$, EA	1.9	3	94.9	0.15
100 $\mu\text{g/mL}$, EA	3.7	9.3	86.8	0.15

Table 5.6. Percentage distribution of HeLa cells exposed to ethyl acetate sub-fraction in the cell cycle.

	Sub G1	G1	S	G2/M
Control	2.35	77.6	11	8.9
50 $\mu\text{g/ml}$, EA	4.3	77.6	8.8	9.15
100 $\mu\text{g/ml}$, EA	9.3	73.6	7.5	9.6

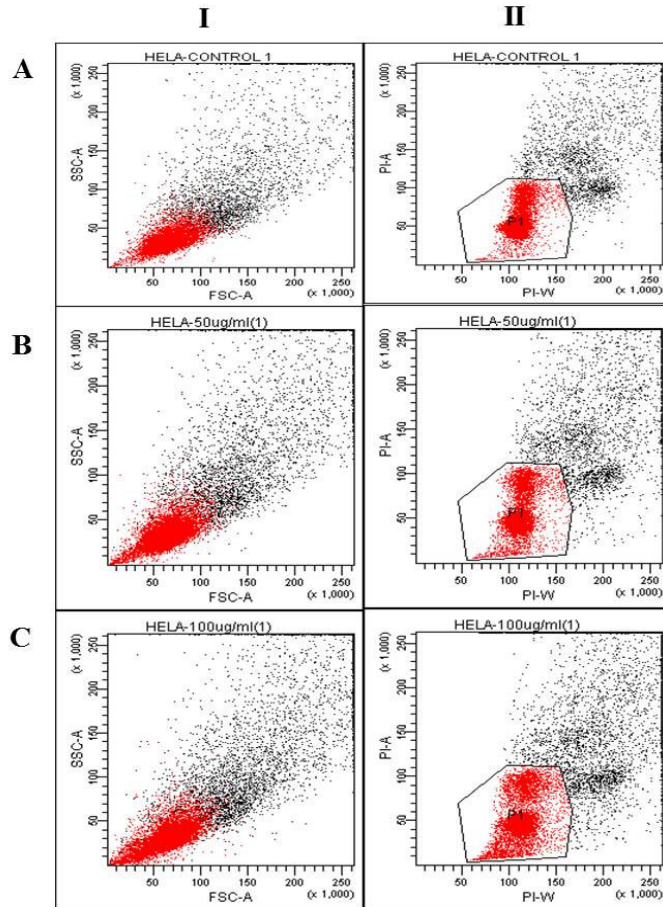


Figure 5.13. EA sub-fraction induces cell cycle arrest in HeLa cells *in vitro*. Flow cytometric cell cycle analysis was performed. HeLa cells at a density of 2×10^5 were plated and incubated for 12 hr followed by incubation for further 24 hr with 50 and 100 $\mu\text{g}/\text{mL}$ of ethyl acetate fraction. Trypsinized cells were washed with pre-chilled PBS and resuspended in ice-cold 70% PBS with 45 min incubation. The PBS washed cells were resuspended in 100 μL of PBS containing RNase A (1mg/mL) for 30min at 37°C. Propidium iodide (10 μL , 1 mg/mL) was added followed by incubation for 15 min in dark and analysis was carried out by BD FACS Aria system. Cell counts in I) Dot plot I represent: Forward scatter (FSC) vs. Side scatter (SSC) with a gate for cells II) PI histogram: - pulse area vs. pulse width. EA sub-fraction (100 $\mu\text{g}/\text{mL}$) treatment decreased the number of cells in S phases and induced a substantial increase in the number of apoptotic cells in the sub-G₁ phase.

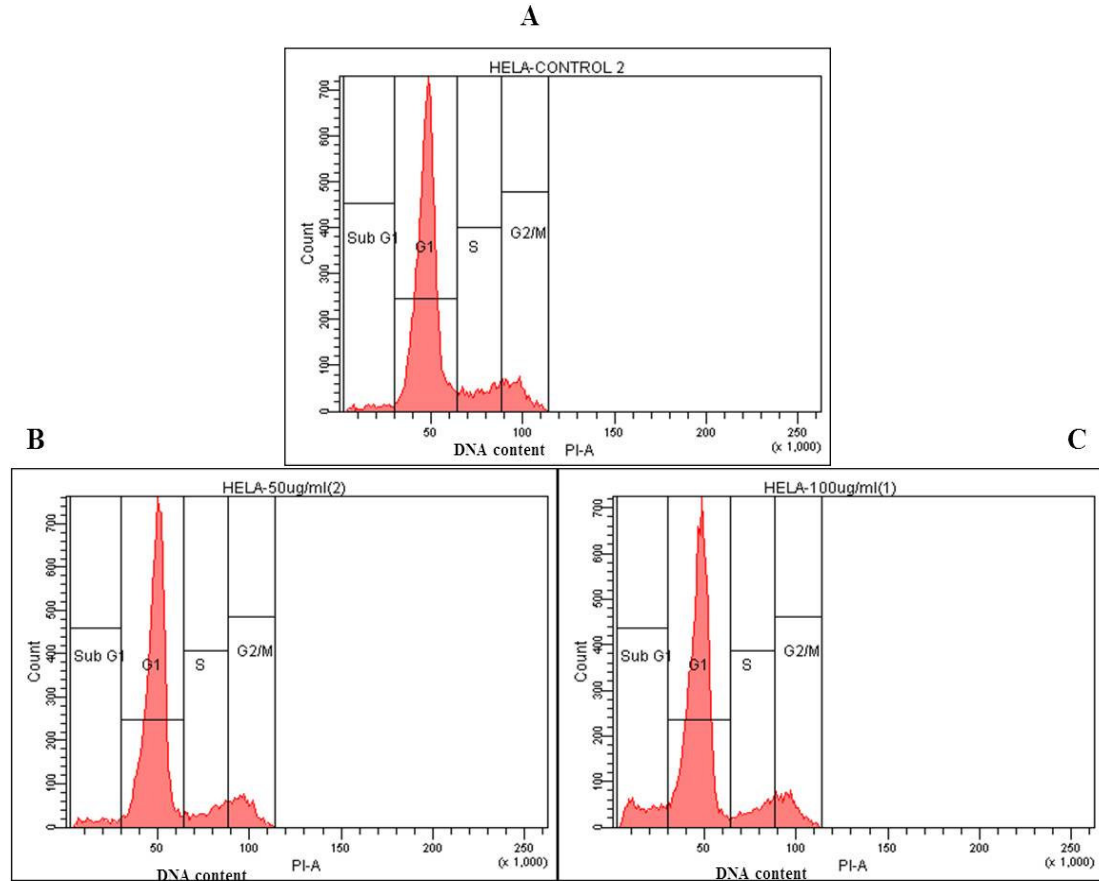


Figure 5.14. (A–C) Cell cycle analysis histograms. (A) Control. (B) 50 and (C) 100 $\mu\text{g}/\text{mL}$ of ethyl acetate sub-fraction. The diagram shows the DNA content flow cytometric histograms of HeLa cells and the distribution of cells in each cell cycle. HeLa cells of density 2×10^5 were plated and incubated for 12hrs followed-by incubation for further 24 hrs with 50 and 100 $\mu\text{g}/\text{mL}$ of ethyl acetate fraction. Trypsinized cells were washed with pre-chilled PBS and resuspended in ice-cold 70% PBS with 45 min incubation. The PBS washed cells were resuspended in 100 μL of PBS containing RNase A (1 mg/mL) for 30 min at 37 °C. Propidium iodide (10 μl , 1mg/ml) was added followed by an incubation of 15 min in dark and analysis was carried out by BD FACS Aria system. The 100 $\mu\text{g}/\text{mL}$ of ethyl acetate sub-fraction decreased the number of cells in S phases and induced a substantial accumulation of a number of apoptotic cells in the sub-G1 phase.

5.3.6. GC-MS analysis of ethyl acetate (EA) sub-fraction of UNPE extract

GC-MS chromatogram of ethyl acetate sub-fraction of the UNPE-methanolic fraction is given in figure 5.15 and list of compounds identified are in table 5.7. There were 45 compounds identified (Figure 5.15 and Table 5.7). The total ion chromatogram showed that the retention time of different phytochemicals ranged from 18.161 to 48.445 minutes. 1-(Benzoyloxy)-2, 2, 6, 6-tetramethyl-4-piperidinyll 4-, celidoniol deoxy-, tetratetracontane are the compounds found with high abundance (peak area percent) in EA Fr (Figure 5.15 and Table 5.7). Tetracosane is a biologically active compound present in EA sub-fraction but not identified in UNPE extract.

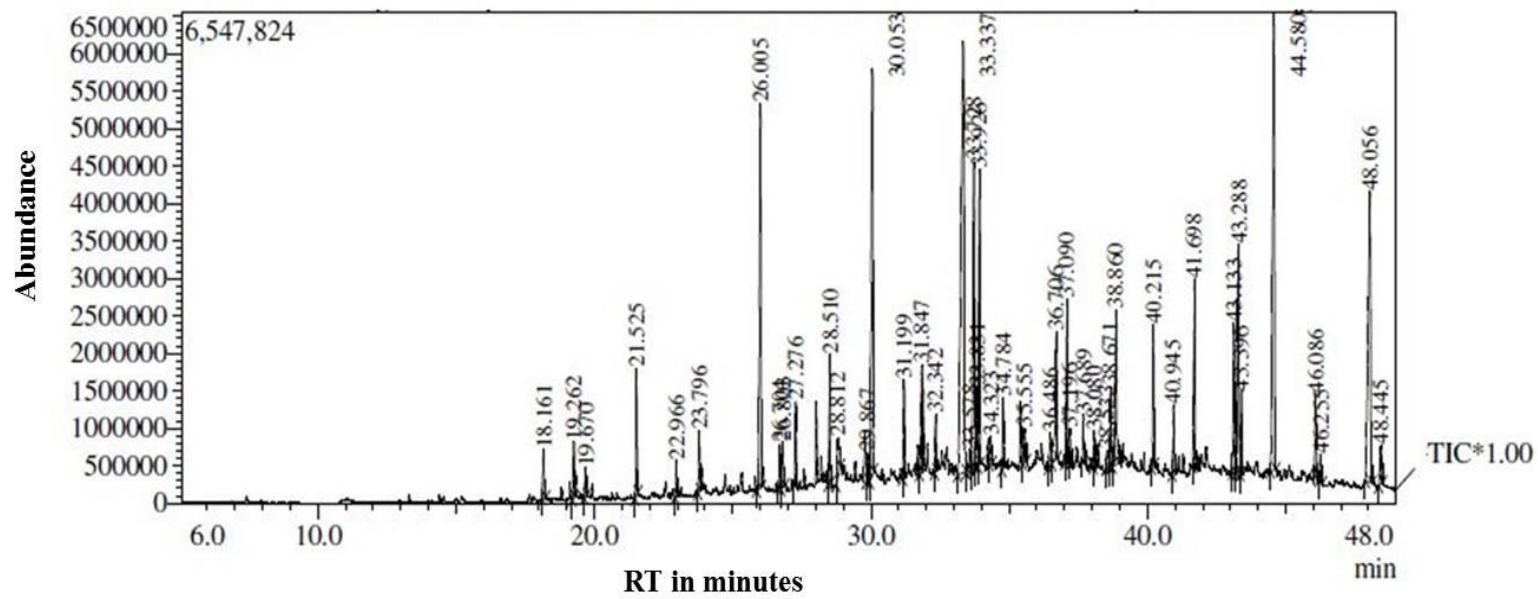


Figure 5.15. Total Ion Chromatogram (TIC) of EA Fr of PE extract of *U.narum* leaves on GC MS analysis

Table 5.7 Compounds reported in the ethyl acetate sub-fraction of UNPE extract through GCMS analysis

Peak	R.Time (min)	Area%	Name	Molecular formula	Molecular mass(kDa)
1	18.161	0.78	Alpha.,.Alpha.'-Dihydroxy-M-Di isopropylbenzene	C ₁₂ H ₁₈ O ₂	194.274
2	19.262	0.87	Alpha.,.Alpha.,.Alpha.',.Alpha.'-Tetramethyl-1,4-Benzene dimethanol	C ₁₀ H ₁₄	134.222
3	19.670	0.40	Cis,Trans-Farnesol	C ₁₅ H ₂₆	222.366
4	21.525	1.50	Hexadecane	C ₁₆ H ₃₄	226.448
5	22.966	0.43	Hexadecane, 2-Methyl-	C ₁₇ H ₃₆	240.468
6	23.796	0.89	Heptadecane	C ₁₇ H ₃₆	420.725
7	26.005	6.03	Nonadecane	C ₁₉ H ₄₀	268.529
8	26.704	0.52	Neophytadiene	C ₂₀ H ₃₈	278.524
9	26.803	0.80	2-Pentadecanone, 6,10,14-Trimethyl-	C ₁₈ H ₃₆ O	268.4778
10	27.276	0.98	Octadecane, 2-Methyl-	C ₁₉ H ₄₀	268.529
11	28.510	1.62	Hexadecanoic Acid, Methyl Ester	C ₁₇ H ₃₄ O ₂	270.457
12	28.812	1.03	Benzothiazole, 2-(2-Hydroxyethylthio)-	C ₇ H ₅ NS ₂	167.244
13	29.867	0.46	1-Octadecene	C ₁₈ H ₃₆	252.486
14	30.053	7.19	Eicosane	C ₂₀ H ₄₂	282.556

