ASSESSMENT OF FULLERENE C_{60}, A CARBON NANOMATERIAL, INDUCED TOXIC RESPONSE IN THE FRESHWATER FISH, *ANABAS TESTUDINEUS* (BLOCH, 1792)

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By

SUMI N.

ENDOCRINOLOGY AND TOXICOLOGY LABORATORY
DEPARTMENT OF ZOOLOGY
UNIVERSITY OF CALICUT
KERALA – 673635, INDIA

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UNIVERSITY OF CALICUT
DEPARTMENT OF ZOOLOGY

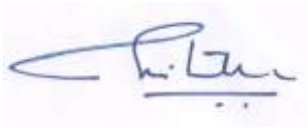
Dr. K. C. Chitra
Associate Professor

Calicut University P. O.
Kerala, India 673 635
Phone : 0494 240 7420
Cell : 09495135330
Email : kcchitra@yahoo.com

25 June, 2020

CERTIFICATE

The adjudicators of the thesis entitled "Assessment of fullerene C_{60}, a carbon nanomaterial, induced toxic response in the freshwater fish, *Anabas testudineus* (Bloch, 1792)" submitted by Ms. N. Sumi have not suggested for any corrections. Hence, as instructed by the Directorate of Research, two copies of thesis and one soft copy of the thesis in PDF format on CD is being sent to the office.



Dr. K. C. Chitra

UNIVERSITY OF CALICUT
DEPARTMENT OF ZOOLOGY

Dr. K. C. Chitra
Associate Professor

Calicut University P. O.
Kerala, India 673 635
Phone : 0494 240 7420
Cell : 09495135330
Email : kcchitra@yahoo.com

30 December, 2019

CERTIFICATE

This is to certify that Ms. Sumi N. has carried out the research work embodied in the present thesis under my supervision and guidance for the full period prescribed under the Ph. D ordinance of this University. I recommend her thesis entitled "Assessment of fullerene C_{60}, a carbon nanomaterial, induced toxic response in the freshwater fish, *Anabas testudineus* (Bloch, 1792)" for submission for the degree of Doctor of Philosophy in this University.

I further certify that this thesis represents the independent work of the candidate under my supervision and no part of the thesis has been presented for the award of any other degree, diploma or associateship in any University.

Dr. K. C. Chitra
Supervisor

DECLARATION

I hereby declare that the work presented in the thesis entitled "Assessment of fullerene C_{60}, a carbon nanomaterial, induced toxic response in the freshwater fish, *Anabas testudineus* (Bloch, 1792)" is a genuine record of research work done carried out by me under the guidance and supervision of Dr. K. C. Chitra, Associate Professor, Department of Zoology, University of Calicut. To the best of my knowledge, no part of this thesis has been previously submitted for the award of any degree, diploma or associateship in any other University.

(Sumi N.)
Candidate

C. U. Campus
30 December, 2019.

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Sumi. N

Dedicated to

My parents & Teachers

CONTENTS

ABSTRACT

Fullerene C_{60}, a carbon-based nanomaterial, induced toxicity evaluation were carried out in the freshwater fish, *Anabas testudineus*. The thesis consists of four chapters concluding the toxic responses of fullerene C_{60} in different aspects. Chapter 1 elaborates the acute toxicity and behavioural modifications induced by fullerene C_{60}. The median lethal concentration based on the acute toxicity test determined by Probit analysis was found as 50 mg/ L. Two sublethal concentrations, such as one-tenth, 5 mg/ L, and one-fifth, 10 mg/ L, were selected and exposed to fish for short-term (24, 48, 72 and 96 h) and long-term (7, 15, 30, and 60 days) durations for further toxicity studies. Fullerene C_{60} exposed at sublethal concentrations altered normal behaviour as well as modified haematological response in the fish. Chapter 2 discussed the effects of fullerene C_{60} on the antioxidant status of testis and ovary, and also correlated the toxic effects on histomorphological damages in gonadal tissues. The study observed the induction of oxidative stress in gonadal tissues thereby indicating reproductive toxicity. The reproductive impairment was further analysed in Chapter 3, which showed hormonal imbalance and induction of vitellogenesis in the fish. Sublethal exposure of fullerene C_{60} altered testicular and ovarian steroidogenesis as evident by decrease in the steroidogenic enzymes in time-dependent manner. Chapter 4 conveyed DNA damage induced by fullerene C_{60} in the fish erythrocytes, gill and liver cells using micronucleus test and comet

assay as the endpoints. Thus the present study evaluated sublethal effects of fullerene C_{60} which was time-dependent, and it was concluded that long-term exposure of nanomaterial impaired behavioural, physiological and reproductive parameters along with genetic damages thereby altering the normal functioning of the animal. The laboratory investigation provides the information for biomonitoring the impact of nanomaterial exposed in the aquatic ecosystem that ultimately affect the health status and survival of fish population, if exposed chronically to sublethal concentration in the natural environment.

പ്രബന്ധ സംഗ്രഹം

ശുദ്ധജല മത്സ്യമായ 'പൊരുക്ക്'/ 'കറൂപ്പ്'ൽ (അനുബസ് ടെസ്സു ടിനിയ സ്) കാർബൺ അധിഷ്ഠിത നാനോമറ്റീരിയലായ ഫുള്ളറിൻ സി-60 മൂലമുള്ള വിഷപ്രതികരണങ്ങൾ ഈ പഠനം വിലയിരുത്തി. ഇത് നാല് അധ്യായങ്ങളിലായി വിശദീകരിച്ചിരിക്കുന്നു. അധ്യായം ഒന്നിൽ ഫുള്ളറിൻ സി-60 യുടെ ഉപയോഗം മൂലം പരീക്ഷണ മത്സ്യത്തിൽ കാരണമായേക്കാവുന്ന തീവ്രവിഷാംശ സാന്ദ്രതയും (അക്യൂട്ട് ടോക്സിസിറ്റി) പെരുമാറ്റ വ്യതിയാനങ്ങളുമാണ് പ്രതിപാദിച്ചിരിക്കുന്നത്. പ്രോബിറ്റ് വിശകലനം ആസ്പദമാക്കിയുള്ള ഉപതീവ്ര സാന്ദ്രതകളായ (സബ് - ലീതൽ) അഞ്ചിലൊന്ന് (10 മി. ഗ്രാം/ലി.), പത്തിലൊന്ന് (5 മി. ഗ്രാം/ലി.), അളവുകൾ തെരഞ്ഞെടുക്കുകയും ഇത് വ്യത്യസ്ത സമയപരിധികളായ ഹ്രസ്വകാല (24, 48, 72, 96 മണിക്കൂർ), ദീർഘകാല (4, 7, 15, 30, 60 ദിവസങ്ങൾ) ദൈർഘ്യങ്ങളിൽ മത്സ്യത്തിനുമേൽ പരീക്ഷിക്കുകയും ചെയ്തു. അനന്തരഫലമായി ഫുള്ളറിൻ സി-60 യുടെ ഉപതീവ്ര സാന്ദ്രതകളുടെ ഉപയോഗം മത്സ്യത്തിന്റെ സാധാരണ സ്വഭാവത്തെയും രക്തഘടകങ്ങളെയും (ഹെമറ്റോളജി) സാരമായി ബാധിച്ചു. അധ്യായം രണ്ടിൽ ഫുള്ളറിൻ സി-60 യുടെ ഉപയോഗം ആൺ, പെൺ, മത്സ്യങ്ങളിലെ പ്രത്യൽപാദന അവയവങ്ങളിലെ ഓക്സീകരണ - നിരോക്സീകരണ (ഓക്സിഡേറ്റീവ് - ആന്റി ഓക്സിഡേറ്റീവ്) നിലയിലും, കോശജാലഘടനയിലും (ഹിസ്റ്റോമോർഫോളജി) മാറ്റമുണ്ടാക്കി. ഫുള്ളറിൻ സി-60 യുടെ ഉപയോഗം പരീക്ഷണ മത്സ്യത്തിൽ കാരണമായേക്കാവുന്ന പ്രത്യുൽപാദന വൈകല്യങ്ങളെ മൂന്നാമധ്യയത്തിൽ കൂടുതൽ വിശകലനങ്ങൾക്ക് വിധേയമാക്കിയിരിക്കുന്നു. അന്തർഗ്രന്ഥിസ്രാവങ്ങളുടെ (ഹോർമോൺ) ഉത്പാദനത്തിനുതകുന്ന ദീപനരസങ്ങളുടെ (എൻസൈമുകൾ) അളവ് കുറയുന്നതിലൂടെ മത്സ്യങ്ങളിൽ അന്തർഗ്രന്ഥിസ്രാവകളുടെ അസന്തുലിതാവസ്ഥയ്ക്ക് കാരണമായി. കൂടാതെ, മത്സ്യങ്ങളെ പ്രത്യുൽപാദനത്തിന് സജ്ജമാക്കുന്ന വിവിധതരം ദീപനരസങ്ങളുടെ നിർമ്മിതിയേയും മാറ്റി മറിച്ചു. ചുവന്നരക്താണുക്കൾ, ചെകിളകൾ, കരൾ, കോശങ്ങൾ എന്നിവയിൽ നടത്തിയ

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Chapter 1 **Fullerene C_{60} induced behavioural and haematological changes in the freshwater fish, *Anabas testudineus***

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LIST OF ABBREVIATIONS

%	Percentage
°C	Degree celcius
A	Ampere
Å	Angstrom
APHA	American Public Health Association guidelines
CADD	Computer Aided Drug Design
cm/sec	Centimeter per sec
cm^{-3}	Cubic centimeter
cm^3/K	Kelvin per cubic centimeter
d	Day
DMSO	Dimethyl sulfoxide
DTNB	Dithiobisnitrobenzoic acid
EC_{50}	Median effective concentration
ED_{50}	Median effective dose
EMEA	European Medicines Evaluation Agency
EPA	The Environmental Protection Agency
eV	Electron volt
FDA	Food and Drug Administration
g	Acceleration due to gravity
g cm^{-3}	Gram per cubic centimeter
g/mol	Gram per mol
Gpa	Giga pascal
h	Hour
H&E	Hematoxylin and Eosin
Hz	Hertz
K	Kelvin
k cal mol^{-1}	Kilocalorie per mole
Kg	Kilogram

kV	Kilovolt
LC_{50}	Median lethal concentration
LD_{50}	Median lethal dose
LOAEL	Lowest observed adverse effect level
mA	Milli ampere
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
mg	Milligram
mg/ L	Milligram per Litter
ng/ kg	Nanogram per kilogram
nm	Nanometer
NOAEL	No observed adverse effect level
OECD	Organisation for Economic Co-operation and Development
ohms m^{-1}	Ohm per meter
OSHA	Occupational Safety and Health Administration
RBC	Red blood corpuscle
SD	Standard Deviation
Sec	Second
UV	Ultraviolet
V	Voltage
vl	Longitudinal sound velocity
Vt	Transverse sound velocity
W/mK	Watts per meter-Kelvin
WBC	White blood corpuscle
µg/ g	Microgram per gram
µg/ kg	Microgram per kilogram
µg/ L	Microgram per litre
µM	Micrometer

Chapter 2: Fullerene C_{60} induced antioxidant and histomorphological changes in testis and ovary of the fish, *Anabas testudineus*

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LIST OF ABBREVIATIONS

%	Percentage
$^{\bullet}OH$	Hydroxyl radical
$^{1}O_2$	Singlet oxygen
5-LOX	5-lipoxygenase
8-OHdG	8-oxo-2′-deoxyguanosine
ANOVA	Analysis of Variance
AO	Atretic oocyte
BAL	Bronchoalveolar lavage
BTB	Blood-testis barrier
Ca^{2+}	Calcium ion
CAT	Catalase
d	Day
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DO	Degenerated oocyte
EDTA	Ethylenediaminetetraacetic acid
Fe	Iron
Fe^{2+}	Ferrous ion
Fe^{3+}	Ferric ion
g	Gravity
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSI	Gonadosomatic index
h	Hour
H_2O_2	Hydrogen peroxide
HOCl	Hypochlorous acid
mg/ L	Milligram per litter
min	Minute
mM	Millimolar
MMC	Melanomacrophage centers
N	Normality
NADPH	Nicotinamide adenine dinucleotide

nM	Nanomolar
nm	Nanometer
NMDA	N-methyl D-aspartate
NO	Nitric oxide
NO_2	Nitrogen dioxide
$NO_2^{\bullet}$	Nitrogen dioxide radical
O_2	Molecular oxygen
$O_2^{\bullet -}$	Superoxide anion
O_3	Ozone
ºC	Degree Celsius
ONOO-	Peroxynitrite radical
$ONOOCO_2^-$	Nitrosoperoxycarbonate radical
PEG	Polyethylene glycol
PUFAs	Polyunsaturated fatty acids
PV	Previtellogenic oocyte
PVP	Polyvinylpyrrolidone
RBC	Red blood cells
RNS	Reactive nitrogen species
RO-	Alkoxyl radical
$RO_2^{\bullet}$	Peroxyl radical
RONS	Reactive oxygen nitrogen species
ROS	Reactive oxygen species
RPZ	Reduced number of spermatozoa
SD	Standard deviation
SOD	Superoxide dismutase
SPG	Spermatogonial cells
SPZ	Spermatozoa
TBARS	Thiobarbituric acid reactive substance
TiO_2	Titanium dioxide
TNF	Tumor necrosis factor
UV	Ultraviolet
V	Vacuoles
VO	Vitellogenic oocyte
w/ v	Weight per volume

Chapter 3: Gonadal toxicity of fullerene C_{60} in the fish, *Anabas testudineus*

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LIST OF ABBREVIATIONS

%	Percentage
11-KT	11-ketotestosterone
17β-HSD	17β-hydroxysteroid dehydrogenase
3β-HSD	3β-hydroxysteroid dehydrogenase
ALP	Alkali labile phosphates
ANOVA	Analysis of variance
cm	Centimeter
d	Day
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E2	Estradiol
ELISA	Enzyme-linked immunosorbent assay
FSH	Follicle-stimulating hormone
g	Gram
g	Acceleration due to gravity
GnRH	Gonadotrophin-releasing hormone
GSH	Reduced glutathione
h	Hour
HPG	Hypothalamo-pituitary-gonadal axis
HPI	Hypothalamo-pituitary-interrenal axis
HRP	Horse radish peroxidase
ICR	Institute of Cancer Research
kDa	Kilodalton
LH	Luteinizing hormone
mg	Milligram
mg/ L	Milligram per liter
MIH	Maturation-inducing hormone
min	Minute
mM	Millimolar
MWCNTs	Multi-walled carbon nanotubes
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
ng/ mg	Nanogram per milligram

ng/ ml	Nanogram per milliliter
nm	Nanometer
NMRI	Naval Medical Research Institute
ºC	Degree Celsius
PBS	Phosphate buffered saline
PVP	Polyvinylpyrrolidone
SD	Standard deviation
sec	Second
SWCNTs	Single-walled carbon nanotubes
w/ v	Weight per volume
µg/ ml	Microgram per milliliter
µl	Microliter

Chapter 4: Cytogenotoxic effects of fullerene C_{60} in the fish, *Anabas testudineus*

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LIST OF ABBREVIATIONS

%	Percentage
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
CDCFH-DA	2′,7′-dichlorodihydrofluorescein diacetate
cm	Centimeter
d	Day
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
g	Gram
GTSP1	Glutathione S-transferase pi1
h	Hour
HBSS	Hank's Balanced Salt Solution
L	Liter
LDH	Lactate dehydrogenase
LMPA	Low melting point agarose
mA	Milliamperes
mg/ L	Milligram per liter
min	Minute
MWCNT	Multi-walled carbon nanotube
Na_2EDTA	Disodium ethylenediaminetetraacetate dihydrate
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide
NF1	Neurofibromatosis type I
NMA	Normal melting point agarose
ºC	Degree Celsius
PBS	Phosphate buffered saline
ROS	Reactive oxygen species
SCGE	Single-cell gel electrophoresis
SD	Standard deviation
SDS	Sodium dodecyl sulfate

SHROOM2	Shroom family member 2
SWCNT	Single-walled carbon nanotube
UV	Ultraviolet
V	Volt
V/ cm	Volt per centimeter
μg/ ml	Microgram per milliliter

LIST OF PUBLICATIONS

- **Sumi, N**., & Chitra, K. C. (2019). Impact of fullerene C_{60} on behavioral and hematological changes in the freshwater fish, *Anabas testudineus* (Bloch, 1792). *Applied nanoscience*, *9*(8), 2147-2167.

- **Sumi, N**., & Chitra, K. C. (2019). Fullerene C_{60} nanomaterial induced oxidative imbalance in gonads of the freshwater fish, *Anabas testudineus* (Bloch, 1792). *Aquatic Toxicology*, *210*, 196-206.

- **Sumi, N**., & Chitra, K. C. (2019). Cytogenotoxic effects of fullerene C_{60} in the freshwater teleostean fish, *Anabas testudineus* (Bloch, 1792). *Genetic Toxicology and Environmental Mutagenesis*, *847*, 503104.

- Sumi, N., & Chitra, K. C. (2020). Possible role of C_{60} fullerene in the induction of reproductive toxicity in the freshwater fish, *Anabas testudineus* (Bloch, 1792). *Environmental Science and Pollution Research.* Accepted Manuscript. Doi: 10.1007/s11356-020-08509-6.

- **Sumi, N**., & Chitra, K. C. (2017). Fullerene ($_{C60}$) induced alteration in the brain antioxidant system of the cichlid fish, *Pseudetroplus maculatus* (Bloch, 1795). *Journal of Global Sciences*, *6*, 4908-4917

- **Sumi. N**., & Chitra, K. C. (2017). Oxidative stress in muscle of the freshwater fish, *Etroplus maculatus* (Bloch, 1795): A

toxic response exposed to Fullerene (C_{60}) nanoparticles. *Asian Fisheries Science*, *30*, 206-214.

- **Sumi, N.,** & Chitra, K. C. (2017). Acute exposure to fullerene (C_{60}) altered antioxidant defence system in hepatocytes of the cichlid fish, *Pseudetroplus maculatus* (Bloch, 1795). *International Journal of Research*, *4*(5), 953-962.

- **Sumi, N**., & Chitra, K. C. (2017). Histopathological alterations in gill, liver and muscle tissues of the freshwater fish, *Pseudetroplus maculatus* exposed to fullerene C_{60}. *International Journal of Fisheries and Aquatic Studies*, 5(3), 604-608.

- **Sumi, N**., & Chitra, K. C. (2016). Effects of fullerene (C_{60}) on antioxidant enzyme activities and lipid peroxidation in gill of the cichlid fish, *Pseudetroplus maculatus* (Bloch, 1795). *The journal of Zoology Studies*, *3*(6), 31-37.

- **Sumi, N**., & Chitra, K. C. (2015). Consequence of fullerene nanoparticle (C_{60}) on oxygen consumption and behavioural modification in the *Etroplus maculatus*. *International Journal of Current Research and review*, *7*(14), 40-44.

1. Pollutants in the environment

Environment is composed of air, water and land, and the interaction among themselves and between the living beings like microbes, plants, animals and humans forms the ecosystems (Kalavathy, 2004). The study of environmental issues in the form of pollutants always lightens-up the present environmental scenario of earth as it is caused due to the unfavourable anthropogenic activities. Pollution is one of the major threats that cause degradation to the environment and its living system (Kazi *et al*., 2009). In every year, more than 10,000 new chemicals are introduced globally, and among which more than 90% are hazardous and toxic that originate as a result of modernization and industrialization. Environmental pollutants reaches the ecosystem either accidently or deliberately due to anthropogenic activities, which adversely affect the aquatic organisms, plants, animals and humans. The pollutants introduced can be grouped as biological, physical or chemical contaminants.

The biological contaminants includes bacteria, fungi, parasites, viruses and toxins from mushrooms, plants and seafood, vapours and gases of biological origin and various organic components dropped by animals. The outbreaks of biological contaminants result in several occupational and non-occupational diseases such as asthma, pneumonia, allergies, inhalation fever and other infectious diseases like tuberculosis, common cold and influenza (Ramachandran, 2005). Physical contaminants are the introduction of foreign objects into the environment that includes plastics, broken glass, dirt, bones, metal staples and so on, which cause serious health problems in organisms in

the form of mutilations in lips, teeth, mouth, gums, and other internal organs, which may lead to severe inflammation and trauma (Ramachandran, 2005).

Among the environmental pollutants, chemical contaminants are of major concern due to its stability and persistence in the environment. It includes chemicals discharged from pharmaceuticals, detergents, plastics, disinfectants, petroleum products, resins, road runoff, deodorants, pesticides and biocides etc. Such contaminants enter into the living organisms through water, soil and air, either from point or non-point sources. Basically, chemical contaminants are classified into two type namely organic and inorganic contaminants. The organic contaminants comprise petroleum and oil spills from concreted areas and roads, pesticides, hormones, fungicides and herbicides originating from agricultural and horticultural industries (Kolpin *et al*., 2002). Inorganic chemical contaminants such as nitrogen, potassium and phosphorus along with metals like zinc, lead, arsenic, iron, chrome, mercury, copper etc are largely released into the waterways as a result of direct discharge from industries, fertilizer runoff from agricultural lands, dairy and meat processing industries, forest processing, energy, mining and wastewater treatment plants (Singh *et al*., 2009).

1.2 Aquatic pollution

Aquatic pollution is of greater global concern than the other types of pollution because water is indispensable for the existence of life. In India, about 70% of surface water and an increasing percentage

of ground water are continuously polluted by various chemical contaminants, which made the water unsafe for consumption, irrigation and industrial purposes. Besides, it was reported that 774 million Indians lack proper toilet facilities, and approximately 14,000 children die every year due to diarrhoea since the natural water bodies are contaminated with hazardous chemicals released from domestic, industrial and other human activities (Million Death Study Collaborators, 2010; WaterAid, 2015). The major aquatic pollutants of recent concern are heavy metals, agricultural and industrial by-products, effluents from wastewater treatment plants, oil spills, xenoestrogens, plasticizers and nano-based pollutants.

Heavy metal pollution in aquatic bodies deteriorate both fauna and flora as the long half-life and non-biodegradability could result in bioaccumulation and biomagnifications, which harmfully affect the living organisms (Sawidis *et al*., 2001; Mapanda *et al*., 2005). The chemicals released from agricultural and industrial sectors make use of dissolved oxygen content in the water bodies for degradation, which lead to oxygen depletion and death of aquatic animals (Miranda *et al*., 2001). Pest controlling by-products used in agricultural fields such as pesticides, fungicides, insecticides, herbicides, rodenticieds and nematocides not only kill or control pests, but causes several adverse effects on organisms like disruption in reproductive behaviour (Beulig and Pilonieta, 2002), inhibition in the growth and death of algae and micro-invertebrates (Nystrom *et al*., 1999; Schulz and Liess, 1999).

The effluents of wastewater treatment plants is a complex mixture of suspended solids, debris, human waste and various

chemicals derived from commercial, residential and industrial sources that introduce various disease causing pathogens, nutrients, endocrine disrupting chemicals, antibiotics etc., into the aquatic ecosystem thereby affect biological organizations at various cellular, tissues, organisms, or community to higher trophic levels (Porter and Janz, 2003). Oil spills also act as aquatic pollutants, which are released from tanker ships, spills of refined petroleum products and waste oil, use of diesel and heavier fuels by large ships, offshore production, refinery operation and drilling wells and rings (Hulsey and Ludivina, 2012). Endocrine disrupting chemicals or xenoestrogens, and plasticizers in the form of microplastics are considered as the serious threat in aquatic ecosystems, as it affect reproduction and development, feminization of fish, reduced gonadal size, delayed sexual maturation, altered and lowered secondary sexual characters in fish, lowered immune system and damages thyroid organ in fish-eating birds (McMaster *et al*., 1991; Servos *et al*., 2001). Recently, nano-pollutants raised global concern owing to the increased production rate, application and discharge into the environment that pose adverse effects on environment and human health (Naghdi *et al*., 2017).