15	31.199	1.24	Eicosane, 2-Methyl-	$C_{21}H_{44}$	296.583
16	31.847	1.86	9-Octadecenoic Acid (Z)-, Methyl Ester	$C_{19}H_{36}O_2$	296.495
17	32.342	0.65	Octadecanoic Acid, Methyl Ester	$C_{19}H_{38}O_2$	298.511
18	33.337	15.97	1-(Benzoyloxy)-2,2,6,6-Tetramethyl-4-Piperidinyl Methylbenzenesulfonate	$C_{23}H_{29}NO_5S$	431.545
19	33.578	0.43	Nonacosanol	$C_{29}H_{60}O$	424.786
20	33.728	4.85	Heneicosane	$C_{21}H_{44}$	296.574
21	33.831	1.22	1-Hexadecanol, Acetate	$C_{18}H_{36}O_2$	284.484
22	33.926	4.12	Phytol, Acetate	$C_{22}H_{42}O_2$	338.568
23	34.323	0.45	Dotriacontane	$C_{32}H_{66}$	450.8664
24	34.784	0.90	Eicosane, 7-Hexyl-	$C_{26}H_{54}$	366.7070
25	35.555	0.54	3-Benzoyl-2-T-Butyl-4-Isopropyl-4-Methyl-Oxazolidin-5-One	$C_{18}H_{25}NO_3$	303.396
26	36.486	0.72	Thiosemicarbazide, 1-Benzoyl-4-(4-Fluorophenyl)-	$C_{14}H_{12}FN_3OS$	289.328
27	36.706	2.69	4-Methylcoumarin-7,8-Diyl Dibenzoate	$C_{24}H_{16}O_6$	400.380
28	37.090	2.24	Tetracosane	$C_{24}H_{50}$	338.654
29	37.196	0.44	Tricosyl Acetate	$C_{25}H_{50}O_2$	382.663
30	37.689	0.64	Guaiacol Benzoate	$C_{14}H_{12}O_3$	228.2433
31	38.080	0.44	Docosane, 9-Butyl-	$C_{26}H_{54}$	366.707

32	38.533	0.44	Decane, 1,9-Bis[(Trimethylsilyl)Oxy]-	C ₁₆ H ₃₈	318.643
33	38.671	1.08	Tricosane	C ₂₃ H ₄₈	324.6272
34	38.860	2.79	Palmitic Acid .Beta.-Monoglyceride	C ₁₉ H ₃₈	330.503
35	40.215	1.71	Octacosane		
36	40.945	0.99	4,4'-((P-Phenylene)Diisopropylidene)Diphenol	C ₂₄ H ₂₆	346.462
37	41.698	2.59	Hexatriacontane	C ₃₆ H ₇₄	506.973
38	43.133	2.13	Pentatriacontane	C ₃₅ H ₇₂	492.946
39	43.288	3.78	Squalene	C ₃₀ H ₅₀	410.718
40	43.396	1.17	Phenacyl 11-Octadecenoate	C ₂₆ H ₄₀ O ₃	400.594
41	44.580	9.56	Celidoniol, Deoxy-	C ₂₉ H ₆₀	408.787
42	46.086	1.19	Docosane	C ₂₂ H ₄₆	310.601
43	46.255	0.41	Hexacosyl Acetate	C ₂₈ H ₅₆	424.743
44	48.056	8.45	Tetratetracontane	C ₄₄ H ₉₀	619.185
45	48.445	0.81	DL-.Alpha.-Tocopherol	C ₂₉ H ₅₀	430.706

5.4. Discussion

Traditional knowledge provides us enormous evidence for the use of plants based on their pharmacological potential. Antitumor efficacy of the plants belonging to the family Annonaceae are well known for many years (Aminimoghdamfarouj *et al.* 2011; Alali *et al.* 1999; Asare *et al.* 2015). *Uvaria* is a well-known species under Annonaceae with reported cytotoxic and antitumor activities ((Buncharoen *et al.* 2016; Chen and Yu 1996; Dai *et al.* 2012). As a plant under *uvaria* species, *U. narum* was the choice for antitumor studies using an animal model.

In these experiments, the oral administration of UNPE extracts at 5 and 10 mg/kg b. wt. causes a significant reduction ($P < 0.01$) in the DLA induced solid tumor volume and increased the lifespan of ascites tumor-bearing mice. These doses, however, are well tolerated by the animals during the entire experimental period (30 days) while maintaining the antitumor efficacy. An interesting finding is that the body weight and total WBC counts do not exhibit much change in the treatment groups. This is a noted advantage over cyclophosphamide (standard) treated animals. Thus, the present study reveals that at concentrations of 5 and 10 mg/kg b.wt., UNPE extract is safe for oral administration and possesses significant antitumor activity against both solid and ascites tumors. Compared to the pretreatment strategy, simultaneous administration achieves significant tumor reduction.

As chemotherapeutic drugs destroy tissues with a high rate of growth, bone marrow could be a susceptible target. Nowadays the foremost aim in the development of anticancer therapeutics is to reduce the chemotherapy-associated side effects (Demain and Vaishnav 2011; Wang *et al.* 2006). Even though cyclophosphamide treatment in this study significantly reduces the tumor in animals, with better efficacy than UNPE extract, there was a considerable reduction in total WBC and weight of animals. This drop in weight or loss in WBC is not seen in animals administered with both doses of UNPE extract.

The cytotoxic and antitumor properties shown by the UNPE extract could be attributed to its phytochemicals. Methanolic and hexane fractions obtained in the initial fractionation of UNPE extract have shown differential toxicity towards DLA and EAC cells and antiproliferative efficacy against various cell lines. Methanolic fraction is found to be more effective than hexane fraction. HPTLC profiling of methanolic fraction gives a number of terpenoid positive bands. Further cytotoxicity based fractionation of the methanolic fraction has resulted in ethyl acetate and toluene sub-fractions. Among this, the sub-fraction EA is found superior to toluene in *in vitro* cytotoxicity and antiproliferative efficacies. Here, all the sub-fractions of UNPE extracts shows antiproliferative/cytotoxic effect, however, EA sub-fraction is found to possess better efficacy.

The mechanistic basis of the toxicity was studied using HeLa cells. The UNPE extract and methanolic fraction of treated cells are seen with green nucleus however with condensed chromatin as seen in merged images with AO/EtBr staining indicating late apoptotic stage. Whereas EA sub-fraction exposed cells are in early apoptosis stage with the blebbing and green nucleus. Apoptosis is a highly regulated and programmed cell death process characterized by numerous cellular alterations. Fluorescent microscopic examination under AO/EtBr is a reliable method of apoptotic detection (Baskic *et al.* 2006; Liu *et al.* 2015a). The fluorescent dye AO (acridine orange) is taken up by both viable and non-viable cells with intercalation into double-stranded DNA leading to the emission of green fluorescence (Leite *et al.* 1999; McGahon *et al.* 1995). Nonviable cells take up ethidium bromide dye which intercalates with DNA leads to the emission of red fluorescence. As a result, viable cells appear with a uniform bright green nucleus. An early apoptotic cell is visible with intact membranes, have a green nucleus with chromatin condensation visible as bright green patches; a late apoptotic cell with bright orange condensed chromatin in the nucleus and a necrotic cell with the bright orange cell (Leite *et al.* 1999; McGahon *et al.* 1995). Accordingly, it has been suggested that the mechanistic basis of cytotoxicity exhibited by UN extract is apoptosis. This has been further ascertained by FACS analysis using Annexin V-FITC staining combined with propidium iodide staining.

This combination is used to distinguish viable, necrotic and different stages of apoptotic cells through differences in plasma membrane integrity and permeability. PI does not stain live or early apoptotic cells due to the presence of an intact plasma membrane but the loss of integrity in late apoptotic and necrotic cells, allow PI to pass through the membranes and intercalate into nucleic acids with the emission of red fluorescence (Rieger *et al.* 2011; Crowley *et al.* 2016; Hodge *et al.* 1999). The results of this study show that HeLa cells when exposed to EA, show PI-stained cells that are increased in Q2 quadrant representing late apoptotic phase. The cells were also subjected to cell cycle analysis, using a combination of PI followed by Hoechst 33342 dye. This combination is an excellent probe to distinguish and sort viable, necrotic, early and late apoptotic cells (Bertho *et al.* 2000; Darzynkiewicz *et al.* 1992). The analysis reveals a slight increase in an apoptotic cell population in EA-treated HeLa cells and cell arrest occurs in the sub-G1 phase.

The GC-MS analysis of UNPE and ethyl acetate fractions revealed the presence of a number of biologically important phytochemicals. There are 53 compounds identified in PE extract of *U.narum* leaves under GC MS analysis among which 19 are shared by EA sub-fraction. Other 24 compounds which are found in the EA fraction are the same that found in UNPE but in the natural derivative form. One of the important compound in UNPE extract, phytol, diterpene alcohol possesses antibacterial and antiradical activity (Pejin *et al.* 2014b) and studies have revealed that it shows remarkable cytotoxicity towards various cancer cells (Pejin *et al.* 2014a). Studies have also revealed that phytol induces apoptosis via the activation of caspase-9 and prevent epithelial-mesenchymal transition in hepatocellular carcinoma cells (Kim *et al.* 2015). Phytol has been documented to reduce IL-1 β and TNF- α levels and oxidative stress thereby inhibiting neutrophil migration and the inflammatory response (Silva *et al.* 2014). The derivative of this phytochemical is documented in the EA sub-fraction of UNPE.

Another compound identified in both UNPE extract and EA sub-fraction is squalene, a natural triterpene and an intermediate in sterol biosynthesis (Spanova and Daum 2011). Previous reports have suggested the chemopreventive, antioxidant and antitumor activities of squalene (against different cancers in rodents like colon, skin, sarcoma, and lung), as dietary supplements for decreasing LDL cholesterol and cardiovascular diseases and as an additive to lipid emulsions as drug carrier in pharmaceutical industries and also in vaccine applications (Spanova and Daum 2011). The research documented that as an isoprenoid antioxidant, squalene has a protective effect on bone marrow progenitor cells against chemotherapy-induced toxicity (Das *et al.* 2008).

Another important compound present in both PE and EA sub-fraction is hexadecane. It is an alkane hydrocarbon already reported for its biological effects such as antibacterial, antioxidant activities (Yogeswari *et al.* 2012). Hexadecanoic acid, methyl ester identified in the EA sub-fraction in our study has been reported to possess antimicrobial, antioxidant, cholesterol reducing, hemolytic, and nematocidal activities (Candra *et al.* 2018).

UNPE crude extract contains another important phytochemical pentadecane an organic molecule reported to be present in many essential oil preparations.

Studies on pentadecane, have revealed its antimicrobial activity against *L.infantum* parasites in culture by arresting cells in the sub G0/G1 and G1 phases of the cell cycle (Bruno *et al.* 2015). Heptadecane, a phytochemical identified both in UNPE extract and EA sub-fraction, is known for its anti-inflammatory activities by modulating NF- κ B in the aged kidney in rats (Kim *et al.* 2013). Eicosane, acyclic alkane, identified in UNPE extract, as well as EA sub-fraction, is a phytochemical with reported anticancer activities.

Previous studies show that eicosane isolated from *Euphorbia kansui* inhibits the growth of human SGC-7901 cancer cells (Yu *et al.* 2005). Additional compounds derived from eicosanes such as eicosane, 2-methyl-, eicosane-7-hexyl- and heneicosane are identified only in EA sub-fraction. Eicosane, 2-methyl- derivative belongs to the family of acyclic alkanes are reported to have antioxidant, cytotoxic, antibacterial and antitumor properties (Phillips *et al.* 2015; Akpuaka *et al.* 2013).

Another important compound in both UNPE and EA sub-fraction is tocopherol. It is reported that tocopherol analogs isolated from the peels of *Citrus unshiu Marcovich* found to have a hepatoprotective and neuroprotective effect (Seo *et al.* 2015). Studies also show that tocopherol acts as efficient singlet oxygen scavengers and potent antioxidant (Jayasinghe *et al.* 2013) (Rastogi *et al.* 2014). Tetracosane is another biologically active compound identified in EA sub-fraction but not in UNPE extract with a high abundance. Tetracosane has been reported to have cytotoxic activity against several cell lines such as AGS, MDA-MB-231, HT-29 and NIH 3T3 cells (Uddin *et al.* 2012; Casuga *et al.* 2016). Methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate is found to be a potent antioxidant (Li *et al.* 2014).

The above studies revealed that the cytotoxic and antitumor activities of UNPE crude extract may be due to the cytotoxic phytochemicals present in the extract. Previous chapter (Chapter 3) has discussed the phytochemical and TLC analysis of UNPE extract which documents that this fraction is rich in terpenoids and steroids. HPTLC as well as GC MS analysis presented in this chapter confirms this finding and revealed a number of cytotoxically active sterol and terpenoid compounds such as phytol, squalene and derivative compounds such as hexadecane and pentadecane, etc. These molecules might be contributing to the apoptosis-inducing ability of the UNPE extract and its fractions.

Together the results of these experiments prove that the mechanism of cytotoxicity of UNPE could be apoptosis by arresting cell cycle at the sub G1 phase. Even though sub-fractions of UNPE possess appreciable antiproliferative/ cytotoxic effect possibly inducing apoptosis, UNPE extract as a whole is observed to be the most potent. Possibly separation of components into different sub-fractions might have reduced the toxic potential. Another possibility is that modification of individual molecules detected in sub-fraction might have reduced the toxic effect. Therefore it is expected that the synergistic effect of phytochemicals in crude UNPE extract provides better cytotoxic efficacy and needs further exploration.