1.3 Nano-pollutants

Nano-sized particles with at least one-dimension size ranged from 1 to 100 nm, found naturally or engineered in the form of nanofilms, nanoplates, nanowires, nanotubes, nanofibres and nanoparticles, and recently gained attention as environmental pollutants (Tiwari *et al*., 2012). The natural sources of nano-pollutants include particles from organic matter, clays and iron oxides in soil and

air, respectively (Klaine *et al*., 2008). Natural nanoparticles are generally classified into manganese-oxides such as vernadite, birnessite, metal sulfides like sephalerite, pyrite and galena, iron-oxides including goethite, hematite, and heavy metal oxides like uraninite. The engineered nanoparticles are classified into five groups consisting of carbon nanomaterials, quantum dots, zero valence nanoparticles, metal oxide nanoparticles and dendrimers (Wigginton *et al*., 2007). The engineered nanomaterials possess exceptional features like large surface area and small size that enables strong adsorption capabilities, reactivities and high mobility to nano-sized materials, which is widely exploited in water and wastewater treatment process for the removal of heavy metals, organic and inorganic pollutants, bacteria, and also used for soil and ground water remediation (Khin *et al*., 2012; Yang *et al*., 2019).

There are wide ranges of nanoparticles incorporated into various consumer products to enhance its performance. Pharmaceuticals-nanoparticle combinations are easily absorbed into the body of organisms. Nano-composite materials are stronger, lighter and resistant to the chemical corrosion compared to the metals thus widely used to synthesize corrosion resistant and improved fuel efficient vehicle parts. Nano-whiskers are used in fabrics mainly in dress materials to make them stain resistant, flame resistant and water resistant without any significant increase in thickness, weight and stiffness of the fabric (Oecotextiles, 2012).

Various nanoparticles such as zinc oxide, titanium dioxide, silica, gold, silver and polymeric nanoparticles are the regular

constituents in various consumer products of day-to-day life (Gupta and Xie, 2018). In addition, several artificial unintentionally generated nanoparticles mainly from the diesel emissions, welding fumes, incinerators, discharges from wastewater treatment plants, landfill sites, accidental spillage during manufacturing, transportation and handling of nanoparticles also cause severe problems in organisms, which include altered gene expression, chromosomal aberrations, alteration in life cycles, cell proliferation activity, embryo-toxicity, and infertility (Warheit *et al*., 2007; Handy *et al*., 2008; Klaine *et al*., 2008).

1.4 Carbon-based nanomaterials

Carbon is the third most abundant element after hydrogen and oxygen having a fascinating ability to bind to itself and with other elements nearby in the periodic table, and forms the base component in deoxyribonucleic acid (Lodish *et al*., 2000). Carbon-based nanomaterials have a sole place in nanoscience and possess exceptional properties, which enable its use in various fields including electrical, chemical, thermal and mechanical, and also extends its applications in energy store and conversion, drug delivery, composite materials, sensors, nanoscale electronic components, and field emission devices (Mauter and Elimelech, 2008; Yan *et al*., 2016). The carbon-based nanomaterials are mainly produced for remarkable commercial interest due to its unique properties including high strength, electrical conductivity, versatility and electron affinity (Astefanei *et al*., 2015).

The carbon nanomaterial includes fullerenes, carbon nanotubes and graphenes having the zero-, one-, and two-dimensions, respectively (Dinadayalane and Leszczynski, 2010). Carbon nanotubes, the most widely used carbon nanomaterial is produced from graphite by arc discharge or chemical vapour deposition into single and multi-walled carbon nanotube (Saito *et al*., 1998). Nanotubes own several distinctive electrical and optical characteristics mainly due to its physical properties including surface functionalization, chirality, length and diameter (Saito *et al*., 1998). Graphene is the earliest allotropes of carbon and the world's thinnest ever known material which is used as the building block for several other carbon materials by rolling and stacking. Graphene possess similar thermal, electrical and optical properties as carbon nanotubes, however, the two-dimensional structure of graphene permits more various electronic properties (Kim *et al*., 2009). Now graphene, the pure form, is replaced by the graphene oxide, which can easily disperse in aqueous medium and its hydrophilic functional groups enables to use in drug delivery systems (Wang *et al*., 2011). Carbon nano-onions are the new phase of allotropic carbon nanomaterials which has potential applications in electromagnetic shielding, gas and energy storage, heterogeneous catalysis, solid lubrication, fuel cells, electro-optical devices, targeted drug delivery, pharmacological therapeutics in cancer cells (Bhinge, 2017).

Carbon nanofibres are the other form of carbon nanomaterials with a cylindrical nanostructure formed from the graphite sheets (Mehdipour *et al*., 2011). The excellent physical or chemical

interactions, especially in the adsorption processes, enables for various applications like hydrogen storage, sensors, functional composites, catalysts and catalytic support and fuel cell systems (Ruiz-Cornejo *et al*., 2018). Carbon quantum dots are the small sized novel class of carbon nanomaterial having a size less than 10 nm generally synthesized from the organic materials like natural polymers, amino acids, grape peel, apple juice and vegetables (Xiao *et al*., 2013; Sachdev and Gopinath, 2015). The outstanding fluorescence emission property of quantum dots are utilized in various fields including bioimaging, photocatalysis, biosensing, drug delivery, optoelectronics and photovoltaic devices (Kleinauskas *et al*., 2013; Zhang *et al*., 2013a).

Carbon black is the pure form of elemental carbon found as colloidal particles with a few nanometer of average particle size formed naturally during the incomplete combustion and thermal decomposition of liquid hydrocarbon or gases under specific conditions (Long *et al*., 2013). Carbon black forms the major component of rubber products, mainly in automotive rubber products like gaskets, hoses and coated tubes and tires. Besides, it also appear as major component in various other products including plastics, electrostatic discharge compounds, high performance coatings, dry-cell batteries, toners and printing inks (Long *et al*., 2013). Nanodiamonds, the nanocrystals of 10 nm size are composed of tetrahedrally bonded carbon atoms produced from graphite by high energy treatment. The surface functionalization property of nanodiamonds permits its uses in

tissue labelling, chemical modification for targeted gene and drug delivery; and also used for cell labelling (Lien *et al*., 2012).

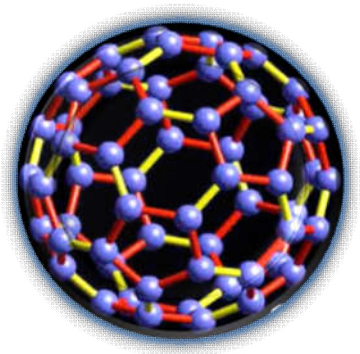
1.5 Fullerene C_{60} nanomaterial

The credit for the discovery of fullerene C_{60} nanomaterial was shared among the group of scientists namely Harold W. Kroto, Robert F. Curl Jr, Richard E. Smalley, and other colleagues in 1985 (Kroto *et al*., 1985). They were honoured as Nobel laureates in the year 1996 sharing Nobel Prize in chemistry for their big discovery. Fullerenes are the third allotrope after diamond and graphite which is purely composed only of carbon elements. The other members of fullerene family are C_{70}, C_{76}, C_{78} and C_{84}, and have wide range of applications in various fields. Fullerenes are produced by two methods namely the arc discharge and resistive heating method through graphite vaporization (Gao and Gao, 1994; Howard *et al*., 1992; Huczko *et al*., 1997). The most commonly used method in industries is arc discharge method for the high production rate of fullerenes. Resistive heating method by vaporization of the graphite rods are achieved at 100-200 A current and 10-20 V voltage between the electrodes (Gao and Gao, 1994).

Another alternative way of fullerene production is by harnessing sunlight where the renewable energy was used, and this method was adopted by a group of scientist at Rice University (Baum, 1993). Besides, there are several other methods available for the production of fullerene, which includes combustion of hydrocarbons (Howard *et al*., 1992), injection of carbon material into a plasma torch (Alexakis *et al*., 1997), microwave synthesis from chloroform (Xie *et*

al., 1999) and dissociation of mixtures of carbon sources and metallic catalyst (Cota-Sanchez *et al*., 2001).

1.5.1 Structure of C_{60} fullerene



Fullerene C_{60} nanomaterial

*(**Source**: https://mathematica.stackexchange.com)*

The structure of fullerenes resembles a geodesic spheroid shape invented by a famous architect, Buckminster Fuller, hence fullerenes was also named as 'buckminster-fullerenes'. It is a highly symmetrical molecule that enables 120 symmetrical operations such as the reflection in a plane and rotation around the axis (Taylor *et al*., 1990). It has the truncated icosahedron structure ($C_{60}H_{60}$) having a mean diameter of 7.1 Å and a van der Waals diameter of 1.1 nm. The truncated icosahedron structure is made up of 12 pentagonal faces, 60 vertices, 20 hexagonal faces and 90 edges (Yannoni *et al*., 1991). C_{60} fullerene is the smallest member in fullerene family that obey the isolated pentagon rule, which predicts that all the isolated pentagons are more stable in fullerene structure than those having adjacent

pentagons (Kroto, 1987). Thus the presence of pentagonal faces in fullerene resulted in the anisotropic electron allocation on the cage. Fullerene C_{60} molecule possess both pentagon (5:6) bonds at the length of 1.45 Å and hexagon (6:6) bonds with a length of 1.40 Å (David *et al*., 1991). The two bonds at 5:6 and 6:6 are also indicated as single and doubled bonds, respectively (Rosseinsky, 1995).

1.5.2 Properties of C_{60} fullerene

Odour	:	Odourless
Colour	:	Black
Shape	:	Needle like crystals
Solubility	:	Soluble in aromatic solvents like carbon disulfide and toluene
Molecular weight	:	720.66 g/ mol
Exact mass	:	720 g/ mol
Monoisotopic mass	:	720 g/ mol
Heavy atom count	:	60
Complexity	:	2030
Formal charge	:	0
Density	:	1.65 g cm^{-3}
Molecular density	:	1.44x1021/ cm^3
Resistivity	:	1014 ohms m^{-1}
Index of refraction	:	2.2 (600 nm)
Bulk modulus	:	14 Gpa
Crystal form	:	Hexagonal cubic

Boiling point	:	Sublimes at 800 K
Standard heat of formation	:	9.08 k cal mol^{-1}
Vapor pressure	:	5 x 10^{-6} torr at room temperature, for crystal form Hexagonal cubic: 8 x 10^{-4} torr at 800K
Standard heat of formation	:	9.08 k cal mol^{-1}
Static dielectric constant	:	4.0-4.5
Structural phase transitions	:	255 K, 90 K
Debye temperature	:	185 K
Phonon mean free path	:	50 Å
Thermal conductivity	:	0.4 W/ mK
Compressibility	:	6.9 x 10^{-12}/ cm^3/ dye
Binding energy per atom	:	7.4 eV
Electron affinity	:	2.65 eV
Thermal expansion	:	6.2 x 10^{-5} cm^3/ K
Ionization potential (1st)	:	7.58 eV
Ionization potential (2nd)	:	11.5eV
Spin-orbit	:	0.0022 eV
Band gap	:	1.7 eV
Velocity of sound vl	:	3.6 x 10^5 cm/ sec
Velocity of sound vt	:	2.1 x 10^5 cm/ sec

***(Source**: Heymann, 1996;*

http://wwc.ch.ic.ac.uk/local/projects/unwin/fullerene.html;

https://www.scsres.com/physical-properties)

1.5.3 Applications of C_{60} fullerene

The outstanding physico-chemical properties of fullerene C_{60} especially in thermal, mechanical, electrical and electronic properties coupled with the chemical properties have incited for broad range of applications in various fields (D'Souza, 2006).

In commercial products:

It is widely used in battery and fuel cell electrodes, strengthening or hardening of materials, optics, gas storage, sensors and as diamond precursors (Withers *et al*., 1997). In addition, it improves photoelectric properties, biological compatibility and act as photosensitizers (Pierrat *et al*., 2009; Zhang *et al*., 2012; Cheng *et al*., 2011).

In biomedical fields:

It shows significant affinity towards certain molecules like nucleic acids, cell receptors such as peptides, proteins and saccharides (Barron, 2016; Jennepalli *et al*., 2014; Vance *et al*., 2016).

In medicine:

Fullerene C_{60} plays an important role in medicine especially in therapeutics. It possesses antiviral activity which can inhibit the replication of human immunodeficiency virus (Friedman *et al*., 1993; Sijbesma *et al*., 1993) as well as the human cytomegalovirus replication (Kotelnikova *et al*., 2003). C_{60} fullerene is used in photodynamic therapy, and magnetic resonance imaging that enable to

target for the treatment of tumors (Mroz *et al*., 2007; Grobmyer and Krishna, 2012). Fullerenes and its derivatives are used in gene and drug delivery (Zakharian *et al*., 2005; Klumpp *et al*., 2007). Fullerene possesses antimicrobial activity which can suppress the adverse effects caused by various bacteria including *Escherichia coli*, *Candida albicans* and *Bacillus subtilis* (Da Ros *et al*., 1996; Mashino *et al*., 1999; Tsao *et al*., 2001). Carboxyfullerene, the fullerene derivative, is used for the treatment of neurodegenerative diseases and amyotrophic lateral sclerosis (Dugan *et al*., 1997).

In dermatology and cosmetics:

Fullerene C_{60} nanoparticle has significant role in dermatological and cosmetic applications (Mousavi *et al*., 2017). It protects skin against the damages caused by UV irradiation (Mousavi *et al*., 2017). It is widely used in the treatment of the chronic inflammatory skin diseases like acne vulgaris (Aoshima *et al*., 2009), as the hair growth stimulator (Zhou *et al*., 2009), and reduces facial skin pores and wrinkles (Kato *et al*., 2010; Inui *et al*., 2014).

1.6 Mode of entry into humans

The global production rate of fullerenes, mainly in nanotechnology, reaches approximately 1500 tonnes annually, and expected to increase by every year (Mitsubishi Chemical Corporation, 2001). Japan leads the world in the production of several commercial and biomedical devices where a plant was opened exclusively for fullerene nanomaterial having the total capacity of 40 tonnes per year (Aitken *et al*., 2006; Fujitani *et al*., 2008). Humans and other animals

are continuously exposed to fullerene and its derivatives from various environmental, commercial, biomedical, pharmaceutical and cosmetic products. Environmental exposure of fullerenes includes hydrocarbon flames, welding and jet engines, metals melting, automobile exhaust etc (Tiwari *et al*., 2016). Besides, occupational exposure is one of the main sources of human exposure that occurs during the large-scale commercial production, either from the synthesis process or the downstream activities. The actual exposure happens during handling of fullerenes after the production by inhalation, accidental ingestion and dermal contact (Fujitani *et al*., 2008).

Fullerenes have small aerodynamic radii of 0.1-1 μm, so that it can easily access the alveolar space and macrophages residing on the airway surface, and finally move into the adjacent bloodstream and circulating monocytes to reach gut and other vital organs, which ultimately cause adverse effects on immune and other physiological systems (Oberdorster *et al*., 2005). The possible intake also occurs through drinking water and food contaminated with fullerenes and reaches the vital organs (Hashizume *et al*., 2015). Nanomaterials released from cosmetic industry form the other major source of fullerene exposure to humans. It has been reported that human skin gets exposed to fullerene from the cosmetic products with a maximum concentration of 26 μg/ kg body weight/ day (Hansen *et al*., 2008).

1.7 Fullerene in aquatic ecosystem

The increased production and continuous use of fullerenes in various fields possibly pollute the aquatic bodies, and pose serious

threat to the aquatic organisms. Fullerenes find their way into aquatic ecosystems through various routes such as accidental spillage, rainwater run-off from the production site, and untreated effluents from wastewater treatment plants. A study reported the presence of high amount of fullerenes discharged from the effluent of wastewater treatment plant in the surface water and sediments reaching up to µg/ L concentration (Farre *et al*., 2010). In the Unites States, the occurrence of fullerenes in sediments reached to 2.5 ng/ kg (Gottschalk *et al*., 2009) whereas in Europe the yearly rise of fullerene deposition in sediments reached approximately 400 ng/ kg (Sun *et al*., 2014). In the aquatic environment, fullerene C_{60} undertakes various transformations including oxidation, photodegradation and biological degradation. The water bodies often contain several natural constituents like dissolved organic matter, which convert fullerene C_{60} into stable form, nano-C_{60} or nC_{60} aggregates (Xie *et al*., 2008). Likewise fullerene C_{60} stays in the water bodies for several days or even months with a concentration ranged up to 100 mg/ L (Deguchi *et al*., 2001; Fortner *et al*., 2005).

The reduced solubility and tendency of aggregation restricts the easy access of fullerenes into the aquatic organisms to some extent (Maynard *et al*., 2004; Brant *et al*., 2005). However, there are several reports stating the toxicity of fullerene C_{60} in various aquatic organisms. Water soluble fullerene, nC_{60} and other derivatives are known to exert toxicity in microbial population, particularly in bacteria by declining the rate of growth and aerobic respiration (Fortner *et al*., 2005). Fullerene C_{60} caused damage to the cuticle fibres, reduced pellet formation and depuration efficiency in the sediment dwelling

oligochaetes, *Lumbriculus variegatus* (Pakarinen, 2011). Several studies have reported the toxicity of fullerene C_{60} in aquatic invertebrates such as *Chironomus riparius* and *Daphnia magna* (Waissi-Leinonen *et al*., 2012; Yan *et al*., 2010). Fullerene C_{60} exposure in freshwater ecosystem caused toxicity in various fish models as juvenile largemouth bass (Oberdorster, 2004), fathead minnow (Zhu *et al*., 2006; Oberdorster *et al*., 2006), Japanese medaka (Seki *et al*., 2008), zebrafish (Usenko *et al*., 2007; Sarasamma *et al*., 2018) and *Pseudetroplus maculatus* (Sumi and Chitra, 2017a).

1.8 Methods of toxicity tests

Several regulatory agencies such as European Medicines Evaluation Agency (EMEA), Food and Drug Administration (FDA), The Organisation for Economic Co-operation and Development (OECD), Occupational Safety and Health Administration (OSHA) and The Environmental Protection Agency (EPA) provides strict guidelines for the use of chemicals and screening of toxicants using a number of *in vitro* and *in vivo* models. According to the OECD guidelines 401, 423 and 425, the use of a drug for clinical purpose was strictly prohibited without proper screening for toxicity. The preliminary toxicity screening was usually conducted in all toxicological studies, which includes subacute, acute, subchronic and chronic exposure studies. In acute toxicity testing, a single dose of the test substance was exposed to animal preferably for a period of 24 h to evaluate the immediate toxic effects. Subacute toxicity test were conducted to assess the effects of toxicants within a period of week while the repeated administration of the sublethal doses to animal for about 15 to

20 d period is subchronic tests. The chronic exposure reveals the mutagenic and carcinogenic potential of a drug, where the different doses of toxicants are exposed to the animal model for 90 d to more than a year.

There are various methods of toxicity testing which includes acute toxicity testing, dermal penetration, carcinogenicity, endocrine disruptors, ecotoxicity, genotoxicity, eye and skin irritation or corrosion test, skin sensitization, pharmacokinetics and metabolism, neurotoxicity, reproductive and developmental toxicity, organ toxicity, phototoxicity and so on. Most of the tests except the acute toxicity test are performed by giving repeated doses to animals over longer periods to assess the nature of toxicity (Eaton and Gallagher, 2010). Recently, toxicologists adopt alternative methods of toxicity testing to avoid distress, pain and death of animal models. *In vitro* cell and tissue culture, computer generated simulations like Computer Aided Drug Design (CADD) software are widely used to predict the potential biological and toxic effects of a drug or a chemical without killing the animal (Doke *et al*., 2015).

Besides, the use of lower vertebrate animal models like microorganisms, invertebrates and fish have some advantages over the higher vertebrate models due to their shorter lifecycle, faster reproduction and large number of progenies. However, both *in vivo* and *in vitro* studies are equally important for the prediction of toxicity, which was further confirmed using the computer models and various softwares (Eisenbrand *et al*., 2002). The toxicokinetic model finally

reveals the absorption, distribution, metabolism and elimination of any toxicants within the body of organism for the selected time and dosage.

1.9 Acute toxicity in ecotoxicology

The median lethal dose or LD_{50} and median lethal concentration or LC_{50} values for specific period of time, usually for 96 h, is considered as the endpoint of acute toxicity testing. The amount of test compound administered by oral or dermal exposure that kills 50% of experimental animal in specific time interval is called LD_{50} value, whereas exposure of test toxicant either through inhalation or in medium is referred as LC_{50} value. The LD_{50} value is often expressed as mg of substance per kg of body weight administered per day, and LC_{50} value is expressed as mg of substance per litre. The other terms frequently used in ecotoxicology are median effective dose or ED_{50}, median effective concentration or EC_{50}, no observed adverse effect level or NOAEL and lowest observed adverse effect level or LOAEL (Rand, 1995).

ED_{50} and EC_{50} are the effective dose and concentration of the test chemical that cause adverse effects on half of the total population in the test group. NOAEL is described as the highest exposure level of the test compound that does not cause any observable toxic effects on the target organism after the repeated dose studies for 28 or 90 d exposure, and/ or other chronic and reproductive studies. NOAEL is usually determined to assess the occupational exposure limit, acceptable range of the daily intake of a chemical and the threshold safety exposure level to humans and other organisms. LOAEL is the lowest exposure level of substance that cause adverse effects on

various functions, morphology, development, growth and lifespan of the target organism (Duffus *et al*., 2007). Acute toxicity test of waterborne chemicals are performed at organisms of various trophic levels, including invertebrates like Daphnia, and vertebrates such as rat, mice, rabbit, guinea pig and fish.

1.10 Behaviour as biomonitoring tool

Behavioural study is one of the ecological biomonitoring tools used to analyze the adjustment of test animal to the altered environmental conditions. It is an efficient method for assessing the water quality as well as general health status of the animal. In addition, behavioural study is an inexpensive method of biomonitoring when compared to the expensive physicochemical stress markers (Gerhardt, 1999; Park *et al*., 2005). The aquatic organisms like fishes and Daphnia are the common indicator organisms for assessing the water quality, because they can easily sense minute changes in the physico-chemical parameters of the environment (Ren and Wang, 2010). Behaviour of animal can be measured by monitoring the supply of electrical field strength (Ren *et al*., 2012) or by computer vision (Kato *et al*., 1996) and recording by video tracking systems (Kuklina *et al*., 2013). Recently, the rapidly developed sophisticated machine learning techniques and deep learning algorithms are widely used for evaluating the abnormal behaviours in aquatic animals.

1.11 Haematology as a diagnostic endpoint

Haematological studies have a crucial role to understand various blood characteristics related to ecological habitat, phylogenetic

position, food selection, pollutants and so on besides the usual functions such as nutrition, oxygenation, acid-base balance and metabolic waste removal. Haematology is considered as a regular diagnostic tool in fish models to study the heath status, metabolic and physiologic conditions of the organism with reference to change in the immediate environment (Rao, 2006). The blood parameters such as erythrocytes, leukocytes, haematocrit, percentage of haemoglobin content, mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV) and mean corpuscular haemoglobin concentration (MCHC) are the commonly used haematological endpoints to analyze effects of toxicants that can directly or indirectly affect the normal oxygen carrying capacity of the aquatic organisms (Wells *et al*., 2005). Moreover, any changes in these parameters are linked to the normal well-being of the organism, and may indirectly affect the survival, growth, reproduction and the population size.