Chapter 6

*Exploration of antioxidant and anti-inflammatory potentials of methanolic extract of *U. narum* (Dunal) Wall. and its partial purification*

6.1. Introduction

Reactive oxygen species are produced in the body as the result of normal metabolic processes or the influence of environmental factors. Minimum levels of ROS generation in the body are important for cell homeostasis and for fighting against pathogens. Overproduction of highly reactive ROS can generate oxidative stress in the system and destroy organelle structures and bio-molecules such as lipid, protein and nucleic acids and alter their functions, which lead to inflammatory responses resulting in the development of different degenerative conditions and diseases including hypertension, neurological disorders, acute respiratory distress syndrome, diabetes, cancer, atherosclerosis, and asthma (Birben *et al.* 2012). If prolonged, excessive oxidative stress generation by macrophages and polymorphonuclear neutrophils at the site of inflammation to eliminate pathogens invading in the body causes endothelial dysfunction and tissue damage. This condition progresses to chronic inflammatory states and associated diseases such as heart diseases and rheumatoid arthritis, inflammatory bowel disease, neurodegenerative, autoimmune diseases, metabolic hormonal disorders and persistent bacterial infections such as tuberculosis. Reactive oxygen species (ROS) are very important molecules that play a major role in the progression of chronic inflammatory disorders (Mittal *et al.* 2014). Normally our body possesses numerous systems with specific mechanisms which include enzymatic and nonenzymatic antioxidants that are usually effective in blocking the deleterious effects of ROS and oxidative stress (Gupta *et al.* 2014). However, if the innate endogenous antioxidants system exceeds its capacity or its limited defensive function, exogenous antioxidants have been shown an important role in the detoxification of the ROS (Davies 1995). Currently, there is a great interest in the possible use of herbs and phytochemical antioxidants for the prevention and treatment of several diseases. Certain compounds derived from herbs were found to be effective against oxidants produced in the body. Significant attention has been paid on the possible protective value of such naturally occurring antioxidant compounds since synthetic products may elicit adverse effects in our body. So it is important to study the antioxidant efficacy of plant extracts *in vivo*. Newer natural antioxidants have to be explored.

The results reported in the previous chapters of this study revealed that *U.narum* extract has significant antioxidant potential when compared to *U.macropoda*. Upon polarity based fractionation of *U.narum*, methanolic extract (UNMET) was found to be more potent antioxidant than other extracts. In this chapter, a detailed investigation of the methanolic extract for its antioxidant potential was carried out. These investigations included studies on the effect of sodium fluoride (NaF) induced oxidative stress in mice and PMA-induced superoxide radical generation in peritoneal macrophages, anti-inflammatory studies using acute and chronic inflammatory mice models and measurement of extravasation of Evans blue dye (EBD) in carrageenan-induced paw edema in mice. Partial purification of methanolic extract was carried out by column chromatography to separate antioxidant principle. EA (ethyl acetate) and AQ (water) fractions were separated. UPLC-Q-TOF-MS analysis of methanolic (UNMET) was done to identify the pharmaceutically important compounds present in it.

6.2. Materials and methods

6.2.1. Collection and preparation of the plant extract

Uvaria narum Wall. leaves were collected and methanolic extract (UNMET) was prepared as per the method is given in chapter 2, section 2.1.5.3 and 2.2.1 and the prepared extract was dissolved in 1% propylene glycol and desired concentrations were prepared for *in vivo* studies.

6.2.2. Animals

Swiss albino and BALB/c female mice (25-30 g) were used for the antioxidant and anti-inflammatory study, respectively and were purchased and maintained as the method mentioned in section 2.1.6. chapter 2.

6.2.3. UPLC Q-TOF-MS analysis of the UNMET extract

UPLC Q-TOF-MS analysis was performed according to the method described in section 2.2.6, Chapter 2. Compounds were identified based on previous MS/MS fragmentation data.

6.2.4. *In vivo* antioxidant activity

6.2.4.1. Determination of antioxidant properties of UNMET in sodium fluoride-induced oxidative stress

Antioxidant effect of UNMET was determined by inducing oxidative stress in Swiss albino mice using sodium fluoride. Thirty-six Swiss albino female mice were divided into six groups of six animals each. The groupings were as follows: Group I- normal without any treatment, Group II: control-NaF (600 ppm) alone through drinking water, Group III: vehicle control- 1% propylene glycol+ NaF (600 ppm), Group IV: Standard - ascorbic acid (10 mg/kg b.wt., p.o.) + NaF (600 ppm), Group V: UNMET-LD-*U.narum* methanolic extract low dose (200 mg/kg b.wt., p.o.) + NaF (600 ppm); Group VI: UNMET-HD - *U.narum* methanolic extract high dose (400 mg/kg b.wt., p.o.) + NaF (600 ppm). NaF administration was through drinking water. The detailed procedure of NaF administration and extract treatment are described in Chapter 2, section 2.2.11. The activity of different antioxidant enzymes such as superoxide dismutase and the levels of glutathione and thiobarbituric acid reacting substances (TBARS) were analyzed as per the procedures mentioned in 2.2.11 and 2.2.11.1.

6.2.4.1.2. Histological analysis of liver

A portion of the liver was excised, washed in phosphate buffered saline and fixed in 10% neutral formalin. Histological sections of 4 μ m thickness were stained with hematoxylin and eosin (H & E) stain and observed under a microscope (200X) to examine the histopathological changes in liver tissues such as necrosis, hemorrhage, and inflammation (chapter 2, section 2.2.18).

6.2.4.2. Determination of the effect of UNMET on PMA-induced superoxide radical generation in peritoneal macrophages.

Twenty four Swiss albino female mice of 4 groups containing 6 animals each were used in this study. Peritoneal macrophages were elicited by injection (i.p) with 5% sodium caseinate. Group I control. Group II: vehicle control, for group III and IV, UNMET-LD (200 mg/kg b.wt.), and UNMET-HD (400 mg/kg b.wt.), respectively were administered. The detailed procedure of elicitation and determination of superoxide generation in macrophages by NBT reduction method are mentioned in Chapter 2, section 2.2.11.2 and 2.2.12.4.

6.2.5. *In vivo* anti-inflammatory studies

6.2.5.1. Acute inflammation study using carrageenan-induced paw edema

The experimental mice (BALB/c female weighing about 25-30g) were divided into 5 groups (6 animals/group). Group, I was control -carrageenan alone (1% w/v) and Group II was vehicle control -1% propylene glycol. Group III: standard diclofenac (25 mg/kg b.wt.) and Group IV and V were UNMET-LD (200 mg/kg b.wt.), and UNMET-HD (400 mg/kg b.wt.) treated, respectively. Detailed procedures for the induction of inflammation and measurement of paw thickness are described in Chapter 2, section 2.2.17.1.

6.2.5.1.1. Measurement of extravasations of Evans blue dye (EBD) in carrageenan-induced paw edema in mice

EBD is used as a marker for determining plasma extravasation. An inflammatory response is accompanied by plasma extravasation. EB binds to serum albumin with high affinity in the inflammatory site and this complex formation is estimated quantitatively to determine the extent of extravasation that accompanies peripheral inflammation. Paw edema was induced by the same procedure as done for acute inflammatory study with carrageenan in BALB/c female mice. The EB dye administration and determination of the concentration of EBD in inflammation site was calculated as per the method of (Gupta *et al.* 2015) (Chapter 2, section 2.2.17.1.1).

6.2.5.2. Acute inflammation study using dextran-induced paw edema

The experimental mice (BALB/c female weighing about 25-30g) were divided into 5 groups (6 animals/group). Group I was control -carrageenan alone (1% w/v) and Group II was vehicle control -1% propylene glycol. Group III: standard diclofenac (25 mg/kg b.wt.) and Group IV and V were UNMET-LD (200 mg/kg b.wt.), and UNMET-HD (400 mg/kg b.wt.) treated, respectively (chapter 2, section 2.2.17.2).

6.2.5.3. Chronic inflammation study using formalin-induced paw edema

The BALB/c female weighing about 25-30g were divided into 5 groups (6 animals/group) and the groups for this study was similar to above the experiments (refer Chapter 2, section, 2.2.17.3).

6.2.5.4. Histological examination of paws

After acute and chronic antiinflammatory experiments, the animals were sacrificed and paws of the animals in all groups were taken aseptically to monitor the inflammatory changes in paws of mice. Paws were washed with normal saline (0.9%) and immediately fixed in 10% buffered formalin solution. Histological processing of tissue was performed and sections of 4 μ m thickness were prepared using microtome. The microtomed sections were further stained with histochemical stains such as hematoxylin and eosin (H & E) stain. The slides were observed under a microscope (200X) to ascertain the degree of inflammation as described in Chapter 2, section 2.2.18.

6.2.6. Partial purification of compounds from UNMET extract using column chromatography

The methanolic extract was subjected to partial purification by bioassay-guided fractionation using column chromatography as described in Chapter 2, section 2.2.7. The crude UNMET extract was subjected to column chromatography in a glass column of dimensions 300 x 10 mm for the purification of an active fraction with potent antioxidant activity. Silica gel (from Merck) of mesh size 60-120 was used as the stationary phase and the solvents used were ethyl acetate and water for the separation of different phytofractions. About 100 mg of the methanolic extract in a minimum required volume of methanol was mixed with 500 mg of silica gel for column chromatography, kept at 40 °C for evaporation to dryness and made it to a

fine powder using mortar and pestle. The column bed was made with silica gel (8 g) and washed with ethyl acetate before the sample. The dried powdered extract was loaded over the column bed and chromatographic separation was carried out using ethyl acetate and water as the mobile phase. Column elute was collected as 100 mL fractions up to a total volume of 200 mL yielding ethyl acetate (EA) and water (AQ) fractions. The fractions were subjected to *in vitro* antioxidant assays as mentioned in Chapter 2, section 2.2.10 and evaluated for its antioxidant activity.

6.2.6.1. *In vitro* antioxidant assay

The screening of EA and AQ fractions for their antioxidant activity was determined by DPPH free radical scavenging assay (chapter 2.section 2.2.10.1) and inhibition of lipid peroxidation (chapter 2.section 2.2.10.3). The more potent antioxidant fraction was selected for the mechanistic study.

6.2.6.2. Measurement of Lipopolysaccharide-induced mouse TNF- α production in Raw264.7 cells

Maintenance of RAW264.7 cells and the treated cells with LPS and extract are described in Chapter 2, section 2.2.17.5. The TNF-alpha levels in control LPS and extract (50 and 100 $\mu\text{g}/\text{mL}$) treated cells were quantitatively determined using Mouse TNF-alpha (Quantikine ELISA Kit, R&D Systems Website Enhancements) as per the method mentioned in Chapter 2 2, section 2.2.17.5.

6.2.6.3.. Quantitative Real-time PCR (qPCR) analysis

cDNA synthesis (using High capacity cDNA reverse transcription kit in the Veriti Thermal cycler Applied Biosystems) and qRT analysis (with Power Up™ SYBR™ Green Master Mix and Step One plus real-time PCR system, Applied Biosystems) were performed according to the procedure described in Chapter 2, section 2.2.17.5.

6.2.6.4. Statistical analysis

Values are expressed as mean \pm SD of 6 animals for all the animal experiments. Statistical analysis for the obtained data was done by one way ANOVA followed by Dunnett post hoc test using Graph Pad InStat 3 software. Results were considered to be statistically significant when the p-value was <0.05 .

6.3. Results

6.3.1. UPLC-Q-TOF-MS analysis of methanolic extract of *U.narum* (UNMET)

UPLC-Q-TOF-MS analysis revealed a number of peaks of phyto compounds. The total ion chromatogram of the methanolic extract contained 31 peaks out of which 21 were identified (Figure 6.1 and Table 6.1). Nine unidentified peaks were obtained in the chromatogram enlisted in Table 6.2. Some of the pharmacologically important compounds such as anonaine (with RT 1.09 min and 265.12 kDa mass), salsolidine (RT, 4.26 min and mass 207.13 kDa), tricetin (hieracin) (RT 7.241, 7.561, 7.889 mins and mass of 302.04 kDa), rutin (7.242 min RT and mass of 610.15), cornuside (with RT 1.093 min and 265.12 kDa mass), artonin L (RT,10.115min and 396.13 kDa mass), mesaconitine (7.24 min RT and mass 632.12 kDa), nicergoline (10.56 min RT and 485.26 kDa mass), quercetin-3-o-beta-glucopyranosyl-6"-acetate (10.56 RT and 507.25 mass), sulfadimidine (12.31 min and 278.09 kDa), 10-hydroxy-camptothecin (17.67 min RT and 364.13 mass) and phthalic acid mono-2- ethyl hexyl ester (18.31, 18.39 mins and 278.15 kDa) were tentatively identified in the methanolic extract of *U.narum* leaves (Table 6.3).

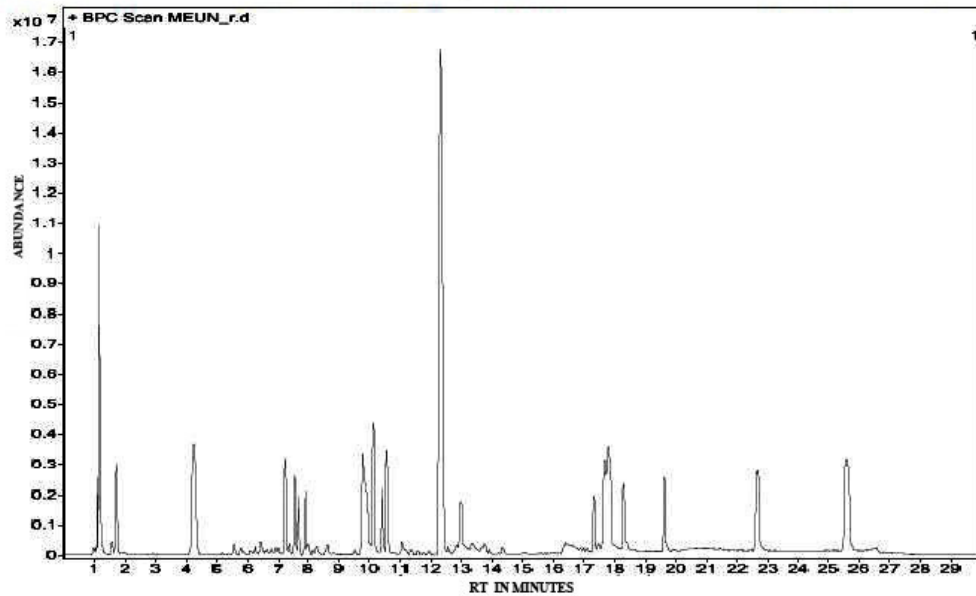


Figure 6.1. shows the total ion chromatogram of the methanolic extract when subjected to UPLC-Q-TOF-MS analysis using Acquity UPLC H class (Waters) system with mobile phase (methanol and 0.1% aqueous formic acid) through BEH C18 column (50 mm \times 2.1 mm \times 1.7 μ m) subjected to gradient elution (0-2 min, 95% acetonitrile; 3-24 min, 0.1% aqueous formic acid and 5% aqueous acetonitrile; 25-30 min 5% methanol). Detection was done by Xevo G2 (Waters, USA) Quadrupole –Time-of-Flight in a wavelength of 210 - 400 nm. The compounds were identified based on their fragmentation and m/z ratio.

Table 6.1. The compounds identified in UNMET by UPLC-Q-TOF-MS analysis

Sl.No.	RT (min)	Mass (kDa)	m/z	Compound name	Molecular Formula
1.	1.093	265.1167	266.124	Anonaine	C ₁₇ H ₁₅ NO ₂
2.	1.162	155.0944	156.1017	Scopoline	C ₈ H ₁₃ N O ₂
3.	1.581	291.1025	314.0926	Gly Ser Glu	C ₁₀ H ₁₇ N ₃ O ₇
4.	1.726	267.0971	268.1045	Deoxyguanosine	C ₁₀ H ₁₃ N ₅ O ₄
5.	4.259	207.1256	208.133	Salsolidine	C ₁₂ H ₁₇ N O ₂
6.	6.439	341.1621	342.1699	5-Hepten-3-(noic Acid, 7-1-Naphthalenyl)Methyl]Amino]- 2,2-Dimethyl-	C ₂₀ H ₂₃ N O ₄
7.	7.241	302.0436	303.0511	Hieracin	C ₁₅ H ₁₀ O ₇
8.	7.242	610.1528	611.1607	Rutin	C ₂₇ H ₃₀ O ₁₆
9.	7.561	302.0428	303.0511	Hieracin	C ₁₅ H ₁₀ O ₇
10.	7.889	302.0431	303.0511	Hieracin	C ₁₅ H ₁₀ O ₇
11.	10.406	327.1153	328.122	His Gly Asp	C ₁₂ H ₁₇ N ₅ O ₆
12.	11.062	454.1687	455.1762	Cys Trp Phe	C ₂₃ H ₂₆ N ₄ O ₄ S
13.	12.311	556.1685	557.1763	7-Dehydrologanin Tetraacetate	C ₂₅ H ₃₂ O ₁₄
14.	12.314	278.079	279.0858	Sulfadimidine	C ₁₂ H ₁₄ N ₄ O ₂ S
15.	17.325	456.2564	457.2619	Sulfolithocholic Acid	C ₂₄ H ₄₀ O ₆ S
16.	18.308	278.1515	279.1588	Phthalic Acid Mono-2- Ethylhexyl Ester	C ₁₆ H ₂₂ O ₄
17.	18.388	278.1511	279.1588	Phthalic Acid Mono-2- Ethylhexyl Ester	C ₁₆ H ₂₂ O ₄
18.	19.635	424.206	425.2138	Ser Arg Tyr	C ₁₈ H ₂₈ N ₆ O ₆
19.	22.668	350.1976	351.2053	2-Pyrrolidinone, 4-(2-Morpholinoethyl)-3,3- Diphenyl-	C ₂₂ H ₂₆ N ₂ O ₂
20.	22.672	349.1813	350.1886	His Pro Pro	C ₁₆ H ₂₃ N ₅ O ₄
21.	25.59	390.2757	413.2654	3beta-Hydroxy-11-Oxo-5beta-Cholan-24-Oic Acid	C ₂₄ H ₃₈ O ₄

Table 6.2. List of unidentified peaks with RF (min), Mass and m/z ratio obtained after UPLC-Q-TOF-MS analysis of UNMET extract

Sl.No	RT(min)	Mass (kDa)	m/z ratio
1.	7.238	632.1178	633.1251
2.	7.523	616.1236	617.1308
3.	7.669	616.1265	617.1338
4.	9.807	543.1568	544.1641
5.	10.115	396.1269	397.1342
6.	10.562	485.2635	486.2708
7.	10.564	507.245	508.2523
8.	12.985	775.2306	776.2379
9.	17.665	364.1278	365.1351

Table 6.3. List of pharmacologically important compounds identified in UNMET by UPLC-Q-TOF-MS analysis

SI NO.	Compound name	Class	Pharmacological activity
1.	Anonaine	Benzoquinolines - alkaloid	antiplasmodial, antibacterial, antifungal, antioxidation, anticancer, antidepressant, and vasorelaxant activity (Li <i>et al.</i> 2013)
2.	Salsolidine	Tetrahydroisoquinoline alkaloids	Catechol O methyltransferase Inhibitors (Sanft and Thomas 1989; Dostert <i>et al.</i> 1988).
3.	Tricetin (Hieracin)	Flavanol	strongest α -glucosidase inhibitory (Wu and Tian 2018); in vitro chemoprevention (Dametto <i>et al.</i> 2017); induces Apoptosis of Human Leukemic HL-60 Cells (Chien <i>et al.</i> 2017); suppresses human oral cancer cell migration (Ho <i>et al.</i> 2013; (Chung <i>et al.</i> 2017); acetylcholinesterase inhibitory (Kuppusamy <i>et al.</i> 2017); antioxidant, antineoplastic and cytotoxic (Singh <i>et al.</i> 2016). suppresses benzo(a)pyrene-induced human nonsmall cell lung cancer and bone metastasis (Hung <i>et al.</i> 2015); Chang <i>et al.</i> 2016); suppresses the migration/invasion of human glioblastoma multiforme (Chao <i>et al.</i> 2015) and human osteosarcoma cells metastasis (Chang <i>et al.</i> 2016).
4.	mesaconitine	diterpenoid alkaloid	antiviral (Xu <i>et al.</i> 2017b), analgesic (Nesterova <i>et al.</i> 2014a), antinociceptive and anti-inflammatory (Chou Lai <i>et al.</i> 2011; Nesterova <i>et al.</i> 2014b), vasorelaxing (Mitamura <i>et al.</i> 2002).
5.	Rutin	flavonoid	Antioxidant (Zhao <i>et al.</i> 2018); Hepatoprotective effects (Liang <i>et al.</i> 2018); protective against lipopolysaccharide-induced mastitis (Su <i>et al.</i> 2018); neuroprotective activity (Giuffre,2018); attenuation of neuroblastoma cell lines (SH-SY5Y) (Khan <i>et al.</i> 2018). suppressing gastric inhibitory polypeptide secretion (Martirosyan <i>et al.</i> 2018). Antiallergic (Hun Jung <i>et al.</i> 2007), Antiproliferative (Yu <i>et al.</i> 2016); Antitumor Effect (Chen <i>et al.</i> 2013; Dixit 2014).

6.	Cornuside	Iridoids	Anti-cholinesterase (Bhakta <i>et al.</i> 2017); inhibits mast cell-mediated allergic response (Li <i>et al.</i> 2016b); attenuates apoptosis in rat cortical neurons (Jiang <i>et al.</i> 2009); anti-inflammatory (Kang <i>et al.</i> 2007; Li <i>et al.</i> 2016c; Choi <i>et al.</i> 2011).
7.	Artonin L	Flavonoid	Apoptosis inducing (Rahman <i>et al.</i> 2016); anticancer (Etti <i>et al.</i> 2017); cytotoxic (Lee <i>et al.</i> 2006); antiinflammatory (Reddy <i>et al.</i> 1991; Wei <i>et al.</i> 2005); Antibacterial (Zajmi <i>et al.</i> 2015; Farooq <i>et al.</i> 2014)
8.	Nicergoline	Alkaloid	Vasodilator (Moore <i>et al.</i> 1982), Adrenergic alpha-antagonists used in the treatment of hypertension, vasospasm, peripheral vascular disease, shock, and pheochromocytoma (Bolli <i>et al.</i> 1983); Nootropic agents facilitate learning or memory, particularly to prevent the cognitive deficits associated with dementias (Fariello 1997); antioxidant (Tanaka <i>et al.</i> 1998); neuroprotective effect (Sortino <i>et al.</i> 1999).
9.	Quercetin-3-O-beta-glucopyranosyl-6"-acetate	Flavonoid O-Glycosides(polyphenol)	Antioxidant (Zheng <i>et al.</i> 2017)
10.	Sulfadimidine	Sulfonamide derivative	Antibacterial (Tella and Obaleye 2010)
11.	10-hydroxy-Camptothecin	Alkaloid	Anticancer (K. Zhao <i>et al.</i> 2018; Yang <i>et al.</i> 2013); anti-angiogenesis and anti-tumor (Song <i>et al.</i> 2018); inducing apoptosis (Dai <i>et al.</i> 2017)
12.	Phthalic acid Mono-2-Ethylhexyl Ester	Benzene dicarboxylic acid ester	plasticizer (Pollack <i>et al.</i> 1985; Thomas <i>et al.</i> 1984); apoptosis-inducing (Richburg and Boekelheide 1996).

6.3.2 *In vivo* antioxidant activity of UNMET extract

6.3.2.1. Effect of UNMET extract on sodium fluoride-induced oxidative stress

Studies on the antioxidant profile of blood showed that administration of NaF significantly reduced GSH level to 1.69 ± 0.08 and 1.83 ± 0.08 nmol/g Hb in control and vehicle control groups, respectively when compared to the normal animals (3.26 ± 0.04 nmol/g Hb). The UNMET-LD (Low dose, 200 mg/kg b.wt.) and UNMET-HD (high dose, 400 mg/kg b.wt.) treated groups showed enhanced GSH concentration to a level of 2.72 ± 0.06 and 2.98 ± 0.05 nmol/g Hb, respectively. The standard ascorbic acid (10 mg/kg b.wt.) treated group restored the blood GSH to 2.99 ± 0.05 nmol/g Hb (Figure 6.2a). Similar results were observed in the activities of blood antioxidant marker enzymes such as SOD and catalase. The reduced activity of SOD after NaF administration (1.69 ± 0.08 U/g Hb) was increased by the treatment with ascorbic acid (2.99 ± 0.01 U/g Hb), UNMET-LD (2.72 ± 0.06 U/g Hb) and UNMET-HD (2.98 ± 0.05 U/g Hb) in a significant manner ($p \leq 0.01$), when compared to the normal GSH level (3.26 ± 0.04 U/g Hb) (Figure 6.2b). The activity of catalase decreased when NaF was administered in control (22.35 ± 2.38 U/g) and vehicle control (23.53 ± 2.41 U/g Hb) groups in comparison to the normal group (36.37 ± 1.02 U/g Hb). The decreased catalase activity was elevated significantly ($p \leq 0.01$) in Vitamin C (34.54 ± 1.34 U/g Hb), UNMET-LD (26.86 ± 2.04 U/g Hb) and UNMET-HD (29.13 ± 3.01 U/g Hb) treated groups (Figure 6.2c).

The antioxidant profile of the liver was altered by NaF administration. Figure 6.3a showed that GSH levels, SOD and catalase activities, and inhibition of lipid peroxidation were increased ($p < 0.05$; $p < 0.01$) in the Vitamin C, UNMET-LD and UNMET-HD groups when compared to those in the control and vehicle control groups after NaF treatment. The GSH content in the liver (15.78 ± 1.20 nmol/mg protein) was reduced in the control group and vehicle control treated animals (9.85 ± 1.07 and 10.0 ± 1.02 nmol/mg protein, respectively). In the groups treated with Vitamin C (14.48 ± 0.9 nmol/mg protein), UNMET-LD (12.99 ± 1.02 nmol/mg protein) and UNMET-HD (13.66 ± 0.97 nmol/mg protein) the GSH levels were restored to normal values and increase observed was significant ($p \leq 0.01$)(Figure 6.3a). The activity of SOD was significantly decreased in the liver of mice treated

with NaF when compared with the normal (1.63 ± 0.21 U/mg protein) group (Figure 6.3b). Treatment of the animals with Vitamin C (10 mg/kg b.wt.), UNMET-LD and UNMET-HD minimized enzyme activity induced by NaF intoxication in the liver by 1.54 ± 0.12 , 1.39 ± 0.11 and 1.46 ± 0.21 U/mg protein, respectively ((Figure 6.3b). The activity of catalase enzyme decreased in NaF treatment animals in comparison to the untreated group (28.20 ± 2.42) (Figure 6.3c). Liver catalase activity was elevated significantly ($p \leq 0.01$) when treated with Vitamin C (10 mg/kg b.wt.) (29.32 ± 2.31 U/mg protein), UNMET-LD (23.28 ± 1.24 U/mg protein) and UNMET-HD (25.87 ± 1.39 U/mg protein) when compared with NaF alone treated (16.72 ± 3.45 U/mg protein) and vehicle control (15.85 ± 1.12 U/mg protein) groups. The MDA contents were significantly elevated in control and vehicle control groups (0.43 ± 0.02 and 0.40 ± 0.03 nmol MDA/mg protein) when compared with the untreated normal group (0.135 ± 0.02 nmol MDA/mg protein). Meanwhile, there noticed a significant decrease in the extent of lipid peroxidation in standard and UNMET extract treated animal groups (Figure 6.3d).

Histopathological examination of the liver revealed necrotic regions in the NaF alone administered control group. Normal cellularity was seen in Vitamin C, UNMET-LD and UNMET-HD treated groups with fewer necrotic cells (Figure 6.4).