1.12 Fish – the sentinel organism

Many plants and animals have been enrolled under the sentinel organism in environmental monitoring programs. Sentinel organisms are the species of plants or animals that used as an indicator of exposure to any toxicant used to assess the impact on human and the environment (Parmar *et al*., 2016). Among the animal models, fish gains prominent role in the risk assessment of aquatic environment. Recently, fish is widely used as sentinel organism in most of the aquatic ecotoxicological studies and environmental risk assessments. Fish occupy the top of food chain and are regarded as the important component in the aquatic ecosystems due to its significant role in

regulating trophic structure and stability, and as an excellent indicator of relative health of aquatic environment (Bahnasawy *et al*., 2009). Fish database provides base-line information about diseases, endocrine functioning, immune responses, and other physiological conditions after xenobiotic exposure before being proved in humans and other higher vertebrates. Thus any changes in biological, chemical and physical features of the water are reflected in health status of fish, which ultimately affect the development of organisms. However, high levels of aquatic pollution are evident from the death of fishes which is feasible for pollution monitoring in the natural environment.

1.13 *Anabas testudineus* - a toxicological model

***Anabas testudineus* (Bloch, 1792)**

The freshwater fish *Anabas testudineus* is commonly named as 'climbing perch' or 'climbing gouramy', based on the myth of climbing trees (Norman, 1975). The fish was also known in other common names as koi, kobhai and kawai. In Kerala, it was known in different places as karuppidy, kalluthi, kallemutty, karuvathy, karup, karikkanny, kallurutty, karatty, kaithakkora, porukku etc. The fish is edible and contains high amount of copper and iron, which is essential

for the haemoglobin synthesis (Sarma *et al*., 2008). *Anabas testudineus* is widely used in toxicological studies due to its easy availability, handling and acclimatization in laboratory conditions.

Anabas can extremely tolerate some unfavourable conditions like unpleasant odour from the hydrogen sulphide rich pools, sewage canals and oxygen depleted water, and hence used as bioindicator to study the rate of pollution in rivers and ponds (Pethiyagoda, 1991). Some of the toxicity endpoints such as haematology, behaviour, histopathology and marker enzymes are tested on the fish species to detect any alteration in the internal environment. The reviewed literatures have reflected the use of Anabas in ecotoxicology as a model, to imitate the environmental damage from molecular to population levels. Fish are exposed to variety of contaminants and their mixtures in the natural environment, and the biological response to the environmental contaminant can be studied using biochemical or cellular modifications. Hence, either the whole organism or a small portion of tissues or cells may be used for the quantification process. Response of fish to the toxicants are usually measured based on three criteria such as biomarkers of exposure, biomarkers of effect and biomarkers of susceptibility where *Anabas testudineus* fulfil most of the features essential for biomonitoring, and hence used as an excellent toxicological model.

1.14 Systematic position and ecology

Phylum : Chordata

Subphylum : Vertebrata

Class	:	Actinopterygii
Order	:	Perciformes
Suborder	:	Anabantoidei
Family	:	Anabantidae
Genus	:	Anabas
Species	:	*Anabas testudineus*

Distribution:

It is distributed worldwide with abundance in Southern China, Taiwan, Indochinese Peninsula, Indonesia and Philippines (Morioka *et al*., 2008; Froese and Pauly, 2018); South and South East Asia like India, Nepal, Pakistan, Bangladesh, Burma, Sri Lanka, Singapore, Thailand and China (Yakupitiyage *et al*., 1998). In India, it is found in Northern states like West Bengal, Bihar, Uttar Pradesh and Orissa, and in Southern states including Kerala, Tamilnadu, Karnataka and Andhra Pradesh (Bersa, 1997).

Habitat:

The fish inhabits mostly in lakes, canals, swamps, ponds, brooks, rivers, flooded water bodies and stagnant water ecosystems like sluggish flowing canals and the ponds in low-lying areas (Bian, 1969).

Appearance:

Anabas testudineus is olive green to dark brown on the dorsal side and pale yellow on the ventral side. Young fish possess transverse dark stripes on the hind body and tail, whereas the longitudinal stripe

runs from the eye to operculum. In juvenile fish, a large dark spot is found at the base of the caudal fin and a small spot at opercular region. Upon maturation, the stripes disappear and the black botches diminish (Yakupitiyage *et al*., 1998). During breeding season, male fish attains a reddish hue on the pectoral and ventral fins with a clear diamond shaped black spot on the caudal peduncle, whereas female have a faint reddish colour with oblong and slightly diffused black spot. The abdomen of female shows prominent bulging and the ventral distance between the two pectoral fin bases is quiet larger than the male fish, which resembles the genital papillae.

Feeding behaviour:

The adult fish is omnivorous and feeds on macrophytic aquatic vegetation, fish fry and shrimps, whereas the young fry and larvae feed on zooplankton and phytoplankton (Pethiyagoda, 1991).

Reproduction:

Anabas testudineus is a seasonal breeder and does not show sexual dimorphism except in the breeding season. They breed on the paddy fields and seasonal ponds, and unlike other anabantid species they do not build bubble nests to protect their eggs. During breeding season, the abdomen of female fish bulges due to the expansion of ovary, and extrude eggs upon gentle pressure applied on abdomen. Similarly, male also extrude white coloured milt on exerting slight pressure on the abdomen (Sakurai *et al*., 1993). The reproductive season varies due to several other factors such as temperature, photoperiod, salinity, rainfall, food supply etc (Lam, 1983; Bromage *et*

al., 2001). Female fish releases 200 to 5000 colourless eggs in natural condition, and the fertilized eggs hatch within 24 h where the fry of 2-3 mm size begins to swim freely.

1.15 Objectives of the study

Fullerene C_{60} nanomaterial possess dual properties, and the toxic effects on aquatic and terrestrial organisms remains in controversy. The present study was undertaken to assess the sublethal effects of C_{60} fullerene in the freshwater fish, *Anabas testudineus* and the following objectives were explored, which will be discussed in the respective sections of the chapters.

- To assess fullerene C_{60} nanomaterial influenced behavioural and haematological changes in the fish, *Anabas testudineus*.
- To evaluate the effects of fullerene C_{60} in the antioxidant status and histopathology of fish gonads.
- To estimate nanomaterial induced changes in the hormonal and reproductive parameters in the fish.
- To determine fullerene C_{60} persuaded genotoxic and cytotoxic effects in the fish.

The abundance of engineered nanomaterials, including all carbon-based nanomaterials, have prompted their use in various fields, and were found as particulates in the atmosphere of urban and industrial areas (Laitinen *et al*., 2014; Encinas and Gomez-de-Balugera, 2018). Occupational exposure of different forms of fullerenes to the workers in the factory has revealed that the exposure rate varies with the size of nanomaterials. It has been reported that during manufacturing and processing, fullerenes are exposed at 10,000 to 15,000 particles/ cm^3 with particle size of 10-50 nm, 7000 particles/ cm^3 for 50-100 nm, and 3000 particles/ cm^3 for 100-200 nm size in the indoor units. While, the outdoor air concentrations near the factory has been estimated as 25,000, 10,000 and 5000 particles/ cm^3 with a particle size of about 10-50 nm, 50-100 nm, and 100-200 nm, respectively (Fujitani *et al*., 2008). The National Institute for Occupational Safety and Health (NIOSH) conducted a survey of quantification of various nanomaterials including fullerenes exposure to the workers of factories and the laboratories by using a newly developed technique called nanoparticle emission assessment technique (NEAT) (Methner *et al*., 2009; Methner *et al*., 2010).

It has been estimated that the average annual increase in the sediment deposits of fullerenes in the United States was approximately 2.5 ng/ kg, whereas in Europe there was a high rise in the sediment deposits to approximately 400 ng/ kg fullerenes (Gottschalk *et al*., 2009). In recent years, the applications of fullerenes and other related carbon nanomaterials have shifted from electronics and industrial to cosmetic industries, with incorporation of fullerenes at 0.2-0.5%

concentrations into liposomes through skin care products (Lens, 2009; Kato *et al*., 2009). Thus it was anticipated that the rate of production of different types of nanomaterials increase to 25 times in global market by 2020 (Wright, 2014).

Thus the enormous production and extensive applications may eventually affect the living organisms inhabiting in the soil, water and the atmospheric air. The toxicity of fullerene C_{60} differ among the organisms, based on the mode of administration, transportation, distribution and elimination process. The toxic potential of C_{60} fullerene in biological system is one of the recent debatable topics. Water miscible fullerene C_{60} with trimethylene methane when administered intravenously to rats at 200-500 mg/ kg dose showed the distribution and persistence of nanomaterial in liver, lung, kidney, spleen, brain, and heart tissues with low elimination rate of about 5.4% after 160 h of injection (Yamago *et al*., 1995). Similarly, another study showed that the intravenous administration of ^{14}C-labelled fullerenes have been shown to rapidly cleared from the circulation and accumulated in liver tissues of rats (Bullard-Dillard *et al*., 1996). The parental administration of fullerene C_{60} has been shown to cause penetration and accumulation in various organs and tissues of mouse embryos along with severe inflammatory response, and dysfunction of vital tissues (Tsuchiya *et al*., 1996).

The acute toxicity of water-soluble polyalkyl sulfonated C_{60}, the fullerene derivative, in mice at 600 mg/ kg dose by intraperitoneal injection revealed 50% death in the exposed group (Chen *et al*., 1998). However, at 500, 750 or 1000 mg/ kg doses have been shown to

rapidly transport from the blood and accumulated in kidney, liver and spleen, causing lysosome-overload nephrosis with diffused degeneration and vacuole formation in kidney. While oral administration of 2500 mg/ kg dose of fullerenes did not elicit toxicity in rats (Chen *et al*., 1998). Similarly, some of the literatures reviewed and stated less or no toxicity of C_{60} fullerenes. In one of the studies, fullerene C_{60} administration has been known to be ingested by the phagocytes like macrophages and other non-phagocytic cells thereby leading to absence of toxicity in organisms. Oral exposure of fullerite, a combination of C_{60} and C_{70}, at a dose of 2000 mg/ kg for 14 d in rats did not show lethality; however alteration in behaviour and weight loss has been observed (Mori *et al*., 2006).

A study evaluated ecotoxicology of carbon-based engineered nanoparticles in which the acute toxicity tests have been performed on several environmentally relevant species such as two freshwater crustaceans, *Daphnia magna* and *Hyalella azteca*, and a marine harpacticoid copepod, and two fish species namely fathead minnow, *Pimephales promelas* and Japanese medaka, *Oryzias latipes*. The study reported variations in the acute toxicity of tested organisms without causing 50% mortality in the invertebrates whereas the reduction in peroxisomal lipid transport protein PMP70 has been observed in fathead minnow, but not in medaka, indicating potential changes in acyl-CoA pathways (Oberdorster *et al*., 2006).

Water-soluble fullerene at 1-100 µg/ ml concentration has been shown to cause cytotoxicity in vascular endothelial cells *in vitro* (Yamawaki and Iwai, 2006). Another *in vitro* study showed adverse effects of fullerene C_{60} on human umbilical vein endothelial cells, and fullerenol exposure showed increased expression of tissue factor

CD142, cell cycle arrest, and apoptosis (Gelderman-Fuhrmann *et al*., 2006). The fullerenes have the ability to enter into the human body through the skin, which has been shown to decrease the cell viability and pro-inflammatory response in human epidermal keratinocytes (Rouse *et al*., 2006). Likewise, pristine-form of fullerene C_{60} easily penetrated into the embryo of zebrafish through chorion and caused 50% mortality at 130 µg/ L, which has been found associated with the embryo concentration or LD_{50} of 0.079 µg/ g of embryo (Isaacson *et al*., 2007). Toxicity of carbon nanotubes has been documented in fish and amphibian larvae, *Ambystoma mexicanum* where the precipitated deposits in the form of black masses has been observed in gills, gut lumen and intestine (Smith *et al*., 2007; Mouchet *et al*., 2007). In a study, C_{60} fullerene exposed through inhalation in male Fischer 344 rats has not been detected in the blood due to the transformation in lung where the half-life of nanoparticles and microparticles were 26 and 29 d, respectively (Baker *et al*., 2008).

Intratracheally instilled C_{60} fullerene at 0.625 and 1mg/ kg doses have been shown to translocate from lung to blood circulation through alveolar capillaries, alveolar lumen and pulmonary lymph nodes in mouse by diffusion and caveolae-mediated pinocytosis mechanism (Naota *et al*., 2009). However, another study has reported the failure of translocation of fullerene C_{60} after intratracheal instillation of nanomaterial for 168 h at 1 and 5 mg/ kg doses from lungs to other organs (Gao *et al*., 2009). Acute toxicity of fullerene C_{60} has been estimated in *Daphnia magna* using immobilization and mortality endpoints, which revealed that the EC_{50} and LC_{50} as 9.344 mg/ L and 10.515 mg/ L, respectively (Zhu *et al*., 2009). Single-walled carbon nanotubes at different degrees of agglomeration has been

shown to enter the nervous system of chicken embryos at high concentrations and caused adverse effects on glial cells and neurons of central and peripheral nervous system (Belyanskaya *et al*., 2009). Inhalation and intratracheal instillation of fullerene C_{60} has been shown to retain in the epithelial cells of lungs for prolonged period, and the clearance pathway was found mainly through phagocytosis by macrophages and tracheobronchial clearance showing half-life for 15-28 d (Shinohara *et al*., 2010).

In some countries, the values of the acceptable exposure concentration of carbon-based nanomaterials such as fullerene and carbon nanotubes has been determined based on several subacute and subchronic inhalation and intratracheal instillation studies. In Japan, the acceptable exposure concentration of fullerene was proposed as 0.39 mg/ m^3. In Europe, the proposed acceptable concentration was 44.4 µg/ m^3 for acute toxicity, and 0.27 µg/ m^3 for chronic toxicity. In Japan, Europe, and the United States, the acceptable exposure concentrations of carbon nanotubes were 0.03, 0.05, and 0.007 mg/ m^3, respectively, and thus helped to understand the effects of nanomaterials on occupational health (Morimoto *et al*., 2012). Systemic administration of carbon-based nanomaterials has been shown to accelerate arteriolar thrombus formation in murine microcirculation independently of their shape without inducing leukocyte-endothelial cell interactions or leukocyte transmigration thereby exerted prothrombotic effects in microvessels (Holzer *et al*., 2014).

Exposure of C_{60} fullerene nanoparticle at 100 µg/ L concentration through feed to the fish, *Etroplus maculatus* for 96 h has been shown to alter the rate of oxygen consumption and behaviour

(Sumi and Chitra, 2015). Interactions of carbon nanotubes with pesticides contaminants, such as carbofuran, polyaromatic hydrocarbons, heavy metals, and dyes has been shown to decline the oxygen consumption and swimming capacity in the freshwater fish, *Oreochromis niloticus* thereby the carbon nanotubes functioned as a pesticide carriers affecting fish survival, metabolism and behaviour (Campos-Garcia *et al*., 2015). Fullerene C_{60} exposed at 0.1 mg/ L concentration to *Pseudetroplus maculatus* for 96 h showed prominent histopathological alterations in gill tissues as evidenced by gill epithelial upliftment, aneurysm, vacuolization, curling and loss of secondary lamellae (Sumi and Chitra, 2017a).

Rainbow trout when injected with corn oil dispersed fullerene C_{60}, single-walled carbon nanotubes, or amorphous carbon black in the caudal vein at 200 µg/ L showed the presence of black material consistent with the injected carbon treatments in kidney of fish without any changes in haematology, or ionoregulatory disturbance in blood plasma (Boyle *et al*., 2018). Aqueous unmodified fullerene C_{60} dispersions at a concentration of 5 mg/ L showed stimulating effects on the activity of electron transport chain of the natural heterotrophic bacterioplanktons in river, while it decreased the bactericidal activity of antibiotics (Mosharova *et al*., 2019). Hence the literature reviewed addressed both positive and negative impacts of carbon-based nanomaterials in various biological systems in different organisms. However, there is a gap area on the impact of carbon nanomaterial, fullerene C_{60} on behavioural and haematological changes in the fish, *Anabas testudineus*, which was evaluated and discussed in the present chapter.

3.1 Animal model

Freshwater fish, *Anabas testudineus,* weighing 11 ± 1 g and measuring 8 ± 1 cm were collected from Pulimugham hatcheries, Alappuzha district, Kerala, India. Fish were transported with least disturbances to the laboratory in well-aerated polythene bags, and acclimatized for two weeks in the laboratory conditions prior to the experiment. During acclimatization, fish were properly fed thrice a day with standard fish pellets and maintained in dechlorinated water under a photoperiod of 12 h light: 12 h dark. The health of fish was continuously monitored and unhealthy fishes were removed from the experimental tanks. Monofilament nets were used to cover the test tanks in order to avoid the escape of fish during the exposure of nanomaterial. Preliminary screening and standardization of physicochemical features of tap water such as water temperature (28 ± 2 °C), pH (6.5 to 7.5) and oxygen saturation (70 and 100%) were carried out following the standardized procedures as prescribed by American Public Health Association guidelines (APHA, 1998), which were maintained throughout the treatment period.

3.2 Chemicals

Fullerene C_{60} (purity >98%, Product Number: 483036, CAS No. 99685-96-8) was purchased from Sigma Aldrich, Germany. Drabkin reagent, Grumwald-Giemsa stain, bromocresol green, 2-oxoglutarate, DL-α-alanine and 2,4-dinitrophenyl hydrazine were obtained from Himedia Research Laboratories, Mumbai, India. Acetylthiocholine iodide and dithiobisnitrobenzoic acid (DTNB) were

obtained from Alfa Aesar, England. All other chemicals used were of analytical grade purchased from local commercial sources.

3.3 Preparation of test chemical

Fullerene C_{60} was dispersed in dimethyl sulfoxide (DMSO) by sonicating in Sonics-Vibracell VX-400 at 35 Hz for 3 h in 30 sec pulse interval using double distilled water so as to ensure an even size distribution before adding to the exposure tanks. The present study was aimed to evaluate the sublethal effects of fullerene C_{60} in a fish model and not to mimic the environmental concentration and its exposure scenario.

3.4 Characterization of nanomaterial

Characterization of fullerene C_{60} nanomaterial was done by X-ray diffraction (XRD) with Cu Kα radiation exposing at 1.54 Å wavelength, 40 kV and 30 mA current. The analysis was performed using PanAlytical X'pert-PRO MRD diffractometer system, Eindhoven, Netherlands. Further, the average crystalline size was determined by Scherrer's formula, $D = 0.94k \lambda / \beta \cos \theta$ where, D, K, λ, β and θ are the average crystal size, Scherrer co-efficient (0.94), x-ray wavelength, Bragg's angle and the full width at half maximum in radians respectively. The size and morphological nature of the fullerene C_{60} nanomaterial was then determined by using a high-resolution transmission electron microscope (JEOL-JEM-200 CX) having 0.23 nm point to point resolution, 0.14 nm lattice resolution at 2000 X-1500000 X magnification.

3.5 Acute toxicity test

Acute toxicity of fullerene C_{60} was tested according to Organization of Economic Co-operation and Development guidelines-203 (OECD, 1992). In order to find the median lethal concentration (semi-static; 96 h-LC_{50}) for 96 h duration, the acclimatized fish were transferred into nine separate tanks. Fish were not fed 24 h prior to the experiment in order to reduce the food and faeces contamination in the test solution. Fullerene C_{60} at seven different concentrations i.e., 20, 30, 40, 50, 60, 70 and 80 mg/ L were exposed to fish maintained in separate tanks, 50 L capacity, holding 10 healthy fish per tank and maintained triplicates under the same conditions. Along with the treatment groups, two controls namely vehicle (1% DMSO) group, and toxicant- and solvent-free group, were also maintained. The mortality and behavioural changes of fish from the experimental and control groups were monitored regularly at every 24 h interval up to 96 h duration. The concentration at which 50% mortality of fish represent the median lethal concentration (96 h-LC_{50}), which was further confirmed using Probit tool of regression analysis with a confident limit of 5% level (Finney, 1971).

3.6 Selection of sublethal concentrations

Based on the median lethal concentration for 96 h duration (i.e., 50 mg/ L), two sublethal concentrations such as 5 mg/ L, representing one-tenth of 96 h-LC_{50}; and 10 mg/ L, representing one-fifth of 96 h-LC_{50} were chosen as sublethal concentrations for further toxicological analysis.

3.7 Grouping of test animal

Fish were grouped in replicates as follows:

Group I	:	Negative control group (without solvent and toxicant)
Group II	:	Vehicle control group (1% DMSO)
Group IIIA	:	Fullerene C_{60} (5 mg/ L) exposed for 24, 48, 72 and 96 h.
Group IIIB	:	Fullerene C_{60} (5 mg/ L) exposed for 7, 15, 30 and 60 d.
Group IIIC	:	Fullerene C_{60} (10 mg/ L) exposed for 24, 48, 72 and 96 h.
Group IIID	:	Fullerene C_{60} (10 mg/ L) exposed for 7, 15, 30 and 60 d.

3.8 Fish behaviour

The behavioural modifications were monitored continuously for 30 min in each experimental tank at every 24 h interval up to 96 h in acute toxicity tests and short-term sublethal exposure groups. Similarly, the changes in the behavioural pattern were observed at 24 h interval for long-term sublethal exposure groups for 60 d. The observation time was changed from morning to evening in order to avoid the changes in the behaviour due to the diurnal fluctuations (Al-Kahem, 1995). The behavioural changes observed in the treatment groups were noted and compared with the control groups.

3.9 Histomorphology of intestine

After the end of the 96 h and 60 d of both sublethal treatment groups, fish were sacrificed and the intestine of fish were dissected out, cleaned with physiological saline (0.9%) and finally fixed in buffered formalin (10%) for 24 to 48 h. The tissue was then dehydrated with ascending grades of alcohol, cleared in xylene and then dipped in molten paraffin wax for an hour for the complete infiltration so as to prepare the tissue blocks. Serial sections were made by using a rotary microtome with a thickness of 4-6 micron, and the sections were double stained with haematoxylin and eosin, and finally mounted with DPX (Roberts and Smail, 2001). The slides were observed under the Carl Zeiss Axioscope-2 plus Trinocular Research Microscope and microphotographs were taken using canon shot camera fitted to the microscope.

3.10 Haematological parameters

Fish were exposed to the sublethal concentrations of fullerene C_{60} for short-term (24, 48, 72 and 96 h) and long-term (7, 15, 30 and 60 d) durations. At the end of each treatment period, fish were gently removed from the experimental tanks, and blood was collected by cardiac puncture using a heparinized syringe. Fish were not anaesthetized during the collection of blood so as to prevent from haemolysis, and the collected blood was used for the estimation of haematological parameters.

3.10.1 Erythrocyte count

Erythrocytes were enumerated in an improved Neubauer haemocytometer, using Hayem's diluting fluid according to the method as described by Rusia and Sood (1992). Briefly, 20 µl of blood was diluted to 2 ml using Hayem's diluting fluid and cells are counted under microscope at 400 X magnifications in a counting chamber within an hour. Total count was expressed as number of red blood cells/ cubic mm of blood.