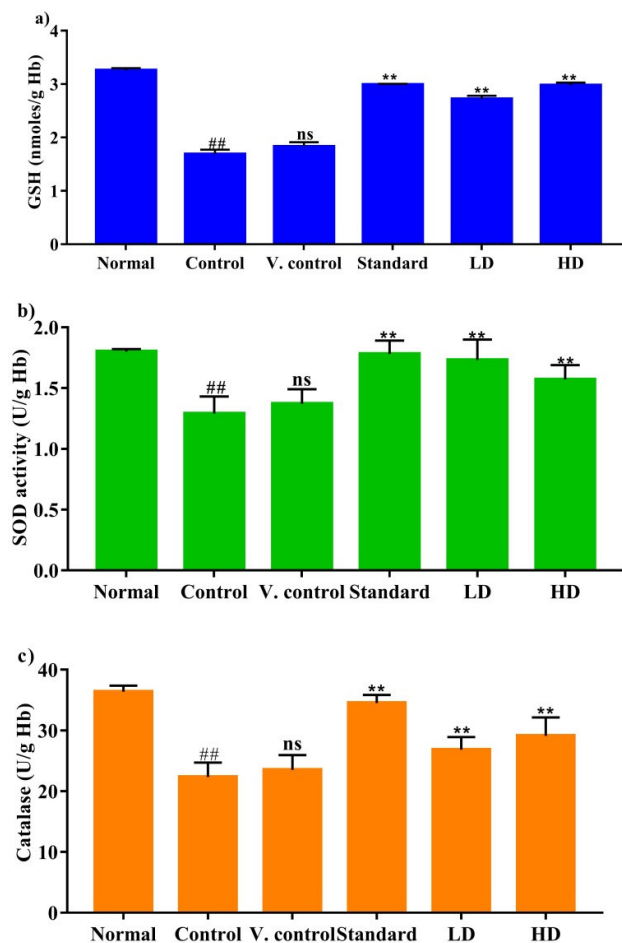


Figure 6.2. Effect of UNMET extract on the antioxidant status of blood following sodium fluoride-induced oxidative stress. Swiss albino female mice were grouped and the animals in group II, III, IV, and V were pretreated for seven days with respective doses of drugs. All the animals except in group I received NaF (600 mg/L) through drinking water from 8th day onwards. After sacrifice on day 15, blood was collected and hemolysate was used for estimation of blood parameters such as hemoglobin, glutathione, the activity of superoxide dismutase (SOD) and catalase. a) GSH b) SOD and c) catalase activity. Values are expressed as mean \pm SD. ##: $p < 0.01$ (control compared to normal), **: $p < 0.01$, (standard, UNMET-LD, 200 mg/kg b.wt. and HD, 400 mg/kg b.wt. compared to control), ns: non-significant, $p > 0.05$, (Vehicle control compared to control)

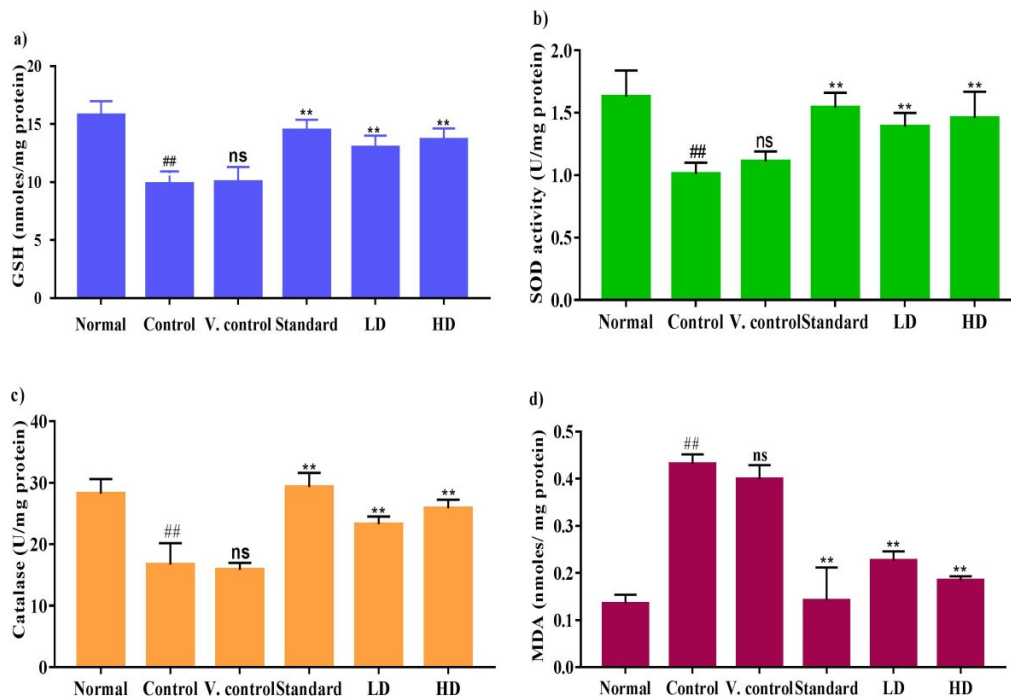


Figure 6.3. Effect of UNMET extract on the antioxidant status in the liver of mice following sodium fluoride-induced oxidative stress. Swiss albino female mice were grouped and the animals in group II, III, IV, and V were pretreated for seven days with respective doses of drugs. All the animals except in group I received NaF (600 mg/L) through drinking water from 8th day onwards. After sacrifice on the day 15, the liver was dissected out and liver homogenate (25% in 0.1M Tris buffer, pH 7) used for estimation of total protein, lipid peroxidation (LPO), SOD and catalase activity, glutathione levels. Values are expressed as mean \pm SD. ##: $p < 0.01$ (control compared to normal), **: $p < 0.01$, (standard, UNMET-LD and UNMET-HD compared to control), ns: non-significant, $p > 0.05$, (Vehicle control compared to control).

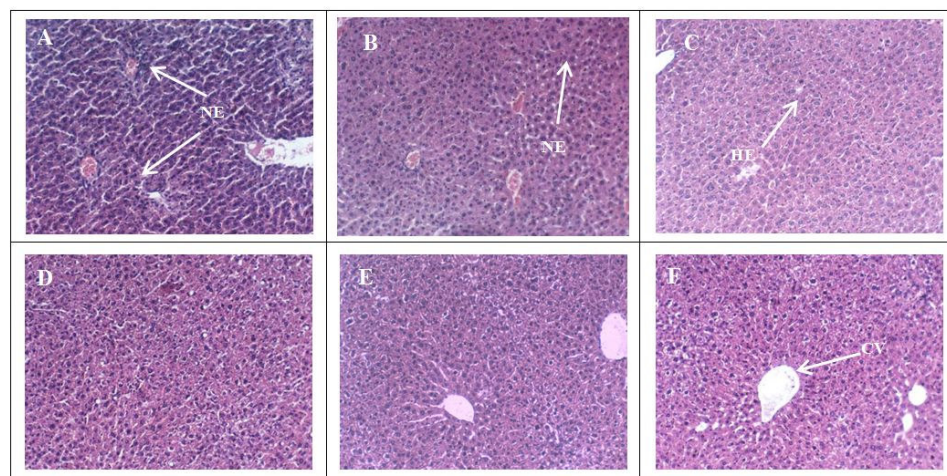


Figure 6.4. Histopathology of liver tissue following NaF induced oxidative stress. A: Control with NaF treated; B: vehicle control; C: normal; D: Vitamin C standard; E: UNMET-LD, 200 mg/kg b.wt. ; F: UNMET-HD, 400 mg/kg b.wt. Swiss albino female mice were grouped and the animals in group II, III, IV, and V were pretreated for seven days with respective doses of drugs. All the animals except in group I received NaF (600 mg/L) through drinking water from 8th day onwards. After sacrifice, a portion of liver was excised, and fix in 10% neutral formalin and histological examination was carried out by hematoxylin and eosin (H & E) staining and observed under a microscope (200 X). Arrows denote necrosis (NE); hepatocytes (HE) and central vein (CV).

6.3.2.2. Effect of UNMET on PMA induced superoxide radical generation in peritoneal macrophages.

Superoxide radicals are generated when sodium caseinate induced macrophages are further activated by PMA. Superoxide radical generation was effectively scavenged by *U.narum* methanolic extract which showed inhibition of 46.37 and 70.88 % at given doses of 200 and 400 mg/kg b.wt. respectively in comparison to the control group. (Figure 6.5 and Table 6.4)

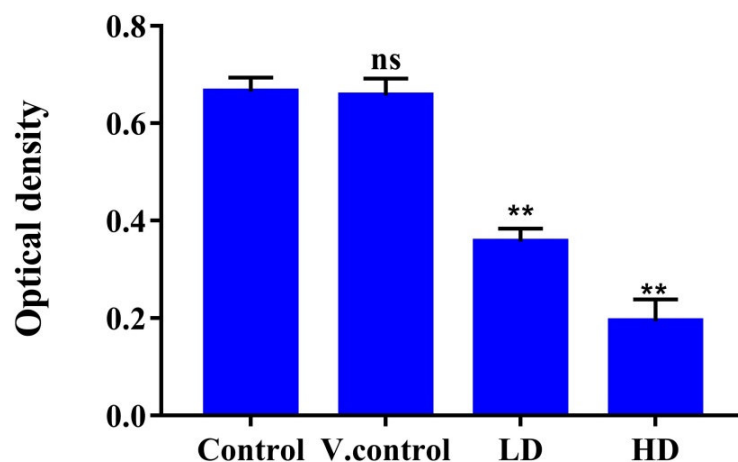


Figure 6.5. Effect of UNMET on PMA induced superoxide radical generation in peritoneal macrophages. Group I control, Group II: vehicle control, for group III and IV, UNMET-LD (200 mg/kg b.wt.), and UNMET-HD (400 mg/kg b.wt.), respectively were administered. On the fifth day, after 1hr of drug administration, sodium caseinate was injected to elicit peritoneal macrophages and the macrophages were activated *in vivo* by injecting PMA (100 mg/animal, i.p.). Peritoneal macrophages were collected after 3hr of PMA injection and centrifuged. The inhibition of superoxide generation in macrophages was determined. Values are expressed as mean \pm SD. **: $p < 0.01$, (standard, UNMET-LD and UNMET-HD compared to control), ns: non-significant, $p > 0.05$, (Vehicle control compared to control).

Table 6.4 Percentage inhibition by UNMET on PMA induced superoxide radical generation in peritoneal macrophages

Groups	Percentage inhibition (%)
UNMET-LD, 200 mg/kg b.wt.	46.37
UNME-HD, 400 mg/kg b.wt.	70.88

6.3.3. *In vivo* anti-inflammatory properties of UNMET extract

6.3.3.1. Effect of UNMET extract in carrageenan-induced acute paw edema inflammation model

The paw edema induced in mice by sub-plantar injection of carrageenan reached the maximum in the untreated group (0.354 ± 0.007 cm) with an increase in paw thickness of 0.165 ± 0.006 cm. However in the UNMET-HD (400 mg/kg b.wt.) and LD (200 mg/kg b.wt) treated animals the increase observed was nominal (0.099 ± 0.005 and 0.110 ± 0.008 cm). Diclofenac treated animals documented an increase in edema volume of 0.071 ± 0.004 cm in the 3rd hr. Paw edema significantly reduced by the administration of UNMET-LD, 200 mg/kg .wt. and UNMET-HD, 400 mg/kg b.wt. extract, respectively. The percentage inhibition of edema formation in 3rd hr was 37.47 and 44.83% in UNMET-LD and UNMET-HD groups, compared to untreated control groups. For the standard diclofenac (25 mg/kg b.wt.) treated groups, the percentage of inhibition was found to be 57.18% (Table 6.5, Figure 6.6 & 6.11).

Table 6.5. Effect of UNMET extract administration on carrageenan-induced acute paw edema.

Group	Initial paw thickness (cm)	Paw thickness in the 3rd hr (cm)	Increase in paw thickness (cm)	% inhibition
Control	0.189 ± 0.003	0.354 ± 0.007	0.165 ± 0.006	-
Vehicle control	$0.186 \pm .006$	0.348 ± 0.009	0.156 ± 0.007	1.6
Diclofenac	0.191 ± 0.004	$0.262 \pm .026$	0.071 ± 0.004	57.18
200 mg/kg b.wt.	0.181 ± 0.018	0.291 ± 0.014	0.110 ± 0.008	37.47
400 mg/kg b.wt.	0.187 ± 0.018	0.286 ± 0.009	0.099 ± 0.005	44.83

Values are expressed as mean \pm SD.

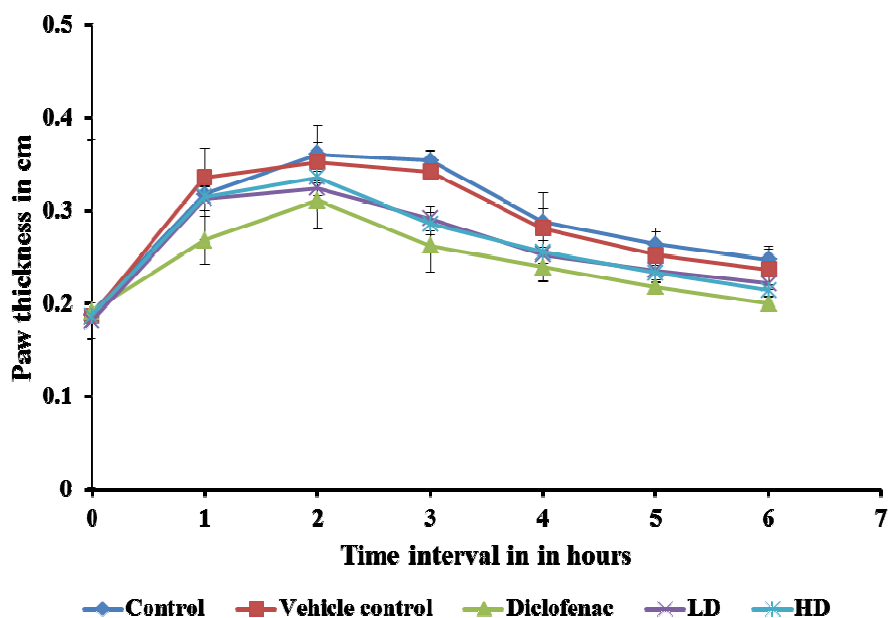


Figure 6.6. *In vivo* anti-inflammatory effect of UNMET extract on carrageenan-induced acute inflammation in murine paw edema. Group I: control -carrageenan alone (1% w/v) and Group II: vehicle control -1% propylene glycol; Group III: standard diclofenac (25 mg/kg b.wt) and Group IV and V were UNMET-LD (200 mg/kg b.wt), and UNMET-HD (400 mg/kg b.wt) treated, respectively. All group except control were pretreated with respective drugs for 4 days. On the 5th day, 1hr after the administration of drugs, acute inflammation was induced by subplantar injection of 0.02 ml of 1% w/v carrageenan in 0.1% carboxymethylcellulose (CMC) in the right hind paw of every mouse. The paw thickness was measured using vernier caliper, 1 hr prior to and for every hour up to 6th hr after carrageenan administration. Values are expressed as mean \pm SD.