3.10.2 Leukocyte count

Blood leukocytes were counted using Turk's diluting fluid by mixing at 1:20 ratio, mixed well and allowed to stand for 2 min. The cells were then counted under the microscope using an improved Neubauer haemocytometer from all 4 "W" marked corner squares (Rusia and Sood, 1992). The total number of leukocyte cells was expressed as number of white blood cells/ cubic mm of blood.

3.10.3 Haemoglobin content

Haemoglobin content in fish blood was done by the standard method as prescribed by Blaxhall and Daisley (1973). Briefly, 20 µl of blood was thoroughly mixed with Drabkin's reagent at room temperature and allowed to stand for 5 min for the full conversion of haemoglobin to cyanmethaemoglobin. The absorbance was read at 540 nm using UV-Visible Spectrophotometer and the values are expressed in g/ dl.

3.10.4 Packed cell volume

The amount of plasma and corpuscles in the fish blood was measured using the method of Hesser (1960). Briefly, the blood was taken in the heparin-coated capillary tube and centrifuged for 2 to 3 min and placed on a haematocrit to determine the volume of packed red cells. The unit of haematocrit or packed cell volume was expressed in percentage.

3.11 Biochemical parameters

After collecting the blood from both sublethal concentrations of fullerene C_{60} exposed fish for short-term (24, 48, 72 and 96 h) and long-term (7, 15, 30 and 60 d) durations. Blood was kept undisturbed and allowed to clot at room temperature for 30-60 min. Blood serum was obtained by centrifugation at 1,000 *g* for 10 min in a cold centrifuge, and used for further biochemical analysis.

3.11.1 Serum total protein

Total soluble protein concentration in the blood serum was determined by the standard protocol (Lowry *et al*., 1951). An aliquot of the test sample was mixed with alkaline copper reagent and vortexed, allowed to stand for 10 min at room temperature. Folin-Ciocalteau reagent (1 N) was added to each of the tubes, vortexed and allowed to stand for another 20 min at room temperature. The optical density was read at 650 nm on a UV-Visible Spectrophotometer. A standard calibration curve was prepared using different concentrations

of bovine serum albumin and the concentration of protein was expressed in mg/ ml.

3.11.2 Serum albumin

Serum albumin was estimated according to the method as described by Doumas *et al*. (1997). Briefly, 5 µl of sample was added to bromocresol green reagent (0.6 mM), and incubated for 10 min at room temperature, and the optical density at 620 nm was measured using UV-Visible Spectrophotometer. The concentration of serum albumin was expressed in mg/ ml.

3.11.3 Serum globulin

Serum globulin was calculated from the known value of protein and albumin concentration using the formula:

Serum globulin = Serum total protein – serum albumin

3.11.4 Serum glucose

Serum glucose was estimated by the method proposed by Trinder (1969). Briefly, the serum sample was mixed to the protein precipitate, centrifuged and the supernatant collected was mixed with the colour reagent. The reagent mixture was incubated at room temperature for 10 min and absorbance was measured at 520 nm in UV-Visible Spectrophotometer and the unit was expressed in mg/ dl.

3.11.5 Activity of serum alanine aminotransferase

Activity of serum alanine aminotransferase was measured in blood serum according to the method of Reitman and Frankel (1957). Briefly, the reaction mixture containing DL-α-alanine (0.2 M) and 2-oxoglutarate (2 mM) dissolved in phosphate buffer (0.1 M; pH 7.4) was vortexed and incubated at 37 ºC for 1 h. After incubation, 250 µl of 2,4-dinitrophenyl hydrazine was added and incubated at room temperature for 20 min. Finally, sodium hydroxide (0.4 N) was added to stop the reaction, and incubated for 10 min at room temperature. The absorbance was read at 510 nm against the blank. A standard calibration was prepared by using different concentrations of sodium pyruvate. The results were expressed as µM pyruvate formed per ml.

3.11.6 Activity of serum aspartate aminotransferase

The activity of aspartate aminotransferase was assayed by the method as described by Reitman and Frankel (1957). The reaction mixture containing aspartate (0.1 M) and 2-oxoglutarate (2 mM) dissolved in phosphate buffer (0.1 M; pH 7.4) was vortexed and incubated at 37 ºC for 1 h. After incubation, 250 µl of 2,4-dinitrophenyl hydrazine was added and incubated at room temperature for 20 min. Finally, to stop the reaction, sodium hydroxide (0.1 N) was added, and maintained in the room temperature for 10 min. The absorbance was read at 510 nm against the blank. A standard calibration was prepared by using different concentrations of sodium pyruvate and the results were expressed as µM glutamate formed per ml.

3.12 Activity of brain acetylcholinesterase

Supernatant of brain tissue homogenate (1% w/ v) from all durations of both sublethal concentrations were used to analyze the activity of acetylcholinesterase enzyme (Ellman *et al*., 1961). Briefly, the supernatant of tissue homogenate in phosphate buffer (0.1 M, pH 8.0) was dissolved in sodium bicarbonate and dithiobisnitrobenzoic acid (DTNB; 0.01 M). The enzyme activity was calculated by measuring yellow colour indicator produced from thiocholine on reaction with dithiobisnitrobenzoate ion. The absorbance was measured at 405 nm against the reagent blank and the activity of enzyme was expressed as nmol acetylthiocholine hydolysed/ min/ mg protein.

3.13 Statistical analysis

The median lethal concentration were analysed by Probit-regression analysis with 95% confidence limit, and then the correlation between the mortality versus concentration were plotted for the best fit line.

The haematological and biochemical data were analyzed using statistical package SPSS 21.0 using one-way analyses of variance (ANOVA). The differences between the mean of control versus treatment groups were determined using Duncan's multiple range as the post-hoc test. The level of significance ($P<0.05$) was represented in asterisks against the control groups. Data were expressed as mean ± standard deviation (SD) for 10 fish in each group.

4.1 Characterization of fullerene C_{60}

Characterization was performed by X-ray diffraction (XRD) and high resolution-transmission electron microscope (HR-TEM) which clearly showed that the nanomaterial is pure and free from other impurities. The XRD analysis showed the face-centered cubic (FCC) system of fullerene C_{60} with distinct reflections having three peaks approximately at 10^{o}, 17^{o} and 20^{o} angles that are equivalent to 111, 220 and 311 crystal planes of FCC respectively (Fig. 1). Hence, the XRD result from this study was same as to the standard cards (JCPDS File No.44-0558) of fullerene C_{60}. Further the size of fullerene C_{60} was analyzed using Scherrer's formula, which calculated the full width at half maximum (FWHM) of the Bragg's angle, where the average particle size was calculated as 34.95 nm. Thus the size and morphological structure of fullerene C_{60} was analyzed by HR-TEM (Fig. 2). From the analysis, the selected area electron diffraction (SAED) pattern showed the crystalline nature of fullerene C_{60}, while the HR-TEM analysis confirmed the morphology of fullerene C_{60} with an average size ranged from 30-60 nm and was in agreement with the XRD result (Fig. 2).

4.2 Acute toxicity of fullerene C_{60}

In *Anabas testudineus*, the acute toxicity was assessed by evaluating the median lethal concentration (LC_{50}-96 h). During the median lethal concentration analysis, there was no mortality observed in the control and vehicle control groups. Fish exposed to C_{60} at 20 mg/ L concentration for 96 h did not show mortality, while the concentrations such as 30, 40, 50 and 60 mg/ L caused 10, 40, 50 and 60% mortality, respectively. The highest mortality rate of 80% was

observed at 70 and 80 mg/ L concentrations of fullerene C_{60} (Table 1). Further, the graph plotted against the concentration and mortality using MS-Excel revealed high degree of positive correlation, i.e., r = +0.978 (Fig. 3). The median lethal concentration, LC_{50}-96 h, of fullerene C_{60} further confirmed by Probit analysis was 50 mg/ L concentration in the freshwater fish, *Anabas testudineus* (Table 2). Based on the value obtained, two sublethal concentrations such as one-tenth of 96 h-LC_{50} (5 mg/ L) and one-fifth of 96 h-LC_{50} (10 mg/ L) were selected to evaluate the sublethal toxicity of fullerene C_{60}.

4.3 Behavioural modification

In both acute toxicity test and sublethal exposure groups, fullerene C_{60} showed prominent behavioural alterations in fish when compared to the respective control groups. During acute toxicity test, immediately after the fullerene exposure fish showed alteration in schooling behaviour, increased surfacing and air engulping. After 24 h, fish was vigorous in swimming, knocked each other and on the walls of treatment tank, loss of equilibrium, and gradually reduced the swimming movements to become lethargic. Some morphological changes like slight haemorrhage and increased mucous secretion were observed on the body surface prior to mortality. The fish death was confirmed by the absence of opercular movements and lack of response towards the gentle prodding with a glass rod.

Behavioural modifications were more prominent during the long-term period of sublethal exposure groups, which include bottom settlement of fish followed by frequent surfacing to engulp atmospheric air. The morphological changes such as slight haemorrhage, increased mucous secretion and descaling were also seen

(Fig. 4). It was interesting to note the accumulation of fullerene C_{60} nanomaterial in the intestinal lumen as black aggregates while exposing the viscera (Fig. 4). However, no lethality was observed during the entire time period of sublethal fullerene C_{60} exposure. Intestinal abnormalities were further assessed by histological analysis, and the altered behavioural pattern was correlated by analyzing the activity of acetylcholinesterase enzyme in brain tissue.

4.4 Activity of brain acetylcholinesterase enzyme

Short-term sublethal exposure of fullerene C_{60} at 5 and 10 mg/ L concentrations showed significant decrease ($P<0.05$) in the enzymatic activity of brain acetylcholinesterase only after 96 h (Fig. 5). However, in long-term exposure groups at both sublethal concentrations there was a significant ($P<0.05$) decrease in acetylcholinesterase activity in all durations, which was duration- and, dose-dependent (Fig. 5).

4.5 Histopathology of intestine

Histology of intestinal tissues obtained from control and vehicle control groups showed normal architecture having compact serosa, lamina propria, muscularis and mucosal layer (Fig. 6). The basal lining of mucosal layer was noted having tall columnar epithelial cells with centrally located nuclei, from which several villi are emerged in to the intestinal lumen. The mucous secreting cells called the goblet cells were less number which was in continuity with the lumen of fish intestine (Fig. 6). The sublethal exposures of fullerene C_{60} after 96 h and 60 d showed prominent alterations like degeneration in the lamina propria and columnar epithelial cells with increased

number of mucous secreting goblet cells (Figs. 7 and 8). The severity of degeneration in intestine found increased with the dose and duration of fullerene exposure.

4.6 Haematological alterations

The short-term exposure of fullerene C_{60} at 5 mg/ L concentration did not show significant changes in RBC count, haemoglobin concentration and the hematocrit value, whereas 10 mg/ L concentration for 96 h showed significant ($P<0.05$) reduction in all parameters (Figs. 9, 11 and 12). Long-term exposure of fullerene C_{60} at both sublethal concentrations showed significant ($P<0.05$) reduction in RBC count, haemoglobin concentration and the hematocrit value (Figs. 9, 11 and 12). Leukocyte count in the fish significantly ($P<0.05$) reduced after 96 h for 5 mg/ L concentration, while at 10 mg/ L concentration showed significant ($P<0.05$) reduction after 72 h onwards (Fig. 10).

4.7 Changes in serum biochemistry

The levels of serum total protein, albumin and globulin remained unchanged after exposure of fullerene C_{60} at 5 mg/ L concentration (Figs. 13-15). However, at 10 mg/ L concentration for 96 h there was a significant ($P<0.05$) reduction in serum total protein and serum albumin while serum globulin increased significantly ($P<0.05$) (Figs. 13-15). In the long-term exposure groups, the levels of serum total protein and albumin showed significant ($P<0.05$) reduction after 7 d at both sublethal concentrations (Figs. 13 and 14). However, fullerene C_{60} exposure at 5 mg/ L concentration showed a significant ($P<0.05$) increase in the serum globulin after 7 and 15 d with

subsequent decline after 30 and 60 d, whereas at 10 mg/ L concentration there was a time-dependent significant ($P<0.05$) reduction in serum globulin level (Fig. 15). Serum glucose increased significantly ($P<0.05$) after 96 h in low sublethal concentration group and after 72 h in high sublethal concentration group (Fig. 16). Similarly, the level of serum glucose was found to increase significantly ($P<0.05$) in time-dependent manner at both sublethal concentrations in long-term exposure groups (Fig. 16). There was a time-dependent significant ($P<0.05$) increase in the activities of alanine and aspartate aminotransferase enzymes in long-term exposure groups without any significant changes in the short-term exposure groups (Figs. 17 and 18).

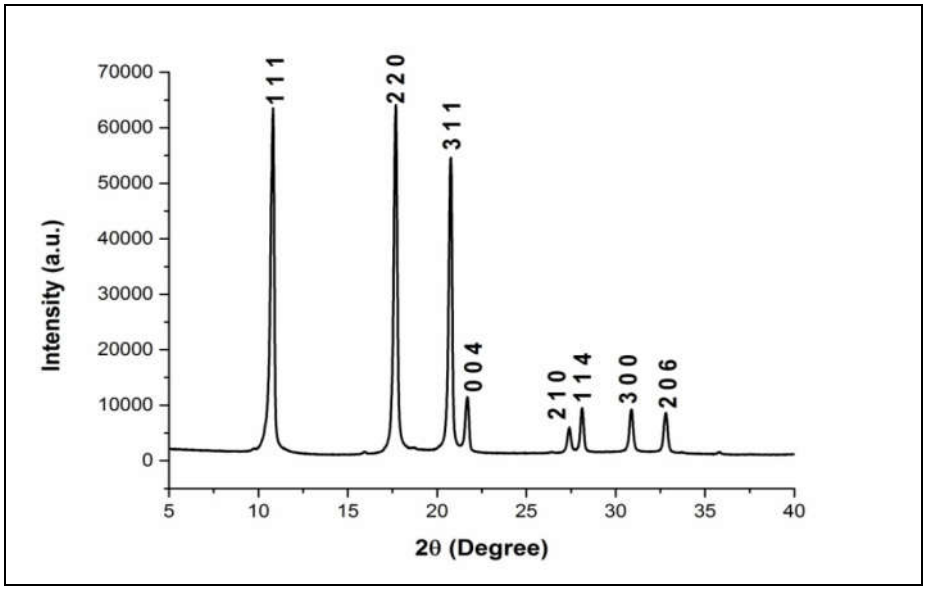


Fig. 1 XRD (PanAlytical X'pert-PRO MRD) image showing structural and crystalline nature of the powdered sample of fullerene C_{60} nanomaterials with particle size of 34.95 nm

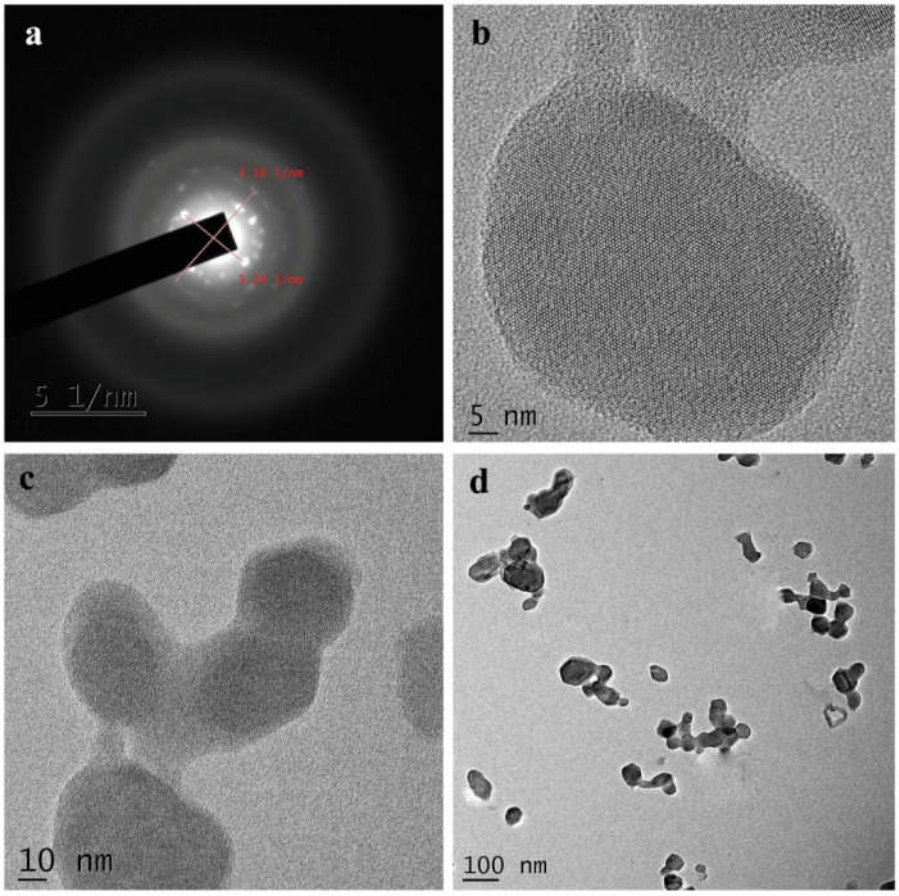


Fig. 2 a- SAED pattern of fullerene C_{60} nanomaterial; b-d-TEM image showing the morphology of fullerene C_{60} aggregates at various nm

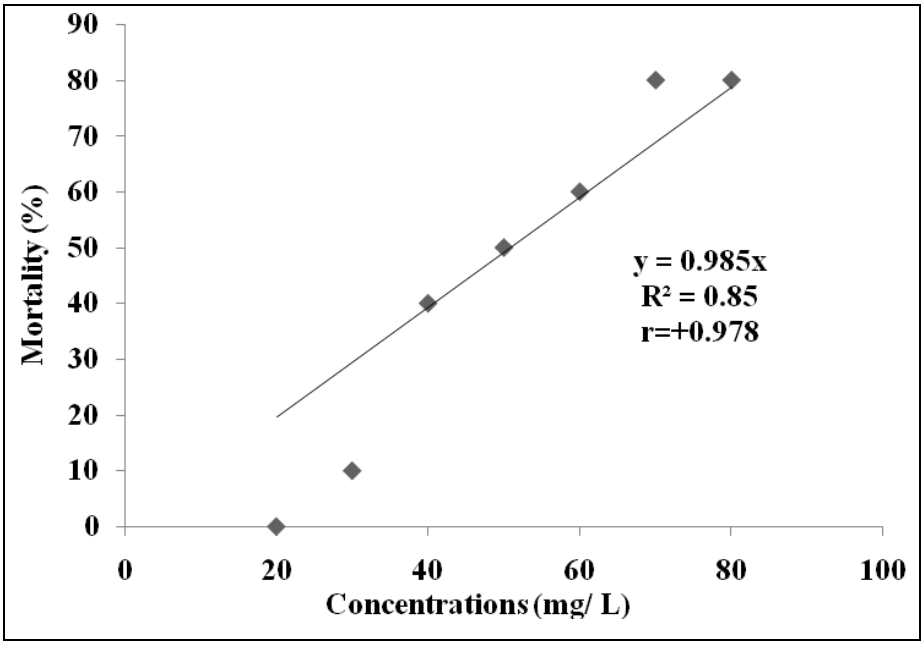


Fig. 3 Median lethal concentration or LC_{50}-96 h of fullerene C_{60} in the freshwater fish, *Anabas testudineus* (n = 10/ group)

Concentration (mg/L)	Mortality (Number of animals)	Mortality (%)	Hour of mortality
Control	0	0	96 h
Solvent control	0	0	96 h
20	0	0	96 h
30	1	10	96 h
40	4	40	96 h
50	5	50	96 h
60	6	60	96 h
70	8	80	96 h
80	8	80	72 h

Table 1 Effect of fullerene C_{60} on the mortality rate of the fish, *Anabas testudineus* (n=10 in replicates)

Probit	Estimate	95 % confidence limits for concentration	
		Upper	Lower
.010	17.723	7.312	25.222
.020	20.045	9.110	27.544
.030	21.673	10.469	29.139
.040	22.984	11.621	30.408
.050	24.109	12.647	31.488
.060	25.110	13.590	32.443
.070	26.022	14.472	33.309
.080	26.866	15.308	34.109
.090	27.658	16.108	34.857
.100	28.407	16.879	35.565
.150	31.731	20.454	38.712
.200	34.649	23.768	41.513
.250	37.365	26.966	44.193
.300	39.985	30.112	46.888
.350	42.577	33.236	49.706
.400	45.191	36.349	52.756
.450	47.873	39.445	56.157
.500	**50.668**	**42.517**	**60.046**
.550	53.626	45.563	64.576
.600	56.808	48.602	69.932
.650	60.296	51.676	76.344
.700	64.204	54.862	84.143
.750	68.706	58.275	93.850
.800	74.092	62.095	106.377
.850	80.904	66.637	123.527
.900	90.373	72.575	149.600
.910	92.821	74.057	156.747
.920	95.557	75.690	164.921
.930	98.657	77.515	174.430
.940	102.239	79.592	185.730
.950	106.483	82.014	199.551
.960	111.695	84.934	217.159
.970	118.454	88.642	241.015
.980	128.075	93.786	276.942
.990	144.850	102.435	344.988

Table 2 Effect of fullerene C_{60} on the median lethal concentration for 96 h (LC_{50}-96 h) in the fish, *Anabas testudineus* by Probit analysis (n=10 in replicates)

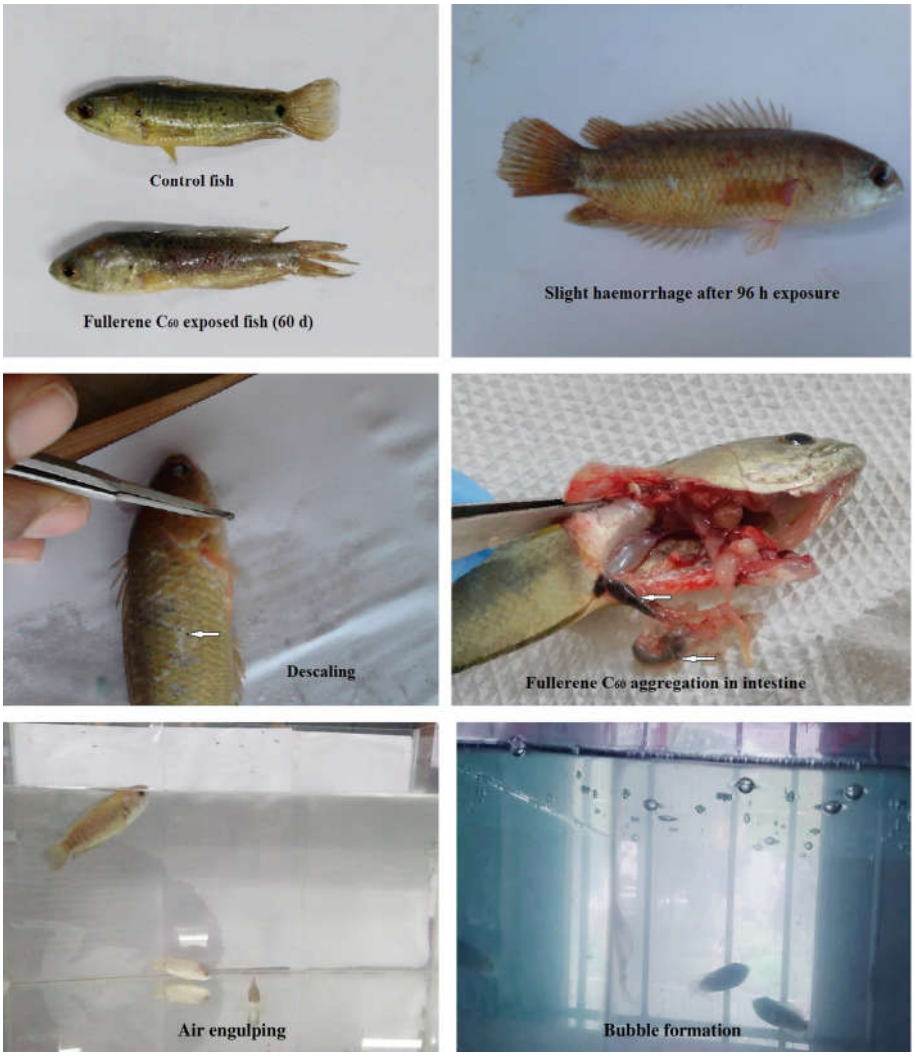


Fig. 4 Fullerene C_{60}-induced behavioural modifications in *Anabas testudineus*

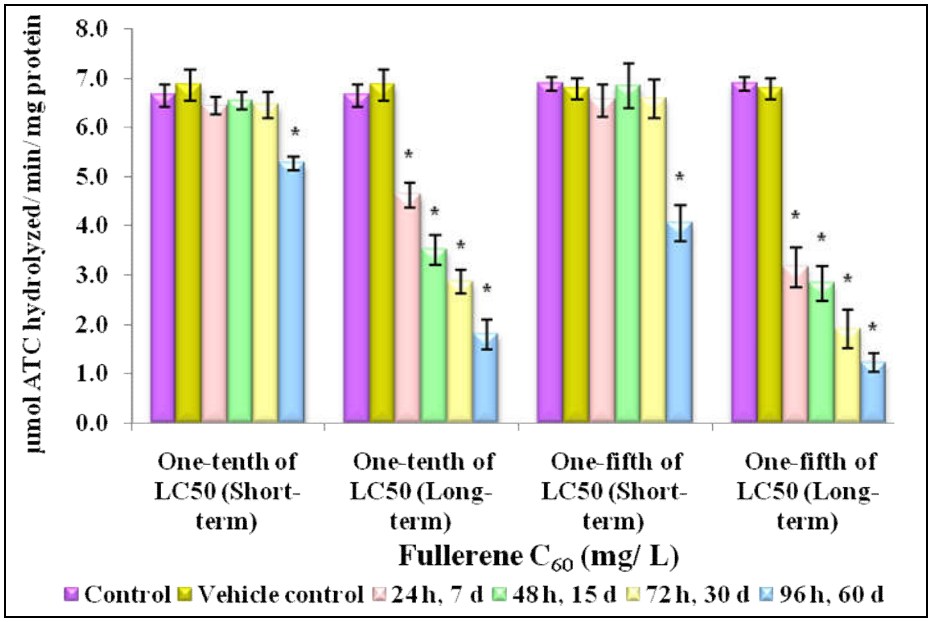


Fig. 5 Effect of fullerene C_{60} on the activity of acetylcholinesterase enzyme in brain of the fish, *Anabas testudineus* (Mean ± SD; n = 10/ group; *P<0.05 against the control groups)

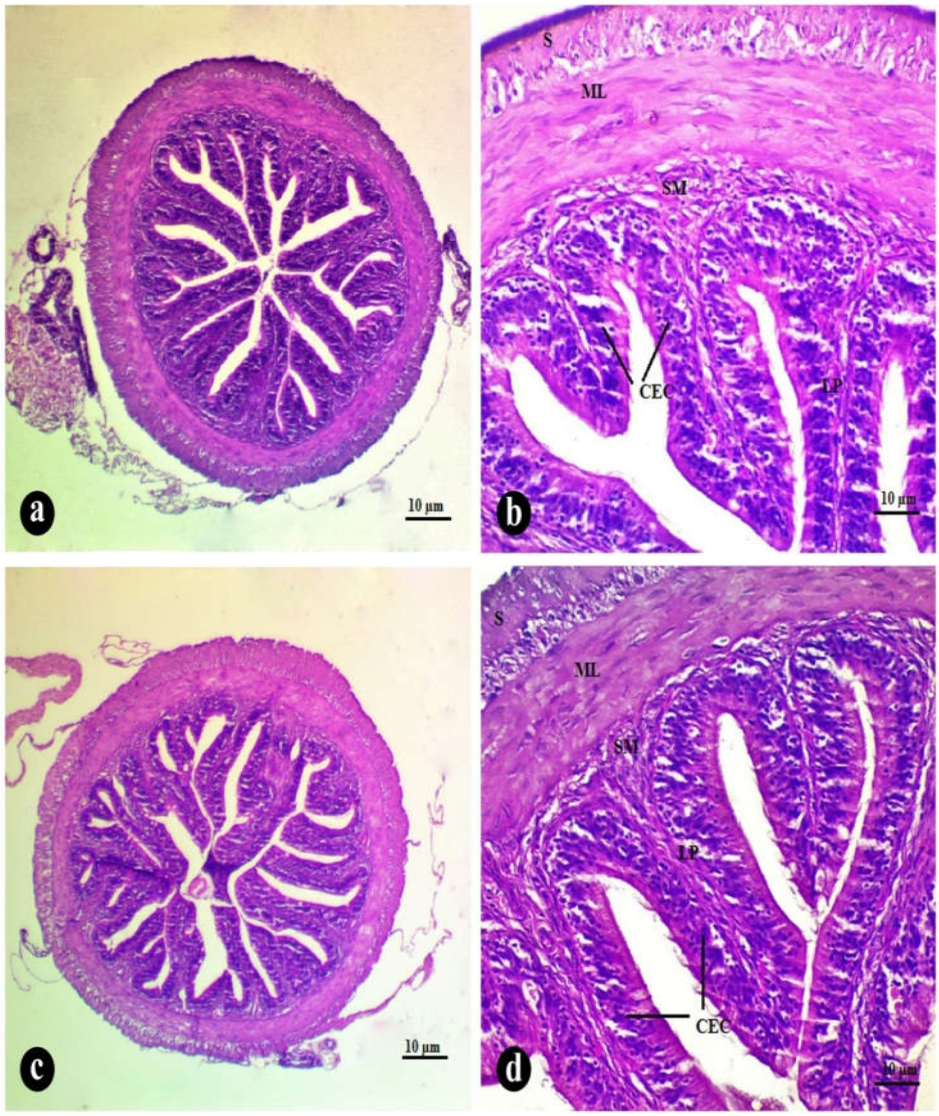


Fig. 6 Photomicrographs of the intestine of *Anabas testudineus* (a-d) (H&E). [a-b] control; [c-d] vehicle control (1% DMSO) showing normal histoarchitecture with serosa (S), muscularis (ML), submucosa (SM), mucosa with lamina propria (LP), columnar epithelial cells (CEC) and intestinal villi

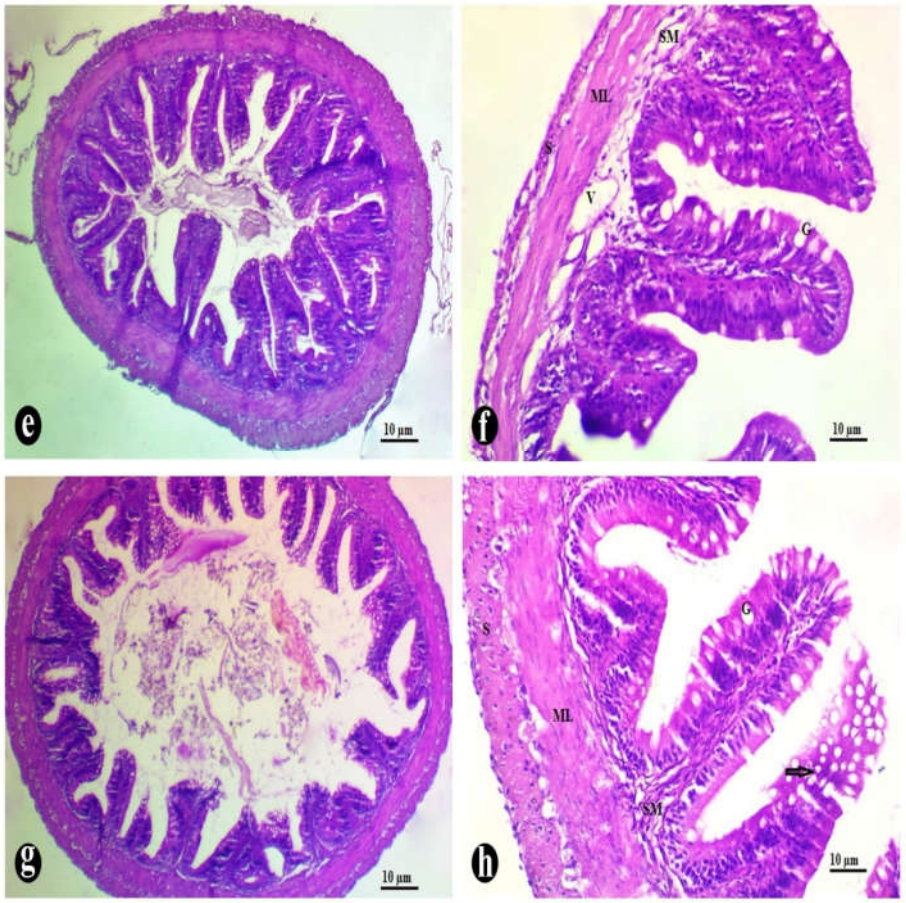


Fig. 7 Photomicrographs of the intestine of *Anabas testudineus* exposed to fullerene C_{60} (e-h) (H&E). [e-f] 5 mg/ L-C_{60} fullerene exposed group for 96 h; [g-h] 10 mg/ L-C_{60} fullerene exposed group for 96 h. The treatment groups showing shortened and fused intestinal villi with disintegration of the submucosa (SM), serosa (S), muscularis (ML), columnar epithelial cells (→), increased number of gastric glands (G) and vacuole formation (V).

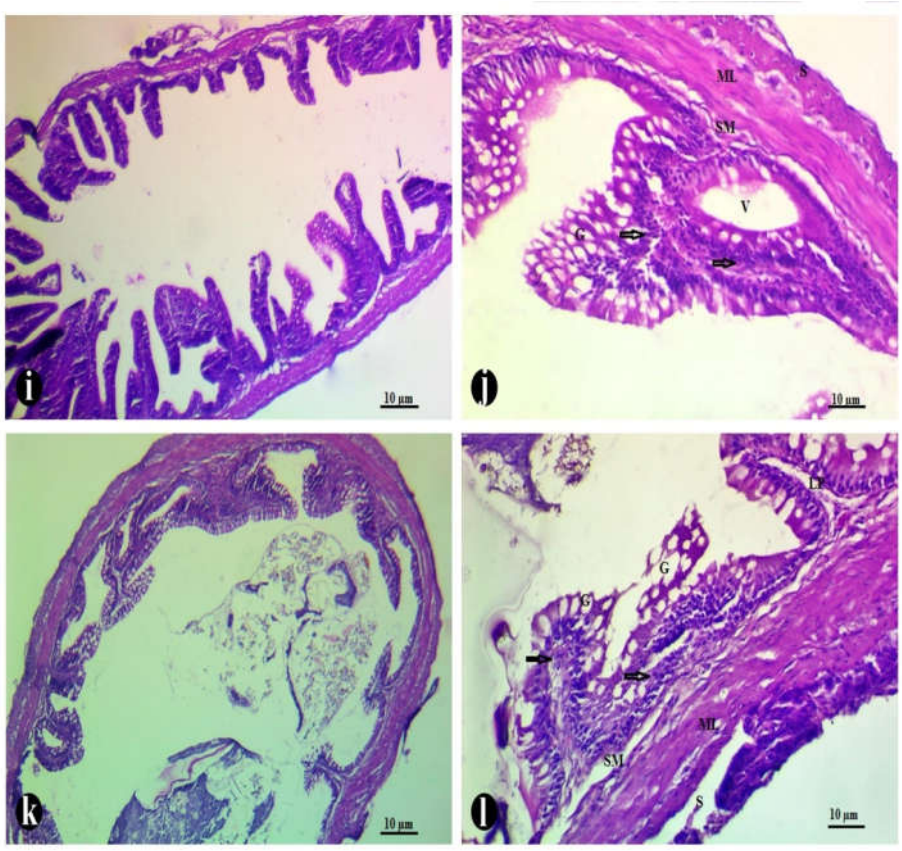


Fig. 8 Photomicrographs of the intestine of *Anabas testudineus* exposed to fullerene C_{60} (i-l) (H&E). [i-j] 5mg/ L-C_{60} fullerene exposed group for 60 d; [k-l] 10 mg/ L-C_{60} fullerene exposed group for 60 d. The treatment groups showing shortened and fused intestinal villi with disintegration of the submucosa (SM), lamina propria (LP) and columnar epithelial cells (→), increased number of gastric glands (G) and vacuole formation (V).

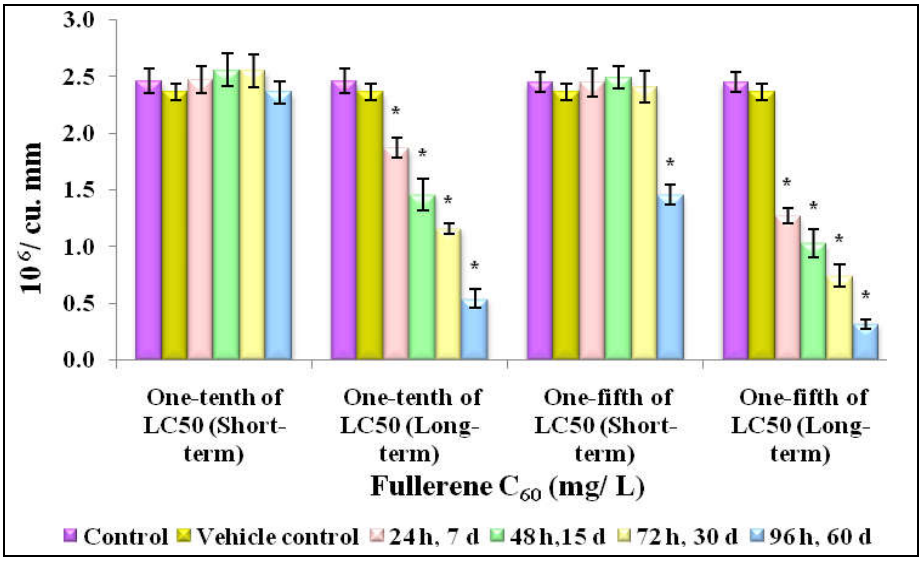


Fig. 9 Effect of fullerene C_{60} on erythrocyte count in the blood of the fish, *Anabas testudineus* (Mean ± SD; n = 10/ group; *P<0.05 against the control groups)

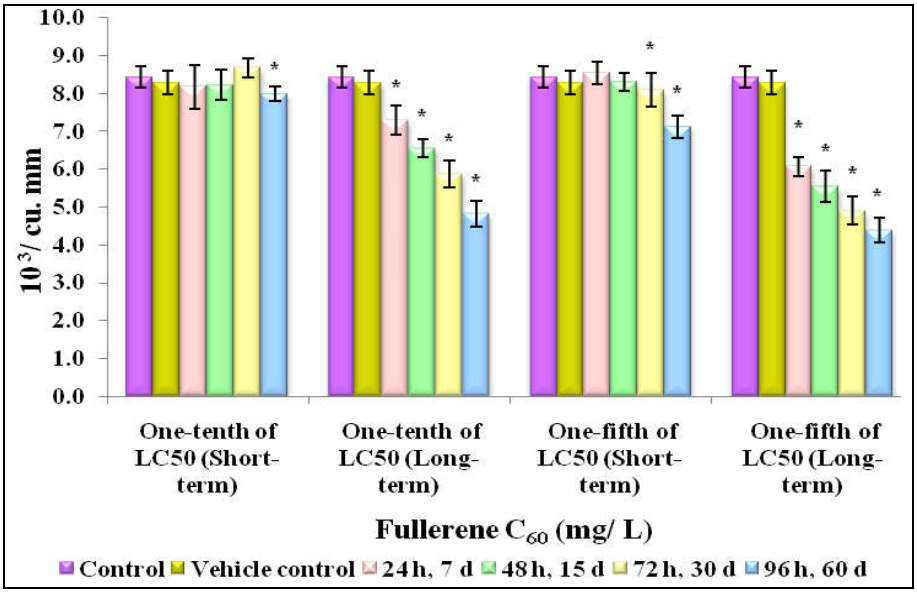


Fig. 10 Effect of fullerene C_{60} on leukocyte count in the blood of the fish, *Anabas testudineus* (Mean ± SD; n = 10/ group; *P<0.05 against the control groups)

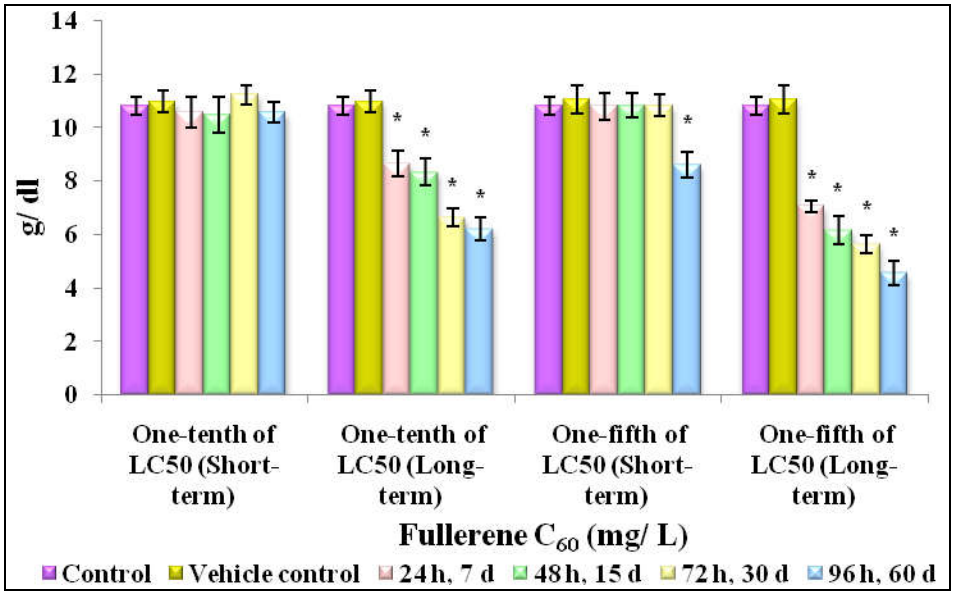


Fig. 11 Effect of fullerene C_{60} on haemoglobin content in the blood of the fish, *Anabas testudineus* (Mean ± SD; n = 10/ group; *P<0.05 against the control groups)

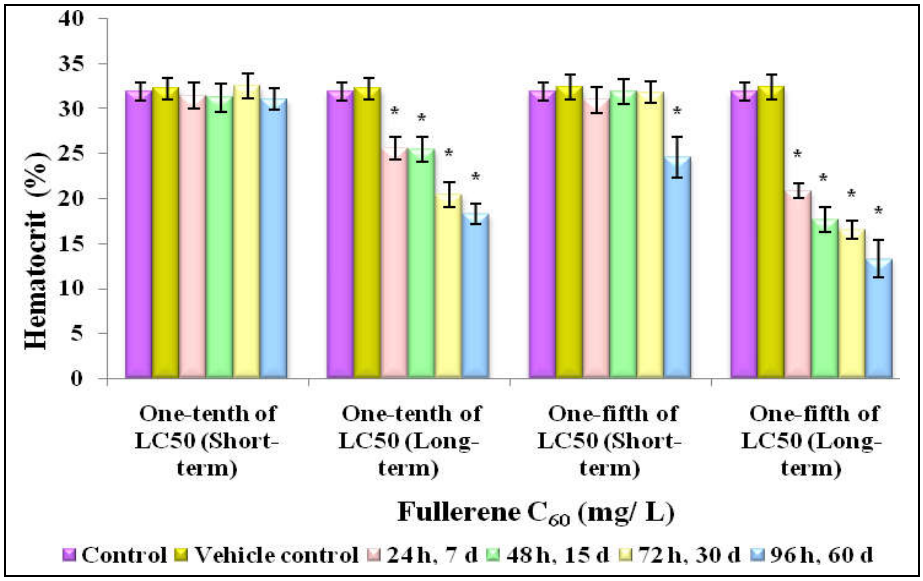


Fig. 12 Effect of fullerene C_{60} on the packed cell volume in the blood of the fish, *Anabas testudineus* (Mean ± SD; n = 10/ group; *P<0.05 against the control groups)

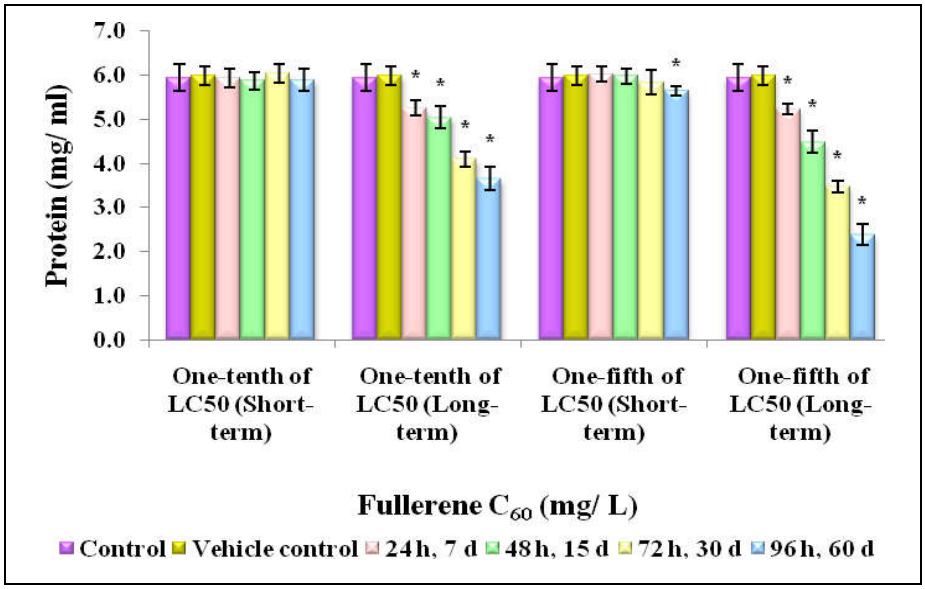


Fig. 13 Effect of fullerene C_{60} on the total protein in serum of the fish, *Anabas testudineus* (Mean ± SD; n = 10/ group; *P<0.05 against the control groups)

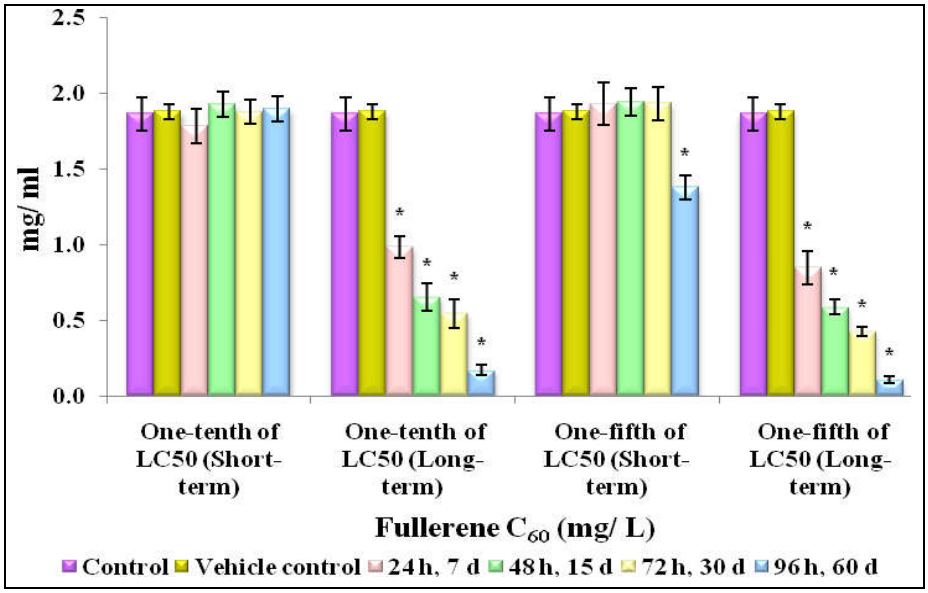


Fig. 14 Effect of fullerene C_{60} on the level of serum albumin in the fish, *Anabas testudineus* (Mean ± SD; n = 10/ group; *P<0.05 against the control groups)