6.3.3.1.1. Effect of UNMET extract on extravasation of Evans blue dye (EBD) in carrageenan-induced paw edema in mice

Evans blue extravasation due to inflammation-induced increase in the vascular permeability was analyzed (Figure 6.7). It was found that pre-treatment with diclofenac (25 mg/kg b.wt.) and UNMET extracts (UNME-LD-200 mg/kg b.wt. and UNME-HD-200 mg/kg b.wt.) significantly reduced ($p \leq 0.01$) extravasation of Evans blue dye. The diclofenac treated groups exhibited 89.35% reduction in dye retention

and UNMET-LD and HD treated group animals showed 75.93 and 88.92 % inhibition when compared to the dye retention in the microenvironment of edema of control group animals. Figures 6.8a and b demonstrate the absorbance of EBD at 620 nm for each animal group and the corresponding concentration of EBD.

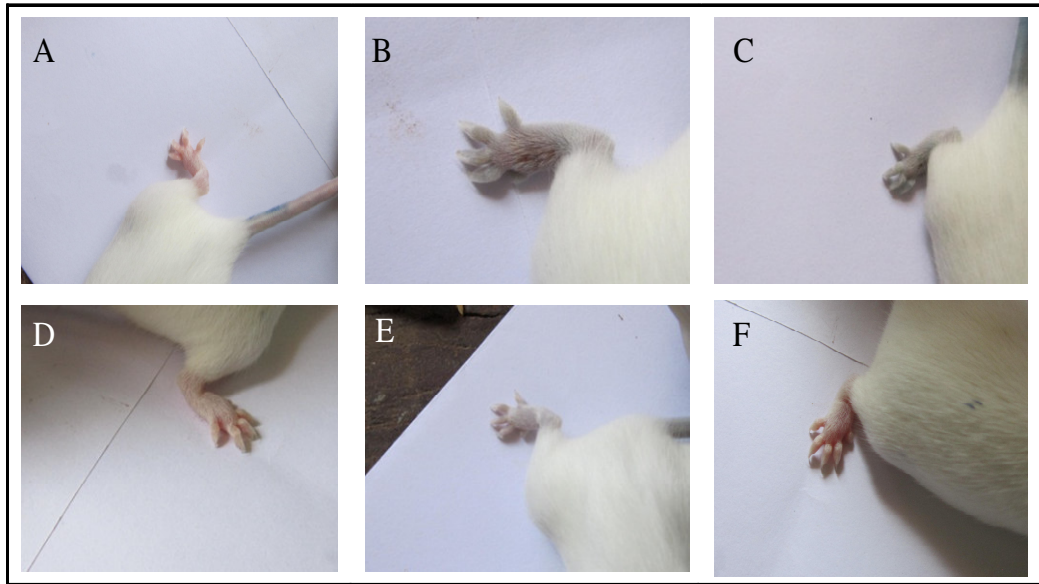


Figure 6.7. Photographs of the hind paw of mice in various experimental groups showing Evans blue retention status. Animals were pre-treated for 5 days with diclofenac and UNMET extracts at desired doses indicated and sub-plantar injection of carrageenan (0.02 mL) was done. After 3.5hr of sub-plantar injection of carrageenan, EB (25 mg/kg b.wt.) was injected into the tail vein and photographed within half hour. A-normal; B- Control (carrageenan treated); C- Vehicle control; D-diclofenac; E- UNMET-LD and F- UNMET-HD treated groups showing leakage of EB dye.

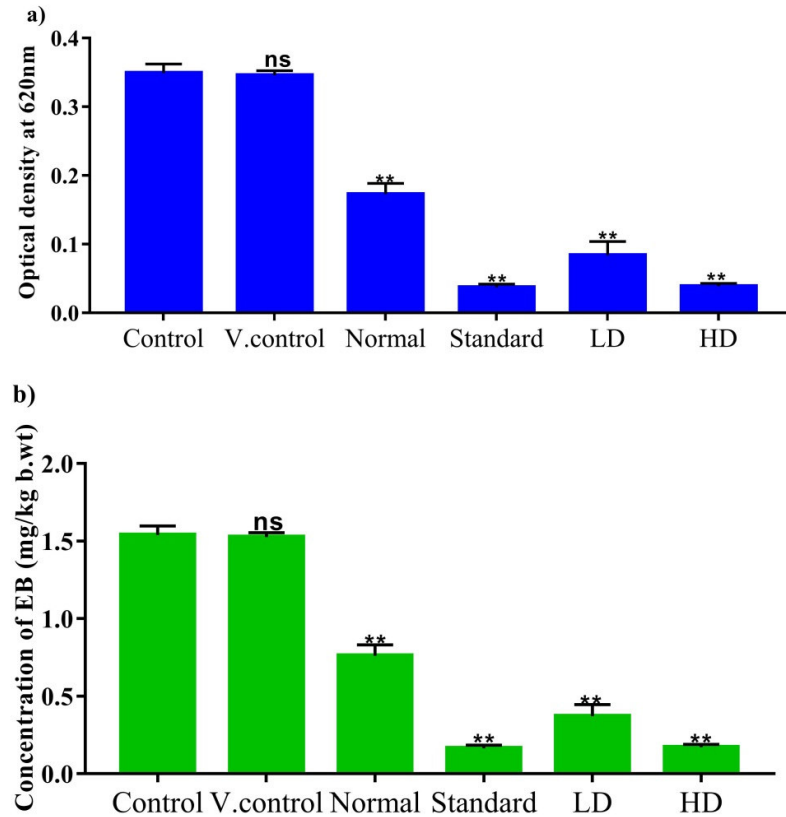


Figure 6.8. Quantitative measurement of Evans blue retained in the edematous paw of animals in the various experimental groups. Animals pre-treated for 5 days with diclofenac and UNMET extracts at doses 200 mg/kg b.wt. and 400 mg/kg b.wt. followed by sub-plantar injection of carrageenan. After 3.5 hr, EB (25 mg/kg b.wt.) was injected into the tail vein and observed within 30 min period for exudation in paws. Edematous tissue was excised out and homogenate prepared in 1mL acetone and sodium sulfate solution (1:4 acetone and 1% sodium sulfate v/v) and kept for 24 h at 37 °C to allow EB dye extraction. Absorbance of supernatant collected after centrifugation was measured at 620 nm using spectrophotometer (a) Concentration was calculated from the standard graph prepared using EB dye (b) Values are expressed as mean \pm SD. **: $p < 0.01$ (Normal, standard, UNMET-LD and UNMET-HD compared to control), ns: non-significant, $p > 0.05$, (Vehicle control compared to control)

6.3.3.2. Effect of UNMET extract on dextran-induced acute inflammation in mice.

The edema volume and percentage change in various treatment groups are given in Figure 6.9 and Table 6.6. Following 3 hr of dextran administration, the edema size reached a maximum in the untreated group (0.341 cm) showing a 45 percent increase. However, in the UNMET-HD (400 mg/kg b.wt.) and LD (200 mg/kg b.wt) treated animals, there was only 35.6 and 38.3% increase observed. Diclofenac treated animals documented a 31.5% increase in edema volume at the same time point. It was found that paw thickness reduced by 25.21% in the 3rd hr in UNMET–HD treated animals when compared to the size of edema in untreated control group animals during the same period. At a dose of 200 mg/kg b.wt. UNMET extract this reduction in the paw edema was only 19.15%. On the other hand, the percentage of inhibition in the diclofenac-treated group was 37.91%.

Table 6.6. Effect of UN methanolic extract administration on dextran-induced acute paw edema.

Group	Initial paw thickness (cm)	Paw thickness in the 3rd hr (cm)	Increase in paw thickness (cm)	% inhibition
Control	0.196 ± 0.003	0.341 ± 0.019	0.146 ± 0.004	-
Vehicle control	0.184 ± 0.002	0.327 ± 0.002	0.143 ± 0.005	1.8
Diclofenac	0.195 ± 0.005	0.285 ± 0.020	0.090 ± 0.010	37.91
200 mg/kg b.wt.	0.190 ± 0.008	0.308 ± 0.017	0.118 ± 0.008	19.15
400 mg/kg b.wt.	0.197 ± 0.003	0.306 ± 0.048	0.109 ± 0.006	25.21

Values are expressed as mean ± SD

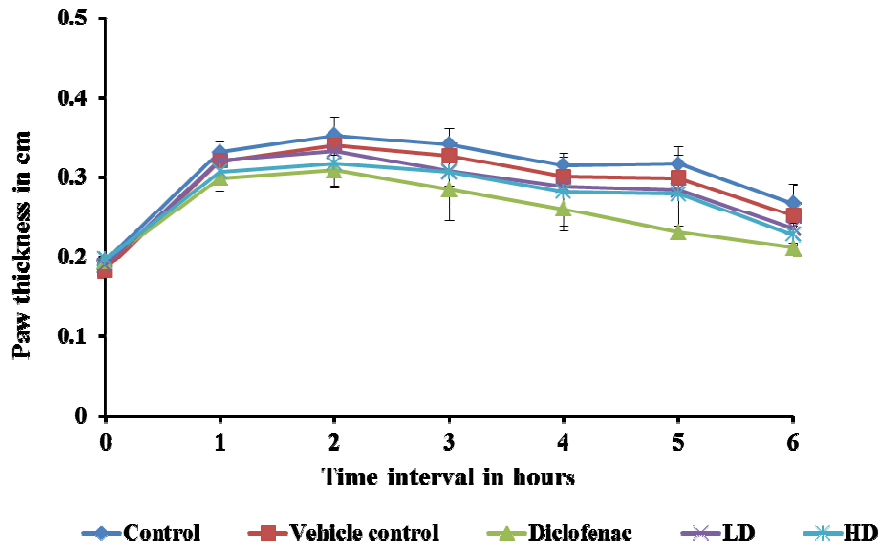


Figure 6.9. *In vivo* anti-inflammatory effect of UNMET extract on dextran-induced acute inflammation in murine paw edema. All group except group I were pretreated with respective drugs for 4 days. On the 5th day, 1h after the administration of drugs, inflammation was induced by subplantar injection of 0.02 ml of 1% dextran in 0.1% carboxymethylcellulose (CMC) in the right hind paw of all animals. The paw thickness was measured using a vernier caliper, 1 hr prior to and for every hour up to a 6th hour after dextran injection. Diclofenac was used as reference drug. Values are expressed as mean \pm SD.

6.3.3.3. Effect of UNMET extract in formalin-induced chronic inflammation

Both lower and higher doses of UNMET extract were found to be effective in reducing the formalin-induced chronic inflammation in mice paw. An increase in paw thickness of 0.251 ± 0.005 cm was observed in control group and in LD and HD of UNMET extracts, the observed increase in paw thickness was 0.190 ± 0.008 and 0.182 ± 0.003 cm, respectively. In the diclofenac administered group the increase was only 0.156 ± 0.006 cm. The percentage inhibition observed for LD and HD of UNMET extracts was 24.37 and 27.49 %, respectively. Diclofenac (25 mg/kg b.wt.) treated animals showed 38.35% inhibition in inflammation (Table 6.7 Figure.6.10 & 6.11).

Table 6.7.Effect of UNMET extract on chronic formalin-induced paw edema

Group	Initial paw thickness (cm)	Paw thickness in the 3rd hr (cm)	Increase in paw thickness (cm)	% inhibition
Control	190 ± 0.011	0.441 ± 0.029	0.251 ± 0.005	-
Vehicle control	0.160 ± 0.016	0.439 ± 0.008	0.279 ± 0.017	-
Diclofenac	0.179 ± 0.011	0.334 ± 0.009	0.156 ± 0.006	38.35
200 mg/kg b.wt.	0.171 ± 0.008	0.360 ± 0.053	0.190 ± 0.008	24.37
400 mg/kg b.wt.	0.169 ± 0.004	0.351 ± 0.009	0.182 ± 0.003	27.49

Values are expressed as mean ± SD.

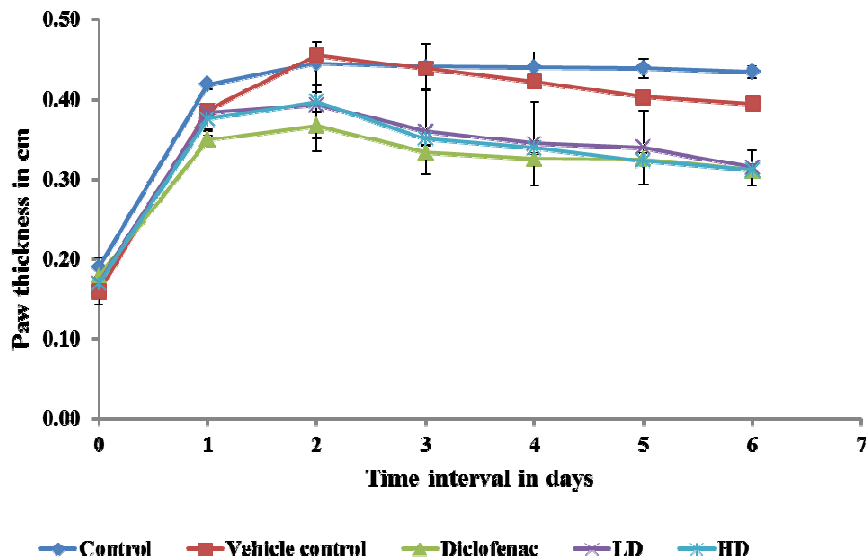


Figure 6.10. *In vivo* anti-inflammatory effect of UNMET extract on formalin-induced chronic inflammation in murine paw edema. The groups except the control group were pretreated with respective drugs for 7 days. On the 8th day, 1h after drug administration, chronic inflammation was induced by subplantar injection of 0.02 ml of 2% formalin on the right hind paw in all animal groups. The paw thickness was measured using vernier caliper, 1 hr prior to and for every day up to 6th day. Diclofenac was used as reference drug. Values are expressed as mean ± SD.