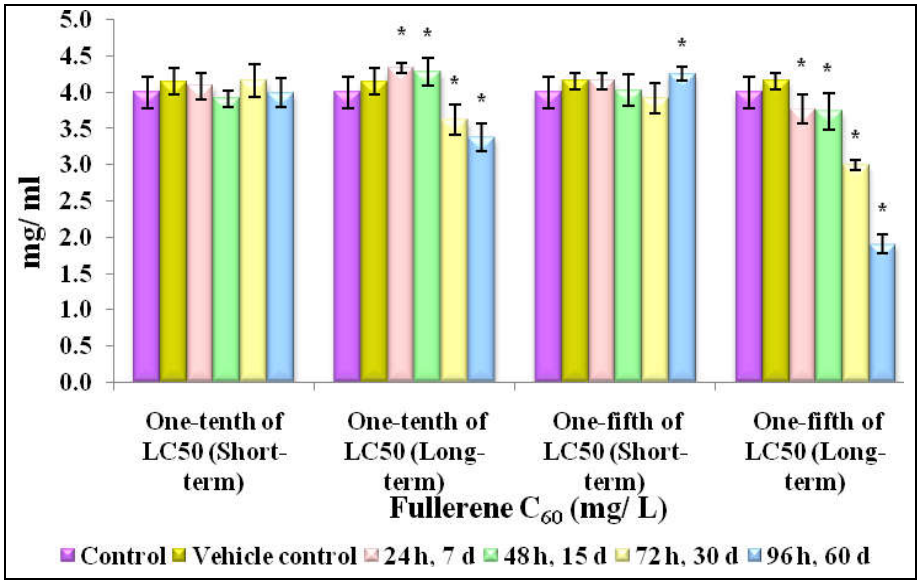


Fig. 15 Effect of fullerene C_{60} on the level of serum globulin in the fish, *Anabas testudineus* (Mean ± SD; n = 10/ group; *P<0.05 against the control groups)

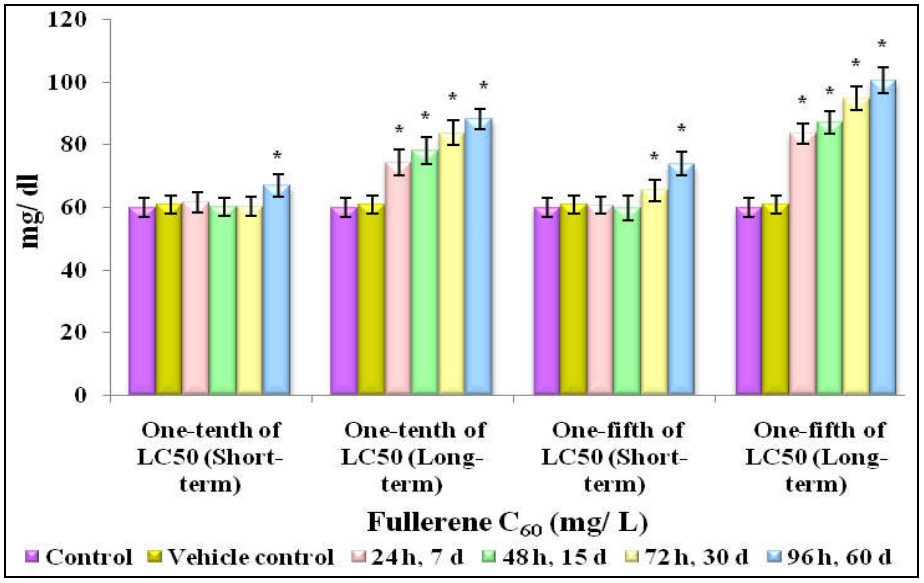


Fig. 16 Effect of fullerene C_{60} on the level of serum glucose in the fish, *Anabas testudineus* (Mean ± SD; n = 10/ group; *P<0.05 against the control groups)

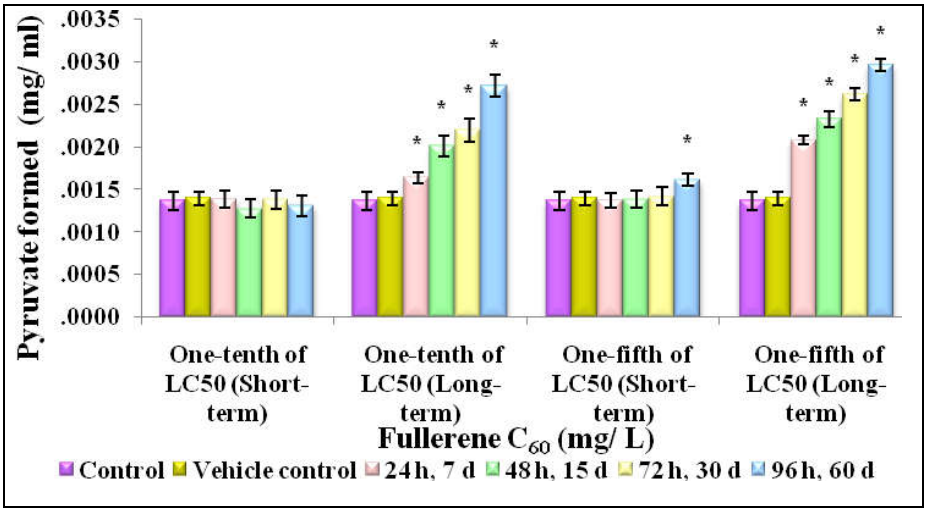


Fig. 17 Effect of fullerene C_{60} on the activity of alanine aminotransferase in serum of the fish, *Anabas testudineus* (Mean ± SD; n = 10/ group; *P<0.05 against the control groups)

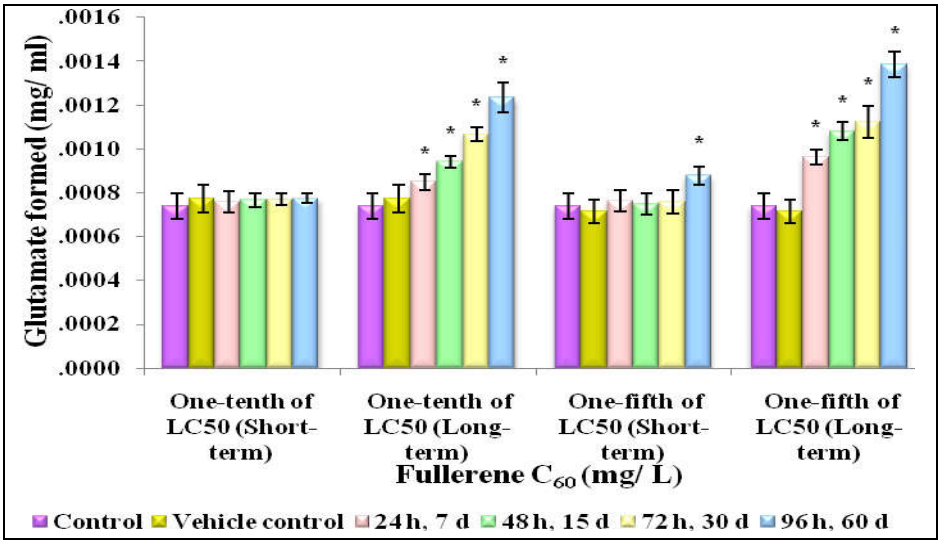


Fig. 18 Effect of fullerene C_{60} on the activity of aspartate aminotransferase in serum of the fish, *Anabas testudineus* (Mean ± SD; n = 10/ group; *P<0.05 against the control groups)

5.1 Characterization of C_{60} fullerene nanomaterial

Nanomaterials are often characterized by its size, surface charge and morphology using the advanced microscopic techniques like atomic force microscopy (AFM), transmission electron microscopy (TEM), scanning electron microscopy (SEM) and X-ray diffraction (XRD). The distribution and physical stability of nanomaterials *in vivo* are affected by various features like size, charge and average particle diameter (Pal *et al*., 2011). The characterization of fullerene C_{60} performed using high resolution-transmission electron microscope (HR-TEM) and X-ray diffraction analysis revealed high purity of nanomaterial, which coincided to the manufacturers' label. The morphology analysed using HR-TEM showed crystalline structure with average particle size of 30-60 nm, while the XRD analysis best fitted to 34.95 nm size.

5.2 Acute toxicity of fullerene C_{60}

Acute toxicity or median lethal concentration (96 h-LC_{50}) of fullerene C_{60} evaluated by Probit analysis was 50 mg/ L. At first, fullerene C_{60} was dispersed in 100% DMSO by sonication so as to obtain 1% DMSO-C_{60} fullerene suspension, which was introduced into the test medium for the toxicity testing in the freshwater fish, *Anabas testudineus*. The method implemented in this study was adopted from another toxicity finding of fullerene C_{60} in the embryo of zebrafish (Usenko *et al*., 2007). Among various organic solvents, DMSO has the ability to penetrate the biological membranes, which enables for the co-transportation of nanomaterial across the membranes (Nortman *et*

al., 2006; Usenko *et al*., 2007). Besides, various studies have evidenced that DMSO exposure did not elicit toxicity in fishes (Asifa and Chitra, 2017; Sumi and Chitra, 2017b; Usenko *et al*., 2007). There are several methods for making insoluble C_{60} fullerene into soluble suspensions that includes mixing of fullerene powder directly with water (Labille *et al*., 2006; Oberdorster *et al*., 2006); dissolving in suitable organic solvents as tetrahydrofuran (THF) (Deguchi *et al*., 2001; Lovern *et al*., 2007), and toluene (Andrievsky *et al*., 1995).

In Daphnia, THF-C_{60} suspension and sonicated-C_{60} suspension showed acute toxicity for 48h-LC_{50} at 460 ppb and 7.9 ppm concentrations, respectively (Lovern and Klaper, 2006). In adult male fathead minnow, exposure of THF-nC_{60} at 0.5 ppm concentration showed 100% mortality within 6-18 h durations. While the same concentration of water stirred-C_{60} exposed for 48 h did not show significant toxicity in the fish (Zhu *et al*., 2006). Another method used for making stable C_{60} suspensions was by dissolving in surfactants or polymers, which act as a shield on the hydrophobic surface of nanomaterial (Yamakoshi *et al*., 1994; Murthy *et al*., 2002). Surface modification also solubilize fullerene by making bond with water solubilizing carboxyl or alcohol groups so as to increase water solubility (Sayes *et al*., 2004).

However, the most common method used in several literatures reviewed was dissolving in DMSO-nano-C_{60} by vigorous stirring or sonication. In zebrafish, acute toxicity of DMSO-C_{60} sonicated suspension was at 200 ppb whereas 100% mortality has been reported above 200 ppb within 48 h of exposure. However, water-soluble

fullerene showed acute toxicity only at 4000 ppb concentration (Usenko *et al*., 2007). The acute toxicity or 96 h-LC_{50} value of DMSO-C_{60} fullerene suspension evaluated by Probit analysis in the present study was 50 mg/ L in the fish, *Anabas testudineus*, the comparatively high value could be due to the resistance of fish species.

5.3 Effect of fullerene C_{60} on behavioural modification in the fish

Behavioural modification is one of the protective mechanisms of aquatic organisms to escape from the unfavourable conditions in the environment (Banaee *et al*., 2008; Al-Ghanim *et al*., 2008). In the present study, alterations in behaviour of the fish, *Anabas testudineus* were noticed after acute C_{60} exposure, which includes disruption in schooling and swimming behaviour, descaling, increased surfacing and mucous secretion. The disrupted schooling behavior noticed immediately after the introduction of fullerene could be the defensive mechanism of the fish to escape from the unpleasant environment (Murthy, 1987). Fullerene C_{60} exposure increased swimming capacity in the fish that lead to increased surfacing to engulp air along with thick mucous deposition throughout the body to prevent the entry of toxicant (Scott and Sloman, 2004; Weis, 2014).

Long-term exposure of fullerene C_{60} resulted in intermittent surfacing where the fish remained in the bottom of tanks over a long period as a result of hypoxia caused due to the toxicant exposure. Other modifications such as descaling, haemorrhage and mucous secretion was observed in the study. Similar observations have been

reported after intraperitoneal injection of hydroxylated fullerene at 20 ppm concentration in fathead minnow, *Pimephales promelas* (Jovanovic *et al*., 2014). Hypoactivity and impairment of circadian rhythm along with less aggressive or anxiety behaviour has been observed after exposure to C_{60} fullerene at 1 and 2 mg/ L concentration in adult zebrafish (Sarasamma *et al*., 2018). Although mucous deposition is considered as a protective mechanism to avoid the entry of fullerene into the body of fish, the toxicity of nanomaterial has been reported in various vital tissues of several organisms. Thus fullerene C_{60} possess the ability to penetrate the skin or muscle as evidenced by the induction of oxidative stress in muscle tissue of the freshwater fish, *Pseudetroplus maculatus* (Sumi and Chitra, 2017c).

5.3.1 Effect of fullerene C_{60} on the activity of acetylcholinesterase enzyme in brain tissue

The changes in swimming behaviour is directly associated with the enzymatic activity of acetylcholinesterase (AChE) enzyme in the brain tissue. Thus the study evaluated the activity of enzyme in brain tissue, which showed significant reduction at both sublethal groups of fullerene C_{60}. AChE is the key enzyme responsible for neurotransmission in brain and muscle tissues (Yang *et al*., 2008). Inhibition of AChE activity by toxicant exposure has been shown to activate the post-synaptic acetylcholine receptors thereby causing hyper-stimulation and other physiological abnormalities including behavioural alterations, respiratory failure and death (Worek *et al*., 2002). Thus the present findings indicated that fullerene C_{60} functions as acetylcholinesterase inhibitor, which could ultimately resulted in the

loss of equilibrium and abnormal behaviour in the test animal. The present results were in agreement with other findings on exposure to carbon-based nanomaterials including carbon nanotubes (Ibrahim *et al*., 2013), and nano and microparticles of fullerene C_{60} in zebrafish (Dal Forno *et al*., 2013; Sarasamma *et al*., 2018).

5.4 Effect of fullerene C_{60} on histopathology of intestine

Sublethal exposure of fullerene C_{60} nanomaterial caused deposition of black aggregates in the intestinal lumen of fish on exposing the viscera, which prompted for the histological analysis. Cellular or organ damage within the gastrointestinal tract enables the absorption of nanoparticles into the body of organism (Buzea *et al*., 2007). The present findings illustrated the entry of nanomaterial through gut besides the access through dermal and gill operculum. The deposition of single-walled nanotubes and water suspended fullerenes has been reported in the intestinal lumen of *Daphnia magna* (Roberts *et al*., 2007; Tervonen *et al*., 2010) and in the stomach and large intestine of rats (Takahashi *et al*., 2012). Histological examination of intestine showed severe lesions such as degenerated columnar epithelial cells, stratum compactum and lamina propria with large number of mucous cells. Such alterations possibly interfere with the intestinal absorption of nutrients however; it increases the quick transfer of fullerene C_{60} into the blood circulation. Similarly, aggregation of hydroxylated fullerene nanomaterial has been noticed in the serosal region of small intestine in the fathead minnow, *Pimephales promelas* (Jovanovic *et al*., 2014).

5.5 Effect of fullerene C_{60} on haematological alterations

One of the major passages of fullerene C_{60} into the major organs of organisms has been stated as through gastrointestinal tract into the blood (Hendrickson *et al*., 2015). Thus analysis of haematology and serum biochemistry is a good tool to monitor the degree of fullerene toxicity in organisms. The present study evaluated some valuable haematological parameters such as RBC and WBC count, haemoglobin concentration and hematocrit value after sublethal exposure of fullerene C_{60}. There was a significant reduction in all observed parameters, which indicated that C_{60} fullerene induced haemolysis and anemia thereby leading to altered physiological conditions and metabolism of the fish. The results showed coincidence with another study on rat when administered with fullerene nano- and microparticle by inhalation decreased RBC count, hemoglobin content and haematocrit showing high degree of toxicity exposed to nanoparticle than the microparticle (Baker *et al*., 2008).

Nano-sized compounds has been known to convert normal haemoglobin into inactive or met-haemoglobin, which induce erythrocyte aggregation, promotes cellular lysis (Kim *et al*., 2016) and suppress oxygen transportation (Pan *et al*., 2016). Haemolytic potential of fullerene C_{60} has been evidenced by shrinkage and crenation of human RBC, which was mediated through oxidative stress (Trpkovic *et al*., 2010). Infiltration of C_{60} has resulted in reduced mechanical strength, weekend tensile resistance and membrane damage in human RBC lipid bilayers (Zhang *et al*., 2013b). Besides the carbon nanomaterials, other metal and metal oxide nanomaterials has been shown to alter haematology by the reduction of RBC and WBC counts, and haemoglobin level in the fish, *Labeo rohita* (Rajkumar *et al*.,

2016; Kanwal *et al*., 2019). Silver, iron oxide and copper nanoparticles exposure to various fish such as *Oreochromis niloticus*, *Labeo rohita* and *Tilapia mossambica*, respectively altered haematological parameters such as RBC count, total WBC counts, platelet count, haematocrit, haemoglobin, MCV, MCH and MCHC levels thereby indicated the haemolytic property of nanomaterials (Thummabancha *et al*., 2016; Keerthika *et al*., 2017; Siddiqui and Noorjahan, 2018).

5.6 Effect of fullerene C_{60} on serum biochemistry

The haemolytic ability of C_{60} fullerene was further substantiated by the evaluation of serum biochemistry, which possibly reflects the abnormal physiological state of fish. Sublethal exposure of fullerene C_{60} to the fish altered serum biochemistry such as total protein, albumin, globulin and glucose in time- and concentration-dependent manner. There are several reasons for the reduction in total protein and albumin found after fullerene exposure, and this could be due to protein degradation, metabolic utilization, and reduction of blood protein production by liver and destruction of the protein synthesizing subcellular structures (Fontana *et al*., 1998). Generally, the hydrophobic nano-sized compounds have the ability to bind with protein by the formation of protein-corona, which may influence the cellular uptake, accumulation, inflammation, degradation and clearance of the nanoparticles (Chellat *et al*., 2005). Besides, protein-corona has been known to denature the proteins thereby cause loss of protein function (Roach *et al*., 2005). Certain docking models have also documented the binding affinity of carboxyfullerene with some of the proteins like HIV protease, bovine serum albumin, fullerene specific antibody and human serum albumin (Benyamini *et al*., 2006).

In the present study, fullerene C_{60} exposure caused increase in the level of serum glucose thereby indicated high energy demand and stress induced by the nanomaterial which subsequently released glucose from the stored glycogen. The result coincided with an inhalation study on fullerene nanoparticle in the serum of rat (Baker *et al*., 2008). In fishes, the impaired osmoregulation also known to cause severe stress, which in turn utilize the energy reserves mainly from the liver and muscle glycogen, with considerable rise in blood glucose level (Vosyliene, 1999). Treatment of titanium dioxide nanoparticle in pregnant rats also showed increase in the fast blood glucose level (Mao *et al*., 2019).

Alanine and aspartate aminotransferases are considered as the conventional tissue marker enzymes to detect liver damage as a result of toxicant exposure. Besides liver, the enzymes were prominent in other tissues like heart, kidney, and skeletal muscles, which then reach the circulatory system after the tissue damage thereby the serum level rises beyond the normal state (Zilva *et al*., 1992). Sublethal exposures of C_{60} fullerene caused significant rise in the levels of serum transaminases, which was supported by another findings from our laboratory showing liver tissue damage in the freshwater fish, *Pseudetroplus maculatus* after fullerene C_{60} exposure (Sumi and Chitra, 2017d). Multi-walled carbon nanotubes have been shown to cause hepatotoxicity in mice by altering serum transaminase activities (Ji *et al*., 2009; Patlolla *et al*., 2011). Similarly, other nanoparticles such as silver, gold and copper nanoparticles treated to different strains of rats and mice showed increase in the activities of serum alanine and aspartate aminotransferases (Abdelhalim and Moussa, 2013; Heydrnejad *et al*., 2015; Tang *et al*., 2019).

1. Characterization of fullerene C_{60} evaluated by X-ray diffraction (XRD) and high resolution-transmission electron microscope (HR-TEM) analysis revealed as highly pure having crystalline morphology with a particle size of about 34.95 nm.

2. Acute toxicity of fullerene C_{60} in the freshwater fish, *Anabas testudineus* evaluated by the median lethal concentration using Probit analysis was 50 mg/ L.

3. Exposure of fullerene C_{60} upsets the normal behavioural pattern of the fish, *Anabas testudineus*.

4. Fullerene C_{60} deposits observed in the intestinal lumen was due to oral uptake of nanomaterial, which is suspected to release into the circulatory system.

5. Entry of fullerene C_{60} into the circulatory system altered haematological parameters and serum biochemistry in the fish, *Anabas testudineus*.

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1.1 Levels of antioxidant defense system

Antioxidants are molecules which inhibits the oxidation of other essential molecules so that it prevents the highly reactive free radical species, which lead to cell damage or cell death. In living organisms, the antioxidant defense system functions at different levels to prevent the damage created by the free radicals. It includes radical prevention, scavenging, damage repair and adaptation. Based on the different mode of actions, antioxidants are categorized into first line, second line, third line and fourth line of antioxidant defense (Ighodaro and Akinloye, 2018).

1.1.1 First line of antioxidant defense

The first line antioxidant defense mechanism includes a group of antioxidants which functions to prevent or suppress the generation of reactive species in cells. It includes both enzymatic and non-enzymatic antioxidants. The major enzymes involved in the first line of antioxidant defense mechanism includes superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and glutathione peroxidase (GPx) (Ighodaro and Akinloye, 2018).

Superoxide dismutase (SOD)

Superoxide dismutase is the prime endogenous antioxidant enzyme in the first line of defense system. It catalyzes the dismutation process which converts two molecules of superoxide anion ($O_2^{\bullet-}$) into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) with metal ion as cofactor (Fridovich, 1997).

$$2O_2^{\bullet-}+2H^+ \rightarrow H_2O_2+O_2$$

Based on the presence of various metal ions as cofactors, namely iron, zinc, copper and manganese, different isoforms of SODs exist. It includes SOD1, SOD2, SOD3, and SOD4. The metalloprotein, SOD1 consist of copper and zinc as the cofactor, and are found predominantly in cytosol, besides it is also localized in nucleus, peroxisomes, and in the lumen between inner and outer membranes of mitochondria (Kira *et al*., 2002). SOD2 is found in the mitochondria with manganese or iron ions are the co-factor (Laukkanen, 2016). It is synthesized in the cytoplasm and transported to the mitochondria through a signal peptide, and involved in the dismutation of reactive superoxides formed in the respiratory chain.

The isomer SOD3 constitute 90-99% of total SODs, and is found mainly in the vascular extracellular spaces with copper and zinc metal ions as the cofactors (Marklund, 1982; Laukkanen, 2016). SOD3 is found abundant in blood vessels, kidneys, lungs, and uterus, with fewer amounts in heart (Stralin *et al*., 1995; Ookawara *et al*., 1998). The isomer SOD4 contains nickel as the cofactor, and found in several aerobic soil bacteria of class Streptomyces (Wuerges *et al*., 2004). SOD is the very crucial enzyme that protects the cells from the dangerous free radicals, oxygen radicals and other injurious agents which promotes the cell death and aging (Dayal *et al*., 2017).

Catalase (CAT)

Catalase is a tetrameric porphyrin-containing enzyme localized mainly in peroxisomes found in all aerobic organisms. The cofactor of

CAT is either manganese or iron that catalyzes the reduction and degradation of hydrogen peroxide into water and molecular oxygen (Chelikani *et al*., 2004). The conversion occurs in two enzymatic steps as follows:

$$\text{CAT-Fe (III)} + H_2O_2 \rightarrow \text{substance I}$$

$$\text{Substance I} + H_2O_2 \rightarrow \text{CAT-Fe (III)} + 2H_2O + O_2$$

In the first step of the above reaction, heme utilizes one molecule of H_2O_2 to oxidize into oxyferryl species and porphyrin cation radical. In the second step, the remaining molecule of H_2O_2 act as reducing agent, and reduces the enzyme into its resting state with subsequent production of water and molecular oxygen (Chelikani *et al*., 2004). High concentration of H_2O_2 is toxic and deleterious to cells (Ercal *et al*., 2001), however, at low concentration H_2O_2 is involved in the regulation of certain physiological processes like carbohydrate metabolism, cell proliferation, cell signaling, mitochondrial function, cell death, platelet activation, and maintain normal thiol redox-balance (Droge, 2002).

Glutathione peroxidase (GPx)

Glutathione peroxidase exists in two forms as selenium-independent and selenium-dependent, each with different subunits and active sites (Rahman, 2007). GPx catalyzes the reduction of H_2O_2 and lipid peroxides into water and alcohols, respectively, which occur more often in mitochondria and also in cytosol to some extend (Cohen and Hochstein, 1963; Goth *et al*., 2004). GPx plays a significant role in the

protection of polyunsaturated fatty acids thereby prevent the cells from lipid peroxidation and subsequent oxidative stress (Gathwala and Aggarwal, 2016). In human, GPx exist in eight isoforms as GPx1, GPx2, GPx3, GPx4, GPx5, GPx6, GPx7 and GPx8 that are encoded in chromosome 3, 14, 5, 19, 6, 6, 1, and 5, respectively (Moron and Castilla-Cortazar, 2012). GPx1 is the most abundant selenium containing enzyme found in almost all cells, whereas, GPx2 is found mainly in the gastrointestinal tract (Baek *et al*., 2007). GPx3 exist primarily in kidney and also in extracellular fluids as the glycoprotein (Burk *et al*., 2011). GPx4 has a mitochondrial isoform which facilitate the apoptotic response to oxidative stress (Liang *et al*., 2009), and also it has a peroxidase independent structural function in the maturation of sperm (Noblanc *et al*., 2011). GPx5 and GPx6 are selenium-independent enzymes which are unable to scavenge H_2O_2 (Ursini *et al.,* 1999).

1.1.2 Second line of antioxidant defense

The second line of antioxidant defense enzymes are called as scavenging antioxidants, which scavenge the active free radicals formed. During scavenging process, the antioxidants donate electron to the free radicals, so that the active free radical become less active or new free radicals with relatively less harmful effects, which are further neutralized by other antioxidants (Ighodaro and Akinloye, 2018). Most of the antioxidant involved in second line of defense are non-enzymatic that includes both hydrophilic and lipophilic antioxidants. Ascorbic acid or vitamin C, glutathione, uric acid, vitamin E, and ubiquinol are some of the powerful antioxidants involved in

scavenging the active free radicals (Arrigoni and De Tullio, 2002). Vitamin C donates a hydrogen atom to the free radical and become a stable form named ascorbyl-free radical. Vitamin C, vitamin E, and β-carotene are also collectively called 'antioxidant vitamins'. They play important role to decrease oxidative stress-induced damages and thus lowers the risk of some chronic diseases. It is evidenced by the fact that insufficiency or lack of vitamin E, β-carotene and vitamin C lead to cardiovascular disorders (Rouhier *et al*., 2008; Joel *et al*., 2019). Vitamin C is also involved in the reduction of Fe^{3+} to Fe^{2+} mainly from the non-heme sources and thereby improves the absorption of iron (Halliwell and Gutteridge, 1999).

1.1.3 Third line of antioxidant defense

The third line of defense antioxidants functions only if the cells or tissues are damaged due to the free radical bursts. It includes *de novo* enzymes, which repair the free radical-induced damages mainly in the biomolecules like protein, DNA and lipids, and also in cell membranes (Ighodaro and Akinloye, 2018). It functions to repair the non-functional and damaged biomolecules like DNA, proteins and lipids so as to prevent from accumulation in cells, which otherwise may prove toxic. The common members of DNA repair enzymes included in this family are glycosylases, polymerases and nucleases, and the proteolytic enzymes such as peptidases, proteinases and proteases, which are found predominantly in mitochondria and cytosol (Ighodaro and Akinloye, 2018; Torregrosa-Munumer *et al*., 2019).

1.1.4 Fourth line of antioxidant defense

The fourth line of antioxidant defense enzymes participates only in the adaptation mechanism where they exploit the signals caused by the generation of free radicals. The same signal is then used to induce the generation and transportation of suitable antioxidant to the appropriate site (Niki, 1993).

1.2 Antioxidant defense system in fish

Fish tissues are enriched with large amount of polyunsaturated fatty acids (PUFAs), which is essential for the membrane function but are highly vulnerable to the oxidative attack (Hsieh and Kinsella, 1989). Like other aerobic organisms, fish also possess a well-equipped antioxidant defense system. Both enzymatic and non-enzymatic antioxidants functions together for the eradication of free radicals. Comparatively, high levels of non-enzymatic antioxidants are found in fishes than the higher vertebrates where the glutathione in RBC functions to protect haemoglobin from spontaneous oxidation to met-haemoglobin (Dafre and Reischl, 1990). The level of antioxidant enzymes are known to vary among the fish species, biotic and abiotic factors, and also based on the phylogenetic position (Tappel *et al*., 1982). The antioxidants level also diverges among different age groups, where the activities of antioxidant enzymes are high in young fish than the adult (Wdzieczak *et al*., 1982). Besides, nutritional factors, feeding behaviour, seasonal changes, environmental conditions and fish behaviour are the factors that play significant role in the fish antioxidant defense system (Morales *et al*., 2004). Fish are sensitive to

the changes in the surrounding environment, particularly suspected to harmful xenobiotics, which ultimately affect the normal antioxidant defense system. Therefore, evaluation of antioxidant defense system is considered as the valuable biomarker to monitor the health status of fish and other aquatic organisms, which is one of the very crucial and hot research areas in ecotoxicology.

1.3 Reactive radical species

Molecular oxygen is essential for all living aerobic organisms for the survival but highly prone to radical formation because of its electronic structure. Reactive oxygen species (ROS) are smaller molecules often derived from molecular oxygen, which include the free oxygen radicals like hydroxyl ($^{\bullet}OH$), superoxide ($O_2^{\bullet}$-), alkoxyl (RO-), and peroxyl ($RO_2^{\bullet}$), as well as the non-radicals like ozone (O_3), hypochlorous acid (HOCl), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). The non-radicals have the ability to convert either to radicals or simply exist as oxidizing agents. Nitrogen dioxide (NO_2), peroxynitrite (ONOO-), and nitric oxide (NO) are the nitrogen-containing oxidants named as reactive nitrogen species (RNS) (Bedard and Krause, 2007). Free radicals like reactive nitrogen and oxygen species are collectively called reactive oxygen nitrogen species (RONS). They are highly reactive because of the unpaired electrons in valance shell or non-static bonds (Salman and Ashraf, 2013). There are different types of free-radical species like nitrogen-, oxygen-, carbon-, and sulphur-centered, which are involved in radical formation (Agarwal *et al*., 2006a; Perrone, 2010). The major intermediates formed during the chain reaction are superoxide anion, hydroxyl

radical and hydrogen peroxide. Superoxide anion is the most common ROS formed by the partial reduction of molecular oxygen (Drew, 2002; Bolisetty and Jaimes, 2013; Salman and Ashraf, 2013). Besides, some oxidative enzymes like aldehyde oxidase, dihydrorotate dehydrogenase, and xanthine oxidase also produce superoxide anion.

H_2O_2 is the least reactive molecule stable at physiological pH and temperature only in the absence of metal ions, and are easily transported to the plasma membrane due to high diffusible property. Moreover, H_2O_2 can combine with superoxide anion by Haber-Weiss reaction to form the hydroxyl radical (Liochev and Fridovich, 2002; Barbusinski, 2009). Fenton reaction also produces hydroxyl radical, the most dangerous and highly reactive molecule, with very short half-life (Lee *et al*., 2004). Singlet oxygen is a non-radical, mild and non-toxic molecule produced during chemical and photosensitization reactions, which directly oxidize proteins, lipids, and DNA, and also participate in cholesterol oxidation and Dielse-Alder reactions (Salman and Ashraf, 2013).

Peroxyl ($ROO^{\bullet}$) and alkoxyl ($RO^{\bullet}$) radicals are the carbon-centered radicals produced during hemolysis of peroxides either in the presence of transition mental ion or UV light irradiation. They are also formed from the biomolecules like proteins, lipids, carbohydrates and DNA during oxidative damage (Salman and Ashraf, 2013). Nitric oxide (NO) is not a high reactive radical but can form other reactive intermediates which damage the biomolecules (Drew and Leeuwenburgh, 2002). Thus NO function as either antioxidant or oxidant in biological systems, and its overproduction can cause RNS

and ROS generation that finally lead to oxidative stress (Bolisetty and Jaimes, 2013).

Peroxynitrite ($OONO^-$) is a type of RNS formed when NO react with O_2- followed by spontaneous decomposition to form $^{\bullet}OH$ and nitrogen dioxide ($NO_2^{\bullet}$) radicals (Radi *et al*., 1993). At physiological pH, peroxynitrite is stronger than NO and O_2- which oxidizes proteins, lipids, DNA and nitrated amino acids (Crow and Beckman, 1995; Pryor and Squadrito, 1995). Peroxynitrate also reacts with carbon dioxide to form nitrosoperoxycarbonate ($ONOOCO_2^-$), which readily decompose to form nitrogen dioxide and carbonate radical that assist in peroxynitrite-induced cellular damages (Szabo *et al*., 2007). Nitrogen dioxide ($NO_2^{\bullet}$) radical formed after the reaction between NO and peroxyl radical oxidizes ascorbic acid, and induce the generation of free radicals resulting in lipid peroxidation (Lee *et al*., 2004).

1.4 Sources and generation of ROS

Mitochondria, peroxisomes, endoplasmic reticulum, plasma membrane, cytosol, and extracellular space are the major regions in which ROS generation occurs (Starkov, 2008; Gross *et al*., 2006). Metabolic process, cellular respiration and radiation are considered as the main sources of ROS generation (Brieger *et al*., 2012). There are several pathways involved in the generation of ROS, which includes mitochondrial 5-lipoxygenase (5-LOX), NADPH oxidase and so on. Besides, several cytosolic enzymes have the ability to generate ROS during the catalytic activity. Smooth endoplasmic reticulum has a chain of electron transport systems which introduce double bonds to

fatty acids during xenobiotic metabolism that results in the generation of ROS. The unsaturated fatty acids in the plasma membrane are also the main source of ROS generation (Babior, 1999). In addition, the conversion of arachidonic acid into various products like thromboxanes, prostaglandins, and leukotrienes by the enzymes such as cyclooxygenase and lipoxygenase produce free radicals (Cho *et al*., 2011).

1.5 Nano-antioxidants

Nano-antioxidants behave as natural antioxidants, which has a crucial role in medical field for the treatment of oxidative stress-mediated diseases (Schubert *et al*., 2006). Studies have been reported regarding the antioxidant properties of nanoparticles (Chen *et al*., 2006; Das *et al*., 2007). Intrinsic antioxidant activity of nanoparticles ranged from metal or metal oxide nanoparticles such as platinum, gold, and iron oxide. The antioxidant capacity of nanoparticles also depends of various factors including chemical composition, nature, surface charge, surface coating and surface to volume ratio (Korsvik *et al*., 2007; Heckert *et al*., 2008). Available literatures illustrated that nano-antioxidants protect cells from oxidative damage, and are fairly nontoxic. The small size of nanoparticles enable for easy entry into the cells by crossing the cell membrane through pinocytosis, and are widely used in targeted drug delivery (Hong *et al*., 2006). Some of the nanoparticles such as platinum, gold, diamond, nickel oxide, iron oxide etc., are known to neutralize ROS thereby used for the treatment of inflammation, diabetes and tumour formation (Barathmanikanth *et al*., 2010; Sharpe *et al*., 2011).

1.6 Nanomaterials as prooxidant

On contrary to the antioxidant properties, some nanomaterials also functions as prooxidants. The engineered nanomaterials generate ROS mainly due to the unique physico-chemical properties such as chemical nature, size, surface area and reactivity (Gonzalez *et al*., 2008) that results in oxidative stress, and also genotoxicity and cytotoxicity (Oberdorster *et al*., 2005). In addition, the shape, oxidation status, bonded surface species, solubility, surface coating, and the degree of agglomeration and aggregation of the nanomaterial also lead to the induction of free radicals (Shaligram and Campbell, 2013).

Nanoparticles-mediated ROS generation lead to several pathological conditions, where the deposition of nanoparticles such as cerium oxide, zinc oxide, silver, and titanium dioxide deposited either in subcellular organelles or in cell surface stimulate oxidative stress (Buzea *et al*., 2007). The small-sized nanoparticles are known to produce more ROS than the same nanoparticles of larger size (Sioutas *et al*., 2005). Surface modification also induce oxidative stress where nanoparticles with large surface area are potentially more toxic where it involve in various reactions including Fenton-like reactions, Fenton reactions, and Haber-Weiss reactions for the production of highly reactive radicals (Petersen and Nelson, 2010). Solubility is another feature of nanoparticles that induce ROS generation, where high solubility enhances particle-cell interactions (Sergeeva *et al*., 2019).

1.7 Fullerene C_{60} as antioxidant

Fullerenes and its derivatives possess dual property as efficient free-radical scavenger and as ROS generator. Based on the scavenging properties, it is also known as "radical sponge" (Krusic *et al*., 1991). Fullerene C_{60} inactivates hydroxyl radicals by binding to its double bonds by two mechanisms (Andrievsky *et al*., 2009). In the first mechanism, fullerene adds ROS on its surface, followed by the regeneration of parent fullerene, and subsequently involved in the catalytic cleavage of ROS. The second mechanism is based on the transfer of outer sphere electrons by reduction and reoxidation reactions between radical species and fullerene (Andrievsky *et al*., 2009).

Fullerene-mediated ROS scavenging ability is widely used in the field of medicine. Fullerene and its derivatives like fullerenol possess antioxidant activity, which has been demonstrated in various *in vivo* and *in vitro* studies (Injac *et al*., 2013). The anionic derivatives of fullerenes possess higher antioxidant property than cationic derivatives, which remove superoxide radicals at a rate similar to that of SOD (Witte *et al*., 2007). C_{60} fullerene resides very close to the interior region of lipid-rich membrane where the chain propagation for the lipid peroxidation begins thus exhibit high antioxidant ability to prevent lipid peroxidation than the natural antioxidants like vitamin E (Wang *et al*., 1999). Some of the water-soluble derivatives of fullerenes like PVP, PEG, isostearic acid, hydroxyl, and γ-cyclodextrin-modified fullerenes also behave as excellent ROS

scavengers in cell cultures, which protects from adverse health effects in human (Xiao *et al*., 2005).

The antioxidant activity of fullerenes is mediated through various signaling pathways, particularly through nuclear factor erythroid 2-like-2/ activator protein-1 (NFE2L2/AP-1) signaling pathway (Cai *et al*., 2008). Fullerene C_{60} can cross the mitochondrial membrane due to its lipophilic property where it scavenges superoxide anion in mitochondria thereby involve in phosphorylation and mild respiratory uncoupling process (Korshunov *et al*., 1997).

1.8 Fullerene C_{60} as free radical generator

One of the common mechanisms of singlet oxygen generation is the photosensitization reaction where energy is transferred to triplet ground state oxygen from an excited photosensitizer having a specific wavelength (Clo *et al*., 2007). The singlet oxygen photosensitization process acquire specific electronic configuration in molecular oxygen. Fullerenes are known to act as an efficient singlet oxygen sensitizer which absorbs energy from both UV and visible spectrum of light (Kratschmer *et al*., 1990; Leach *et al.,* 1992). During light excitation, C_{60} from the singlet excited state ($^1C_{60}^{\bullet}$) through the intersystem crossing and reaches into the triplet state ($^3C_{60}^{\bullet}$), where it quenches molecular oxygen to produce huge amount of singlet oxygen (Arbogast *et al*., 1991). The triplet excited state of fullerene behave as an excellent electron acceptor, while the reduced fullerene triplet ($^3C_{60}$) quickly transfer its one electron to the molecular oxygen and to form superoxide-anion radical (Yamakoshi *et al*., 1998).

In water, fullerene exists as aggregates having unique photochemical properties that differ from other molecular fullerenes,

and are not involved in the production of ROS (Lee *et al*., 2008). However, C_{60}-water suspension effectively produces singlet oxygen on prolonged light irradiation (Hou and Jafvert, 2009). Aqueous fullerenol is more efficient than C_{60}-aqueous suspension to generate singlet oxygen and superoxide anion (Chae *et al*., 2011). Fullerene-mediated singlet oxygen generation by type II photochemical mechanism quickly reacts with amino acids and polyunsaturated fatty acids to undergo lipid peroxidation, membrane damage, and DNA damage (Briviba *et al*., 1997; Davies, 2003). The superoxide anion free radical formed by fullerene through type II photochemical mechanism is readily converted into other forms of ROS, including hydroxyl radical and hydrogen peroxide finally leading to cell damage (Ray *et al*., 2012).

1.9 Role of ROS in reproduction

ROS like superoxide anion, hydroxyl radical, hydrogen peroxide and other free radicals are continuously generated in the body of organism during the metabolic process, which has significant role in normal development and reproduction (Birben *et al*., 2012). In male and female reproduction, ROS functions as a double-edged sword. ROS generation at physiological concentration is essential for capacitation, acrosome reaction, sperm-egg fusion, and hyperactivation in male reproductive system (Agarwal *et al*., 2014a) whereas at high concentration, it adversely affect the sperm function leading to male infertility (Agarwal *et al.,* 2014b). In female reproductive system, the physiological concentration of ROS is involved in ovarian steroidogenesis, folliculogenesis, oocyte maturation, luteolysis, and ovulation (Agarwal *et al*., 2006b; Esfandiari *et al*., 2005), while high

concentration inhibits oocyte development and vitellogenesis (Agarwal *et al*., 2014a).

Several nanoparticles have been known to directly involve in the induction of ROS generation and thereby oxidative stress in reproductive tissues. Nanoparticles also cross the blood-testis barrier (BTB) which then disrupts Sertoli cells, influenced by the induction of oxidative stress (Braydich-Stolle *et al*., 2010). Besides, other male reproductive toxicity includes altered morphology of seminiferous tubules, reduction in size of Leydig cells, apoptosis in germ cells, DNA damage, low sperm motility, viability, count and morphology (Braydich-Stolle *et al*., 2010; Gromadzka-Ostrowska *et al*., 2012; Garcia *et al*., 2014). The most frequently observed alteration in sperm morphology associated to nanoparticles exposure includes folded, amorphous spermatozoa, sperm with elongated or undulating heads with small hook (Chen *et al*., 2013).

Nanoparticles also exhibit significant toxicity in female reproductive tissues through ROS generation and oxidative stress. It was well recognized by exposure to several nanoparticles including titanium dioxide, nickel, zinc oxide and other nanoparticles to different organisms are shown to alter the morphology of follicles, reduction in the number of mature oocytes, swelling and rupturing of mitochondria, irregular nuclear membrane, and chromatin condensation in oocytes, decline in yolk lipid content and apoptosis in ovarian cells (Gao *et al*., 2012; Liu *et al*., 2017; Wang *et al*., 2018).

1.10 Oxidative stress and histopathology as biomarkers

Oxidative stress adversely affects the normal functioning in the body of organisms. Primary and secondary products formed as a result

of free radical formation are used as biomarker to monitor the oxidative stress. Free radicals mainly affect the biological membranes by the induction of lipid peroxidation, where malondialdehyde is formed as the secondary byproduct. Oxidative stress also cause hydroxylation and specific modifications in pyrimidine and purine bases of DNA, and the products formed is also considered as the biomarkers (Valavanidis *et al*., 2006). In aquatic organisms, the amount of 8-oxo-2′-deoxyguanosine (8-OHdG) formed as the result of pollutant exposure is used as solid biomarker of oxidative stress (Rodriguez-Ariza *et al*., 1999). Besides, the free radical-induced damage in protein oxidation is also measured by the evaluation of protein carbonyl and its derivatives (Huggins *et al*., 1993). Fluctuations in the level of antioxidant enzymes in organ systems are associated with exposure of different stressors in the form of xenobiotics, which are suspected to cause adverse effects on the exposed organisms. Therefore, the present study was focused to evaluate the antioxidant status in gonads of the freshwater fish *Anabas testudineus* after sublethal exposure of fullerene C_{60} nanomaterial.

In addition to the biochemical analysis, monitoring histological changes in tissues are highly precise way to assess the effect of toxicants on organisms. It is the potential biomarker to assess the target organ toxicity and thus referred as the gold standard. The microscopic examination of tissues is used to evaluate the effects of pollutants at organism or tissue level. It is also used as an indicator of contamination in aquatic ecosystems, as the morphological changes in the tissues negatively reflect the health status of the ecosystem. Thus the study also confirmed the intensity of nanomaterial-induced toxicity by assessing histology of gonadal tissues.

Toxicity of nanoparticles largely depends on its physico-chemical features where the surface area is the key factor to induce adverse effects on humans and other organisms. The engineered nanomaterials exert toxicity through various pathways, in which ROS formation is one of the mechanisms which could cause oxidative stress. Interaction of nanoparticles with biological systems also trigger a cascade of molecular events including genetic damage, inflammation, inhibition of cell division, apoptosis, damage to membranes, protein and DNA (Stone *et al*., 2007; Li *et al*., 2008; Johnston *et al*., 2010). However, most of the researches focused on ROS-mediated toxicity of nanoparticles on various organisms. Toxicity of the carbon-based nanomaterial, fullerene C_{60} is always a controversial topic as it behave as both prooxidant and antioxidant in various biological systems (Markovic and Trajkovic, 2008). The present study reviewed the prooxidant and antioxidant properties of fullerene C_{60} and its derivatives in detail.