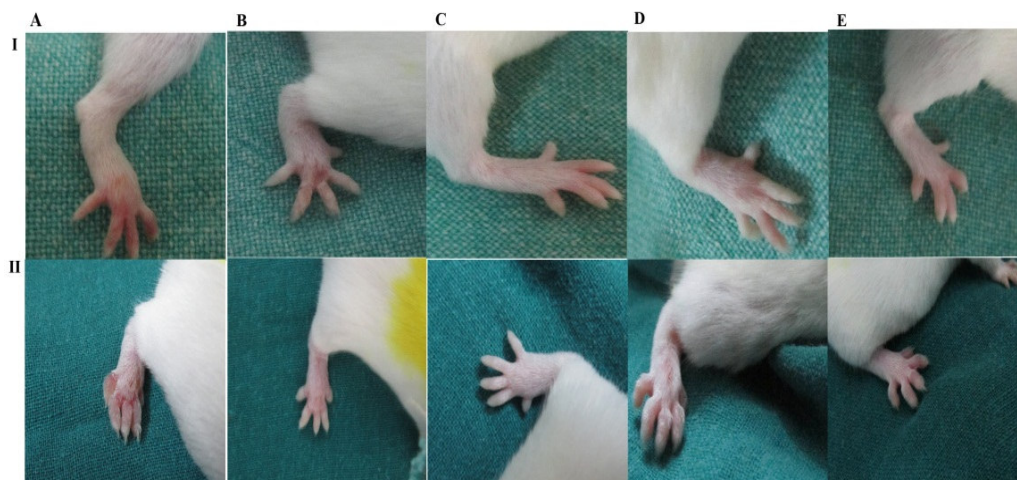


Figure 6.11. Photographs of animals in the study of *in vivo* anti-inflammatory effect of UNMET extract on I) carrageenan and II) formalin-induced inflammation of paw edema in mice. A: Control; B: vehicle control; C: Diclofenac; D: UNMELD-200 mg/kg b.wt. E: UNMEHD-200 mg/kg b.wt.

6.3.3.4. Effect of UNMET extract on histopathology of the hind paw in murine antiinflammatory models.

Intense paw edema, characterized by a presence of prominent infiltrations of inflammatory cells (neutrophils) was produced by sub-plantar injection of carrageenan or formaldehyde into the hind paw. Standard group pretreated with diclofenac (25 mg/kg b.wt.) showed a significant decrease in paw edema and the infiltration of inflammatory cells. Pre-treatment with UNMET extract was found to reduce the infiltration of neutrophils in both acute and chronic edema models (Figure 6.12).

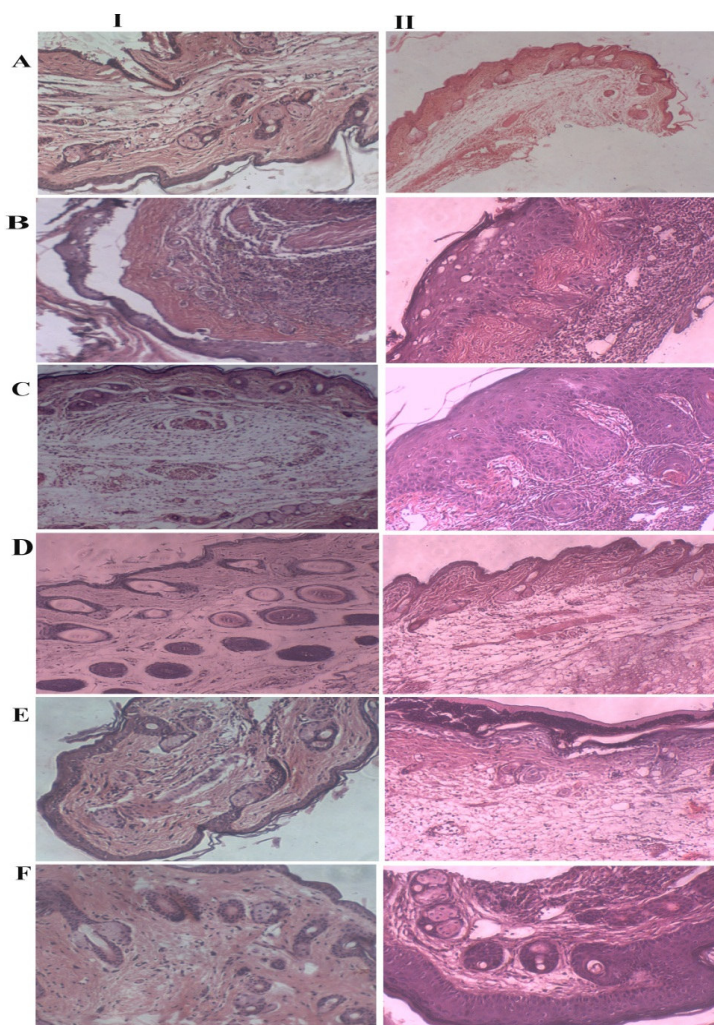


Figure 6.12. Effect of UNMET extract on histological changes in skin tissues of the plantar region of mouse paws. I) carrageenan and II) formalin-induced paw edema models. A: normal without any treatment; B: Control; C: vehicle control; D: Diclofenac; E: UNMELD-200 mg/kg b.wt. F: UNMEHD-200 mg/kg b.wt. After acute and chronic antiinflammatory experiments, the animals were sacrificed and paws of all groups were taken aseptically to confirm the inflammatory changes in paws of mice. Paws were washed, fixed in 10% buffered formalin solution and histological examination of tissue was performed using histochemical stains such as hematoxylin and eosin (H & E) stain and the tissue sections were observed under a microscope (200X) to ascertain the degree of inflammation.

6.4. Partial purification of compounds from methanolic extracts using column chromatography

Further purification of the UNMET using column chromatography yielded partially purified ethyl acetate (EA) and water (AQ) fractions. These fractions were then analyzed for antioxidant efficacy *in vitro*.

6.4.1. *In vitro* antioxidant activity of sub-fractions of UNMET extract

6.4.1.1. DPPH radical scavenging activity

Comparison of DPPH radical scavenging activity of UNMET-EA and UNMET- AQ fractions were performed and the results are depicted in Figure 6.13A. A dose-dependent reduction of DPPH free radical was observed within the concentration range of 10-100 $\mu\text{g/mL}$ of the fractions added to the reaction. UNMET-AQ fraction needed for 50% reduction (IC 50 value) of DPPH radical was found to be $68.49 \pm 2.97 \mu\text{g/mL}$. However, for UNMET- EA fraction the IC₅₀ was $>100 \mu\text{g/mL}$.

6.4.1.2. Inhibition of lipid peroxidation

There was a dose-dependent inhibition in iron/ ascorbate system induced tissue lipid peroxidation by the addition of UNMET- EA and AQ fractions. The UNMET-AQ fraction showed the highest inhibitory potential with a documented IC₅₀ value of $70.42 \pm 3.47 \mu\text{g/mL}$. The EA fraction, on the other hand, inhibited the peroxidation process with a comparatively higher IC₅₀ value of $99.14 \pm 1.79 \mu\text{g/mL}$ (Figure 6.13.B).

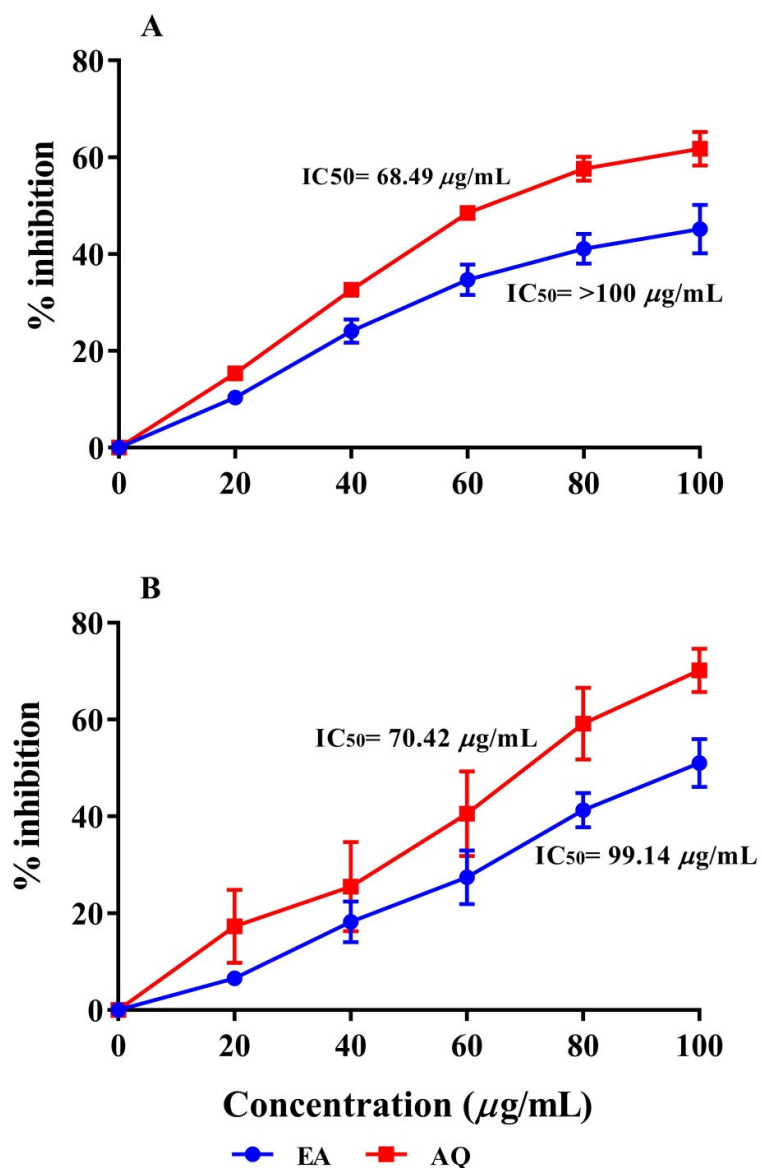


Figure 6.13. Antioxidant potentials of *U.narum* methanolic extract fractions such as EA and AQ fractions A) DPPH free radical scavenging assay; B) Inhibition of lipid peroxidation. Data are the mean \pm S.D.

6.4.2. Effect of UNMETAQ fraction on Lipopolysaccharide-induced mouse TNF- α production.

LPS induced TNF alpha production by RAW264.7 macrophages in various experimental conditions are depicted in Figure 6.14. In the untreated normal or

UNMET-AQ pretreated RAW264.7 cells the TNF alpha level was not sensitive enough to be detected. However, RAW264.7 cells exposed to LPS had TNF alpha level of 342.5 ± 28.93 pg/ mL) in the extracellular medium. On the other hand, UNMET-AQ pretreated cells (50 and 100 $\mu\text{g}/\text{mL}$), when exposed to LPS, showed significantly reduced levels of TNF alpha (226.17 ± 15.14 and 137 ± 15.22 pg/ mL, respectively).

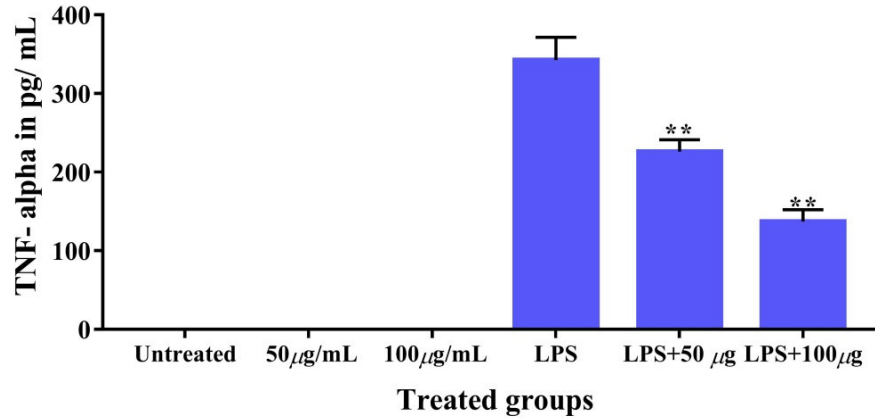


Figure 6. 14. Effect of UNMET-AQ fraction on LPS induced TNF- α production in RAW264.7 cells: Cells (1×10^4 cells/ mL DMEM) were seeded in 96 well plates. At 80% confluence, cells were exposed to UNMET-AQ fractions (50 and 100 $\mu\text{g}/\text{mL}$) for 12hrs. LPS (1 $\mu\text{g}/\text{mL}$) was then added and incubation continued for an additional 12hrs. TNF- alpha levels in the supernatant was quantitatively determined using Mouse TNF-alpha Quantikine ELISA Kit. **: $p < 0.01$ (UNMET-AQ, 50 and 100 $\mu\text{g}/\text{mL}$ compared to control).

6.4.3. Quantitative real-time PCR (qRT PCR) analysis

Figure 6.15 shows mRNA level expression of TNF- α . The fold change in expression of TNF alpha mRNA with respect to GAPDH (housekeeping gene) in normal untreated RAW264.7 cells was found to be 1.03 ± 0.04 . However in LPS alone treated cells, the observed fold change was 1.91 ± 0.17 . When these cells were pretreated with UNMET-AQ fraction, (50 and 100 $\mu\text{g}/\text{mL}$) and challenged with LPS, the fold changes in cells decreased to 1.87 ± 0.10 and 0.44 ± 0.03 . The decrease in fold change in the UNMET - AQ, 100 $\mu\text{g}/\text{mL}$ exposed cells was highly significant ($P < 0.01$).

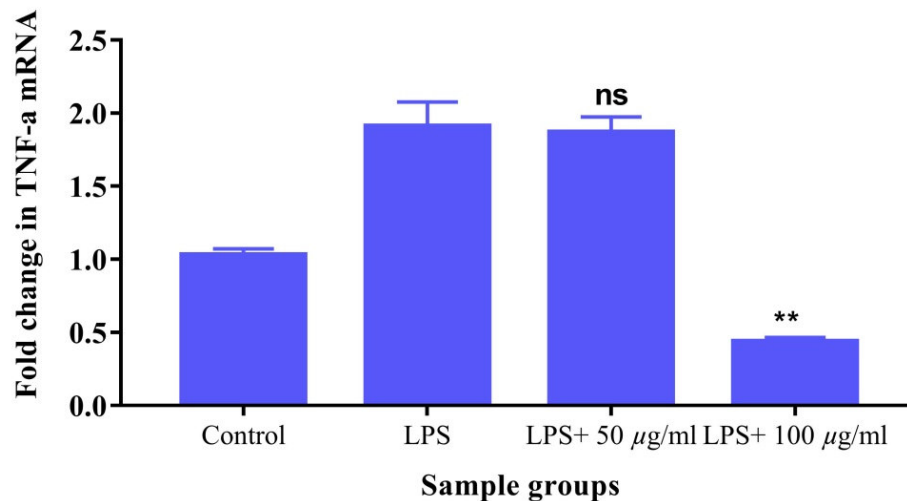


Figure 6.15. Expressions of TNF- α in LPS stimulated RAW264.7 cells exposed to UNMET-AQ fraction. The cells were treated with UNMET-AQ extract in 50 and 100 $\mu\text{g/mL}$ concentrations and cDNA synthesized from the total RNA of treated and untreated cells were amplified using specific primers for GAPDH and TNF- α genes. The gene expression was compared by relative quantification using the $\Delta\Delta\text{CT}$ method and the fold change (FC) was calculated as $2^{-\Delta\Delta\text{CT}}$. Values are expressed as mean \pm SD. **: $p < 0.01$ (UNMET-AQ, 100 $\mu\text{g/mL}$, ns: nonsignificant compared to LPS control).

6.4. Discussion

Fluoride toxicity affects the normal homeostasis of the body by inducing overwhelming ROS production leading to oxidative stress and redox imbalance. In the present study, the administration of NaF decreased the activity of antioxidant enzymes such as SOD, catalase and GSH levels in the blood as well as liver tissues of mice. Concomitantly a significant increase in the extent of lipid peroxidation in liver tissue was observed. However, in UNMET-LD and UNMET-HD administered animals, significant improvement in the antioxidant enzyme activity and GSH level was noticed. The lipid peroxidation status was also found to be reduced. This indicates that the UNMET extract reverses NaF induced disturbance in the redox status of animals. NaF is known to exert its pro-oxidant effect via fluoride radicals that chelate iron molecules in the active centers of antioxidant enzymes. This reduces the antioxidant enzyme status and increases lipid peroxidation as seen in the present

study. As described in the previous chapter (Chapter 3) UNMET extract possesses radical reducing, scavenging and lipid peroxidation inhibition properties. Supporting this, UPLC-Q-TOF-MS analysis in the present study revealed that the extract contained quercetin-3-O-beta-glucopyranosyl-6"-acetate, rutin and anonaine which are known antioxidants with free radical scavenging efficacy (Zhao *et al.* 2018); Li *et al.* 2013;(Zheng *et al.* 2017). So it is likely that the extract may contain antioxidant phytochemicals that scavenged the fluoride generated radicals and inhibited its chelation to iron molecules of enzyme proteins. This could be the possible reason for the improved catalase, SOD activity in the UNMET extract treated animals. The reason for the improved GSH level in these animals is difficult to explain at present. However, it is possible to assume that the enzymes involved in maintaining GSH levels in the body may be activated by the extract thereby promoting GSH synthesis. This assumption is based on the fact that antioxidant molecules are known to modulate the transcription factor (nuclear factor 2 related factor 2) Nrf2; the activity of which improves the synthesis of the tripeptide GSH and the GSH dependent enzymes. The drop in lipid peroxidation observed in the UNMET extract treated animals also supports this assumption. Together, the results suggest that the protective ability of UNMET extract against NaF induced toxicity in animals is mediated through its antioxidant efficacy.

In this study, the UNMET extract showed effective inhibition in the formation of paw edema in carrageenan, and dextran-induced acute inflammation as well as formalin-induced chronic inflammation when compared to untreated control mice. Evans blue extravasation under carrageenan-induced acute inflammation was found to be reduced significantly in animals treated with UNMET extracts as evidenced by the less retention of dye at the edematous site compared to control animals. Increased vascular permeability at inflammatory sites causes edema and Evans blue dye injected through tail vein thus get retained at this site. The observed reduction in Evans blue retention at carrageenan-induced edema site could be due to reduced blood vessel permeability. This is a visible indication for the conclusion that UNMET extract prevents carrageenan induced vascular permeability.

Sub-plantar injection of carrageenan, a highly sulfated polysaccharide increases the protein expression levels of interleukin 1 (IL-1) β and tumor necrosis factor α (TNF-

α), prostaglandin E2 (PGE2) and nitric oxide (NO) and iNOS and COX-2 in paw (Mansouri *et al.* 2015). It causes increased blood flow and infiltration of neutrophils due to vasodilation of capillaries beneath the paw skin causing redness and swelling. On the other hand, polysaccharide dextran while injecting to mice paw, promotes anaphylactic reaction with extravasation due to the secretion of histamine and serotonin from mast cells and forms edema (Sapna Sachan and Singh 2013).

The chronic dose of formalin-induced swelling of the paw which succeeds in two phases with two periods of vascular permeability induction (Damas and Liegeois 1999). Formalin-induced inflammation and edema are said to be biphasic, with an early neurogenic part along with a later tissue-mediated response. Early phase nociception cause rapid onset of edema and is associated with activity in primary afferent neurons and axon reflexes. Later stages, inflammatory response get down-regulated by descending neuronal pathways and by the principal action of tissue-mediated components involving secondary humoral component. (Wheeler-Aceto and Cowan 1991). The histological details of all the edematous tissue from UNMET extract treated animals also reveal less infiltration of inflammatory cells. It is thus unequivocally demonstrated that the anti-inflammatory potential of the UNMET extract is through reduced vascular permeability and subsequent recruitment of inflammatory cells and their secretion.

Further purification of UNMET extract projected an aqueous fraction (UNMET-AQ) with the highest antioxidant activity. In RAW264.7 macrophages exposed to UNMET-AQ was found to inhibit LPS induced TNF- α production both at mRNA and protein level. Lipopolysaccharide (LPS), PMA and TNF- α induce I κ B α , an inhibitory regulator of NF- κ B to dissociate from NF- κ B and thereby activate NF- κ B (Nuclear factor kappa B) a transcription factor that regulates inflammatory gene expression that mediates cell survival, cellular adhesion, cell cycle activation, cell proliferation and angiogenesis (Miller and Hunt 1998). Stimulation of cells with these factors leads to the activation and the translocation of p50/65 (subunits of NF-kappa B) into the nucleus and binds to nuclear receptors for initiating gene transcription (Beg *et al.* 1993). The resynthesis of I κ B α is the very important action of antioxidants stimulating the regulatory mechanism which involved in the functioning of NF-kappa B (Beg *et al.* 1993).

The anti-inflammatory effect of UNMET extract may be due to the presence of synergistic action of phytochemicals. Compounds present in UNMET extract such as rutin and cornuside as detected in LCMS analysis are known inhibitors of NF kappa B signaling pathway. Rutin has been reported to regulate the expression of NF- κ B and p38, thereby inhibiting the development of lung cancer (Li *et al.* 2017). Studies have also reported that cornuside inhibits NF kappa B and LPS induced inflammatory cytokines in RAW 264.7 macrophages (Choi *et al.* 2011; Li *et al.* 2016a). Since TNF-alpha-induced inflammation is mediated by NF κ B pathway, the active components in UNMET-AQ fraction thus could be a good anti-inflammatory molecule with cancer preventive potential. A detailed study on the inhibitory effects of UNMET on TNF-alpha and NF-kappa B signaling pathways are further required for elucidating the mechanism of action.

SUMMARY

Summary

From the preceding studies, the plant *Uvaria narum* has been found to be a good source of pharmacologically important phytochemicals. The petroleum ether (UNPE) extract documenting cytotoxic and tumor reducing efficacy is expected to have chemotherapeutic potential and methanolic (UNMET) extract with its observed antioxidant and anti-inflammatory efficacy both in *in vitro* and *in vivo* are considered to have cancer-preventing potential.

Even though all extracts of *U.narum* show remarkable cytotoxicity /antiproliferative effect, UNPE extract is found to be the most promising as its toxic effect is directed more towards neoplastic cells than to normal cells. In mice, the tolerable limit of this extract is found to be 5, 7.5 and 10 mg/ Kg b.wt. as elucidated from acute and sub-acute toxicity studies. Oral administration of UNPE extract at these doses have achieved a significant reduction in tumor volume and increased the lifespan of tumor-bearing mice. The tumor reducing the efficacy of UNPE extract is observed to be well below that of standard cyclophosphamide treated groups. However, animals in the standard group showed a significant reduction in total WBC counts in comparison to animals in UNPE extract treated group. This is considered to be an advantage of UNPE extract over cyclophosphamide. When the mechanistic basis of the toxicity assessed in HeLa cells exposed to UNPE extract and its active subfraction, using AO/EtBr dual staining and FACS analysis (using Annexin V-FITC that combined propidium iodide stain) showed the features characteristic of apoptosis thereby suggesting the apoptosis-inducing mechanism of the UNPE extract. Cell cycle analysis confirms the same and shows that cell cycle arrest occurred in the sub-G1 phase. It is interesting to note that the UNPE extract as such exerts higher toxicity than its subfractions. The important cytotoxic class of compounds revealed by phytochemical, TLC and GC-MS methods have been found segregated into the subfractions. Moreover, some of these compounds detected in the subfractions are in the modified form. It is thus assumed that the loss in the cytotoxic potential of the UNPE during its fractionation might be due to this segregation of individual phytocompounds and thus a synergism of compounds in UNPE is likely to contribute to its cytotoxic and antitumor property. UNPE extract can be studied further as a most

potent candidate in chemotherapeutic drug development and requires further tumor model studies and detailing of its apoptotic mode of death.

On the other hand, the most promising antioxidant and the anti-inflammatory UNMET extract are found safe upon administration upto 2000 mg/ Kg b.wt in acute toxicity assessment. Oral administration of UNMET extract at doses 200, 300 and 400 mg/ kg b.wt. has not elicited any toxic effect in sub-acute toxicity analysis for 28 days. This is confirmed from the unaltered hemogram, liver and kidney function marker enzyme activities and histological details of internal organs between normal and treated animals. Further, the UNMET extract could reverse the NaF induced loss in glutathione, SOD and catalase activities and also hike in TBARS, thereby restoring redox balance in mice and also inhibit PMA induced superoxide radical generation in peritoneal macrophages. These results strongly suggest the antioxidant potential of the UNMET extract. In addition, the UNMET extract is found to possess significant anti-inflammatory activity, as it significantly inhibits carrageenan and dextran-induced acute inflammation as well as formalin-induced chronic inflammation in mice. Reduced extravasation of Evans blue dye in the carrageenan-induced paw edema model by UNMET extract confirms its anti-inflammatory efficacy. The antioxidant phytochemicals quercetin-3-O-beta-glucopyranosyl-6"-acetate, rutin and anonaine documented in the UNMET extract by LC-MS analysis are likely to be responsible for its antioxidant and anti-inflammatory activities. Subsequent fractionation of UNMET extract for the purification of active antioxidant and anti-inflammatory principle has resulted in the separation of ethyl acetate (EA) and aqueous (AQ) fractions, of which UNMET-AQ fraction shows better antioxidant potential *in vitro*. Quantitative evaluation on lipopolysaccharide (LPS) –induced TNF- α secretion in RAW264.7 cells shows that pre-treatment of the UNMET-AQ fraction (50 and 100 μ g/mL) significantly ($P < 0.01$) reduces TNF alpha production. Supporting this result, there observes a significant reduction in the expression of TNF alpha mRNA with respect to GAPDH (housekeeping gene). This indicates that UNMET-AQ fraction contains phytochemicals capable of altering TNF alpha signaling which forms the basis for the anti-inflammatory potential of the UNMET extract. Overall the study concludes that *U.narum* is a source of important anticancer phytochemicals which need to be further assessed using additional pre-clinical and clinical studies.

Future perspectives

This study gives an insight into the mechanistic basis for the antiproliferative efficacy of petroleum ether extract and the antioxidant efficacy of methanolic extract of *Uvaria narum* leaves. To the extent, possible active components have been identified using GC-MS and LC-MS analysis. The UNPE extract which shows promising cytotoxic and antitumor potential needs to be further explored using more animals models especially in cancers of reproductive organs. The toxicity of higher dose of UNPE and UNMET extract towards the testis is thought to be due to the presence of developmental and reproductive toxic phytochemicals. Sometime *Uvaria narum* could be specifically used in reproductive cancers which need to be tested using cell culture and animal model studies. The organ-specific toxic compound can be purified or isolated from this extract which can be used in experimental purposes as a targeting agent. Additionally, a detailed pharmacological assessment has to be undertaken before bringing them into clinical trials. Purification and characterization of the antioxidant and anti-inflammatory molecule from the UNMET extract and assessment of its inhibitory effect on TNF-alpha signaling is another important work to be carried out, which may lead to the development of antineoplastic drug candidates.

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1. **Smitha K Ramavarma**, Ansa P Unnikrishnan, Babu. T. Devassy and Achuthan C Raghavamenon (2018). *In vitro* cytotoxic and antiproliferative activities of *Uvaria narum* seed oil (UNSO). International Journal of Advanced Research (**Accepted**).
2. **K. R. Smitha**, T.D. Babu and C. R. Achuthan (2014). Phytochemical screening and *in vitro* cytotoxicity analysis of *Uvaria narum* (Dunal) Wall. Asian Journal of Phytomedicine and Clinical Research, **2 (2)** 40-47.