One of the earlier studies has reported that carboxy-fullerene prevents the apoptotic injury of the cortical cell cultures induced by N-methyl D-aspartate (NMDA) without interfering with Ca^{2+} influx induced by NMDA in cell culture (Dugan *et al*., 1996). Fullerene derivative, monomalonic acid C_{60} has been shown to inhibit nitric oxide-dependent relaxation of aortic smooth muscle in rat (Satoh *et al*., 1997). An *in vitro* study on the rat hippocampal slices has reported that the polyhydroxylated fullerenol blocks the inhibition of population spikes in the hippocampus slices of rat induced by hydrogen peroxide

and cumene hydroperoxide (Tsai *et al.,* 1997). Carboxyfullerene, a water-soluble carboxylic acid derivative of a fullerene, has been reported as nontoxic protective agent against iron-induced oxidative stress in the nigrostriatal dopaminergic system of rats (Lin *et al*., 1999). Similarly, C_{60} and other water-soluble fullerene derivatives have been proved as antioxidants against radical-initiated lipid peroxidation observed by fluorescence quenching process (Wang *et al*., 1999).

A water-soluble C_{60} derivative, hexasulfobutyl[60]fullerene has been shown to act as a potent free radical scavenger by efficiently protecting plasma lipid from oxidation in association with lipoprotein in both the aqueous and lipophilic phases (Hsu *et al*., 2000). The antioxidant property of hexasulfobutylated C_{60} has been reported on Long Evans rat where it functioned as a neuroprotective agent on focal cerebral ischemia (Huang *et al*., 2001). Water-soluble derivative of fullerene C_{60} in combination with ascorbic acid when applied to chromaffin cell cultures exposed to levodopa has been shown to increase the survival of adrenal chromaffin cells and prevented cell death thereby proved as a potent neuroprotective agent (Corona-Morales *et al*., 2003).

Aqueous fullerene C_{60} suspension has been considered as a powerful liver-protective agent in carbon tetrachloride intoxicated rats as it protected the liver from injury as evidenced by histological examinations (Gharbi *et al*., 2005). Polyvinylpyrrolidone-fullerene C_{60}

derivative has been reported as a shield against the damage induced by ROS production and oxidative stress in human keratinocytes as a result of ultraviolet light irradiation (Xiao *et al*., 2006). In an *in vitro* study on a novel cystine C(60) derivative a protective effect of nanoparticle has been observed upon the hydrogen peroxide-induced apoptosis in rat pheochromocytoma PC12 cells (Hu *et al*., 2007). Water-soluble C_{60} fullerene has a potential therapeutic property as evidenced by its protection against the articular cartilage in osteoarthritis through the down-regulation of chondrocyte catabolic activity and inhibition of cartilage degeneration *in vitro* and *in vivo* (Yudoh *et al*., 2007).

Fullerene C_{60}/C_{70} nanocrystalline suspension suppressed oxidative stress and mitochondrial depolarization induced by proinflammatory cytokine tumor necrosis factor (TNF), and has been suggested for the use of TNF-based cancer therapy or prevention of TNF-dependent tissue damage in mouse L929 fibroblast cell line (Harhaji *et al*., 2008). C_{60} nanoparticles has been shown to reduce sodium nitroprusside-induced apoptotic cell death by preventing mitochondrial depolarization, caspase activation, cell membrane phosphatidylserine exposure and DNA fragmentation thereby prevented nitric oxide-mediated cell injury in inflammatory or autoimmune disorders examined in mouse L929 fibroblasts (Misirkic *et al*., 2009). Similarly, in an adjuvant-induced arthritic rats administered to water soluble C_{60} nanoparticles has been shown to downregulate the receptor activator NFkappaB (RANK) signaling pathway-induced osteoclast differentiation, and suggested as a

potential therapeutic agent for the inhibition of osteoclastic bone destruction in arthritis (Yudoh *et al*., 2009).

A potential bactericidal effect of the organic nanomaterial fullerene C_{60} has been documented in gram-negative bacteria grown in mucous secretions of common carp, *Cyprinus carpio*, which showed that the nanomaterial inhibited the growth of bacterial communities within 3 h of exposure (Letts *et al*., 2011). Administration of hydrated C_{60} fullerene has been shown to reduce diabetes-induced oxidative stress and associated complications such as testicular dysfunction and spermatogenic disruption in Wistar strain male albino rats (Bal *et al*., 2011). Fullerene C_{60} and its modified water-soluble derivative has been recommended as a potential medicines against the bronchial asthma as it was known to inhibit the free radical-induced lipid peroxidation in lung and spleen tissues of mice (Bobrova *et al*., 2012).

Oral administration of C_{60} fullerene in olive oil suspension has been shown to delay senescence in rats (Baati *et al*., 2012). Fullerene C_{60} has showed protective antioxidant effect by scavenging titanium dioxide (TiO_2)-photocatalyzed ROS generation in the skin surface and therefore effectively used for the functional improvement of TiO_2-containing sunscreens (Kato *et al*., 2014). Carboxylic acid C_{60} derivatives have shown to exert neuroprotective effects regulating mitochondrial dynamics and functions in microglial cells, thus illustrated the neuroprotective properties of C_{60} derivatives in BV-2 microglial cell model (Ye *et al*., 2015). Fullerene C_{60} was found to

inhibit free radical formation and destructive processes in connective tissue during adjuvant arthritis in rats thereby function as a feasible therapeutic agent (Mamontova *et al*., 2015). Reduced glutathione C_{60} derivative has showed protective effect against hydrogen peroxide-induced cell apoptosis by scavenging free radicals and maintaining intracellular calcium homeostasis without the evidence of toxicity in cultured HEK 293T cells (Huang *et al*., 2016).

The pristine C_{60} fullerene aqueous colloid solution has shown to reduce the recovery time of the muscle contraction force and increased the time of active muscle functioning thus enabled muscle endurance by possible maintenance of prooxidant-antioxidant muscle tissue homeostasis in rat (Prylutskyy *et al*., 2017). In one of the studies, polyhydroxylated fullerene C_{60} exposure has radio-protective ability on human erythrocytes as evidenced after irradiation with high-energy electrons (Grebowski *et al*., 2018). Fullerene C_{60} nanoparticles exposure showed hepatoprotective effect against cyclophosphamide-induced toxicities in albino rats, and suggested for monotherapy against chemotherapeutic agent in clinical trials (Elshater *et al*., 2018). Unmodified hydrated C_{60} fullerene molecules has shown to exhibit antioxidant properties as revealed by prevention against DNA and protein damage associated to ROS thus protected mice against injuries caused by ionizing radiation-induced oxidative stress (Gudkov *et al*., 2019). Recently, studies have illustrated that the antioxidant effect of fullerene derivatives depends on their chemical structure (Sergeeva *et al*., 2019), and aggregation behaviour (Chen *et al*., 2019). One of the

recent literatures further added the evidence of therapeutic application of water-soluble polyhydroxylated fullerene C_{60} by inhibiting the inflammation of synovial membranes and the damage towards the cartilage chondrocytes in osteoarthritis joints (Pei *et al*., 2019).

Although several literatures portrayed the antioxidant properties of fullerene and its derivatives in various animal and cell culture models, the negative effects of fullerenes on various organisms have been reported. A study reported that fullerene C_{60} has promoted the growth inhibition of the human keratinocytes up to 50-60% in both light and dark conditions for a period of 8 d (Bullard-Dillard *et al*., 1996). Fullerenol-1 has been shown to suppress the levels of the microsomal enzymes in mice pretreated intraperitoneally, and decreased the activities of P450-dependent monooxygenase and mitochondrial oxidative phosphorylation in liver microsomes (Ueng *et al*., 1997). Polyalkylsulfonated C_{60} administered to female Sprague-Dawley CD rats in a single-dose for acute toxicity study and daily for 12 d for subacute toxicity study revealed a suppression in the activities of liver cytochrome P450-dependent monooxygenase enzymes, including cytochrome P450, cytochrome b5, and benzo(a)pyrene hydroxylase, but an increased level of kidney cytochrome P450-dependent monooxygenase activities, including NADPH-cytochrome P450 reductase has been observed (Chen *et al*., 1998).

Fullerene C_{60}, on photosensitization, has shown to induce significant oxidative damage by the induction of lipid peroxidation in

lipid-rich membranes of rat liver (Kamat *et al*., 1998). Nano-C_{60} exposure has been reported to cause a significant increase in the ROS generation, lipid peroxidation and cell death in human dermal fibroblasts and human liver carcinoma cells (Sayes *et al*., 2004). An *in vivo* study on the freshwater fish, largemouth bass showed that C_{60} fullerene (nC_{60}) exposure for 48 h resulted in significant oxidative stress and lipid peroxidation in the brain, and reduced GSH activity in the gill tissue (Oberdorster, 2004). Similarly, elevated cell membrane lipid peroxidation and its associated cytotoxicity have been observed after 48 h of C_{60} colloid exposure on various human cell lines such as neuronal astrocytes, liver carcinoma cells and dermal fibroblasts (Sayes *et al*., 2005).

The fullerol, $C_{60}(OH)_{22–26}$ exposure generated ROS along with the stimulation of heme oxygenase 1 expression, glutathione depletion, and other ROS-mediated damages like increased calcium level, mitochondrial damage and internalization of fullerol in the macrophages (Xia *et al*., 2006). Exposure of pure C_{60} fullerene suspension to various cell lines including rat C6 glioma, mouse L929 fibrosarcoma and human U251 glioma cells showed increased ROS generation, ROS-dependent necrosis and membrane damage within few hours than the water-soluble polyhydroxylated fullerene exposure (Isakovic *et al*., 2006). Treatment of different forms of nC_{60} suspensions namely EtOH/nC_{60}, THF/nC_{60}, and aqu/nC_{60} on various cell lines as normal human dermal fibroblasts, human keratinocyte (NTCC 2544), mouse melanoma (B16) and mouse fibrosarcoma

(L929) induced toxicity and membrane damage in all cell lines with more toxicity on THF/nC_{60} exposure (Markovic *et al*., 2007).

An *in vivo* pulmonary toxicity study on rats showed increased lipid peroxidation and neutrophil count in BAL fluids after the intratracheal instillation of water-soluble derivative of fullerene C_{60} (Sayes *et al*., 2007). Fullerene C_{60} exposed to embryonic zebrafish altered the expression of several key stress response genes such as glutathione-S-transferase, glutamate cysteine ligase, ferritin, alpha-tocopherol transport protein and heat shock protein 70 thereby resulted in oxidative stress (Usenko *et al*., 2008). The human cell line namely human umbilical vein endothelial cells exposed to both nC_{60} and fullerenol, $C_{60}(OH)_{24}$, caused cell cycle arrest at G1 stage, elevated intracellular calcium level, apoptosis and pro-inflammatory responses (Gelderman *et al*., 2008). The uptake of fullerol, $C_{60}(OH)_{22\text{-}26}$ by human lens epithelial cells, HLE-B3 showed both cytotoxic and phototoxic damages including low metabolic activity, increased lactate dehydrogenase release, ROS generation and apoptosis (Roberts *et al*., 2008).

Chronic toxicity of C_{60} fullerene has been illustrated by the induction of oxidative stress and growth inhibition in the freshwater fish, *Carassius auratus* (Zhu *et al*., 2008). Inhalation exposure of C_{60} fullerene to rats for a month upregulated certain key genes associated with inflammatory responses, apoptosis, oxidative stress and metallo endopeptidase expression (Fujita *et al*., 2009). Intragastric application

of fullerene C_{60} in rats has reported to induce oxidative degeneration ultimately leading to DNA lesions in the liver and lungs (Folkmann *et al*., 2009). The defect in human retinal pigment epithelial cells has been demonstrated by the induction of apoptosis, necrosis, singlet oxygen formation and lipid peroxidation after fullerol exposure (Wielgus *et al*., 2010). Fullerene C_{60} also induced inflammatory responses in the lung of mice as evidenced by cell infiltration, expression of tissue damage in related genes (Park *et al*., 2010).

Intraperitoneal exposure to nano- or microparticles of fullerene C_{60} has been shown to increase the activity of acetylcholinesterase and lipid peroxidation in the brain tissue of adult zebrafish, *Danio rerio* (Dal Forno *et al*., 2013). Hydroxylated fullerenes exposed to fathead minnow, *Pimephales promelas* induced histopathologic changes as loss of interstitial cellularity in kidney, loss of intracytoplasmic glycogen in liver, variable number of leukocytes, macrophages, and fewer heterophils and rodlet cells in the coelom (Jovanovic *et al*., 2014). Aqueous C_{60} fullerene has been shown to induce apoptosis in peritoneal macrophage of mouse by changing the mitochondrial membrane potential (Zhang *et al*., 2015). Carbon-based nanoparticles has been found to induce brain and gonadal alterations determined by biospectroscopy techniques in zebrafish, *Danio rerio* (Li *et al*., 2015).

Previous studies from our laboratory have demonstrated the toxicity of fullerene C_{60}-DMSO suspension by alteration in the antioxidant status of various tissues such as gill, brain, liver and

muscle of the freshwater fish, *Pseudetroplus maculatus* (Sumi and Chitra, 2016; 2017a; 2017b; 2017c). Another study has reported that fullerene C_{60}-induced oxidative stress and subsequent reduction in the antioxidative capacity in zebrafish (Sarasamma *et al*., 2018). Recently, the National Toxicology Program has revealed the accumulation of fullerene C_{60} in the body occurs through intratracheal, instillation and intravenous administration in male Fischer 344 rats so that induced detrimental health effects (Shipkowski *et al*., 2019). Thus the dual properties of fullerene persuaded for the evaluation of antioxidant status in gonads of the freshwater fish, *Anabas testudineus*.

3.1 Test animal

Healthy, mature freshwater fish, *Anabas testudineus* (11 ± 1 g; 8 ± 1 cm) collected from hatcheries were acclimatized in the laboratory, and experiment was conducted during May to August so as to obtain the mature phase of gonads.

3.2 Chemicals

Fullerene C_{60} (purity >98%, Product Number: 483036, CAS No. 99685-96-8) was procured from Sigma Aldrich, Germany. Malondialdehyde, Folin-Ciocalteau reagent, NADPH, glutathione reduced, glutathione oxidized, horseradish peroxidase, thiobarbituric acid, ethylenediaminetetraacetic acid (EDTA), sodium azide, glutathione reductase and pyrogallol were obtained from Himedia Research Laboratories, Mumbai, India. All other chemicals used were of analytical grade purchased from local commercial sources.

3.3 Preparation of fullerene C_{60} suspension

The characterization of nanomaterial performed earlier was found similar to the manufacturer's description. The uniform suspension of fullerene C_{60} was prepared in the organic solvent, dimethyl sulfoxide (DMSO), by sonicating for 1-3 h (Usenko *et al*., 2007). DMSO (1%) was used as vehicle control to dissolve the nanomaterial, and our previous study has reported that the organic solvent did not exert toxicity in fish model (Asifa and Chitra, 2017).

3.4 Experimental design

Fullerene C_{60} at two sublethal concentrations such as one-tenth of LC_{50}-96 h (5 mg/ L) and one-fifth of LC_{50}-96 h (10 mg/ L) were chosen as the test concentrations. Fish were exposed for short-term

(24, 48, 72 and 96 h) and long-term (7, 15, 30 and 60 d) periods. After 60 d of fullerene C_{60} exposure at both sublethal concentrations, fish were maintained devoid of the toxicant and vehicle exposure in well-aerated, clean and fresh, dechlorinated tapwater for another 60 d, which was considered as treatment withdrawal group. Grouping was done as follows:

<table>
<tr><th>Group I</th><th>Group II</th><th colspan="4">Group III & Group IV</th><th>Group V</th><th>Group VI</th></tr>
<tr><td></td><td></td><td colspan="4">Fullerene C_{60} nanomaterial</td><td colspan="2">Withdrawal group</td></tr>
<tr><td rowspan="2">Negative control group</td><td rowspan="2">Vehicle control group</td><td colspan="4">Short-term exposure group (24, 48, 72 and 96 h) & Long-term exposure group (7, 15, 30 and 60 d)</td><td rowspan="2">after 5 mg/L fullerene C_{60} exposure (60 d)</td><td rowspan="2">after 10 mg/L fullerene C_{60} exposure (60 d)</td></tr>
<tr><td>without solvent and toxicant</td><td>1% DMSO</td><td>5 mg/L</td><td>10 mg/L</td><td>5 mg/L</td><td>10 mg/L</td></tr>
</table>

3.5 Collection of tissues

After the end of every treatment period, fish were caught using a small dip net without giving stress to the animal. Body weights were recorded with and without mucous deposition, and decapitated. Gonads from control, vehicle, and treatment and withdrawal groups were dissected, weighed separately and stored at -80 ºC until the biochemical analysis were performed.

3.6 Gonadosomatic index

The gonadosomatic index (GSI) of ovary and testes was evaluated by the following formula:

$$GSI = (\text{Gonad weight (g)} / \text{Fish weight (g)}) \times 100.$$

3.7 Preparation of tissue samples

Tissue homogenates (1% w/ v) from ovaries and testes were prepared in 0.9% ice-cold normal saline using a motor-driven glass teflon homogenizer on crushed ice for 1 min. Then tissue homogenates were centrifuged at 800 *g* for 15 min at 4 ºC, the supernatants were separated and used for the biochemical analysis.

3.8 Biochemical analysis

3.8.1 Determination of protein

Protein contents were determined according to the method of Lowry *et al*. (1951). An aliquot of the test sample was vortexed with alkaline copper reagent, and were allowed to stand for 10 min at room temperature. Folin-Ciocalteau reagent (1 N) was added to each of the tubes, vortexed and allowed to stand for 20 min at room temperature. The optical density was read at 610 nm on a Shimadzu UV-Visible Spectrophotometer. A standard calibration curve was prepared using different concentrations of bovine serum albumin.

3.8.2 Superoxide dismutase

Superoxide dismutase (EC 1.15.1.1) was assayed by the method of Marklund and Marklund (1974). The assay mixture contained tris hydrochloric acid buffer (50 mM), EDTA (1 mM), pyrogallol (0.2 mM) and test sample. Increase in the absorbance was measured immediately after the addition of sample at 420 nm against enzyme blank at 1 min interval for 3 min on a Shimadzu UV-Visible Spectrophotometer. Activity of enzyme was expressed as nmol pyrogallol oxidised/ min/ mg protein.

3.8.3 Catalase

Catalase (EC. 1.11.1.6) was determined according to the method as described by Claiborne (1985). The reaction mixture contained phosphate buffer, hydrogen peroxide (19 mM) and test sample. Decrease in absorbance was measured immediately at 240 nm against the enzyme blank at 1 min intervals for 3 min on a Shimadzu UV-Visible Spectrophotometer. Activity of enzyme was expressed as μmol hydrogen peroxide consumed/ min/ mg protein.

3.8.4 Glutathione reductase

Glutathione reductase (EC. 1.6.4.2) was assayed by the method of Carlberg and Mannervik (1985). The assay mixture contained phosphate buffer (100 mM), NADPH (200 mM), EDTA (10 mM), glutathione oxidized (20 mM) and test sample. Disappearance of NADPH was measured immediately at 340 nm against the enzyme blank at 1 min intervals for 3 min on a Shimadzu UV-Visible Spectrophotometer. The unit of enzyme activity was expressed in μmol NADPH oxidised/ min/ mg protein.

3.8.5 Glutathione peroxidase

Glutathione peroxidase (EC.1.11.1.9) was assessed by the standard protocol as described by Mohandas *et al*. (1984). The reaction mixture contained phosphate buffer (100 mM), EDTA (10 mM), sodium azide, glutathione reductase, glutathione reduced, NADPH (200 mM), hydrogen peroxide and test sample. Disappearance of NADPH was measured immediately at 340 nm against enzyme blank at 10 sec intervals for 3 min on a Shimadzu UV-

Visible Spectrophotometer. Activity of enzyme was expressed as μmol NADPH oxidised/ min/ mg protein.

3.8.6 Hydrogen peroxide generation assay

Hydrogen peroxide generation was assayed by the method of Pick and Keisari (1981). The assay mixture contained phosphate buffer (50 mM), horseradish peroxidase (8.5 units/ ml), phenol red (0.28 nM), dextrose (5.5 nM) and test sample. It was incubated at 32 ºC for 30 min, followed by the addition of sodium hydroxide (10 N) to terminate the reaction. The absorbance of reaction mixture was read at 610 nm against enzyme blank on a Shimadzu UV-Visible Spectrophotometer. The quantity of hydrogen peroxide produced was expressed as nmol hydrogen peroxide generated/ mg protein. For the preparation of standard curve, known amount of hydrogen peroxide and all the above reagents except sample were incubated for 30 min at 32 ºC and then sodium hydroxide (10 N) was added to stop the reaction.

3.8.7 Lipid peroxidation

A breakdown product of lipid peroxidation, thiobarbituric acid reactive substance (TBARS), was measured by the method of Ohkawa *et al*. (1979). The stock solution contained equal volumes of trichloroacetic acid (15% w/ v), hydrochloric acid (0.25 N) and 2-thiobarbituric acid (0.37% w/ v). One volume of the test sample and two volumes of stock reagent were mixed in a screw-capped centrifuge tube, vortexed and heated for 15 min in a boiling water bath. After cooling on ice the precipitate was removed by centrifugation at 800 *g* for 15 min and absorbance was measured at 532 nm against the sample. The values were expressed as μmol of malondialdehyde

formed/ mg protein. A standard curve was prepared with the known amount of malondialdehyde and all the above reagents except enzyme source.

3.9 Histomorphological analysis

Gonadal tissues were dissected out from both sublethal exposure groups of fullerene C_{60} nanomaterial treated for 96 h and 60 d, and also from the treatment withdrawal groups. The tissues were rinsed thoroughly in physiological saline to remove the debris and clotted blood, and were fixed in 10% buffered formalin for 24-48 h. The tissues were dehydrated in ascending grades of alcohol and cleared in xylene until it become translucent. The tissue blocks were prepared by embedding in molten paraffin wax for an hour. Sections were made using rotary microtome at 4 to 6 micron thickness, which were double stained with haematoxylin and eosin stains, and finally mounted in DPX (Roberts and Smail, 2001). Slides were examined and compared with the corresponding control tissues. Photographs were captured using a canon shot camera fitted to the Carl Zeiss Axioscope-2 plus Trinocular Research Microscope.

3.10 Statistical analyses

Statistical analyses were performed using SPSS 21.0 statistical tool. In each group, ten animals were maintained and were triplicated to minimize the statistical errors. All data are expressed in Mean ± standard deviation (SD) per group. The differences between and among the control, treatment and withdrawal groups were tested using One-way Analysis of Variance (ANOVA) followed by Duncan's Multiple Range as post-hoc test. The values were set significant at $P<0.05$ against the control groups.

4.1 Sublethal effects of fullerene C_{60} nanomaterial on the body weight of the fish, *Anabas testudineus*

The body weight of *Anabas testudineus* was measured after exposure to two sublethal concentrations, 5 and 10 mg/ L, of fullerene C_{60} for short-term and long-term durations. Exposure to fullerene C_{60} at low sublethal concentration (5 mg/ L) did not cause significant changes in the body weight after the short-term durations, whereas the long-term exposures caused significant ($P<0.05$) reduction after 7 d onwards in time-dependent manner (Fig. 1). Exposure of fullerene C_{60} at high sublethal concentration (10 mg/ L) caused significant ($P<0.05$) reduction in the body weight after 96 h onwards, which was duration-dependent (Fig. 1). The body weight of fish exposed to the vehicle control (1% DMSO) remained unchanged in all durations throughout the experiment, and was found similar to that of negative control group. Treatment of fullerene C_{60} was withdrawn after 60 d of exposure, and was retained in toxicant- and vehicle-free water for another 60 d, which showed regain in the body weight of fish (Fig. 1).

4.2 Effects of fullerene C_{60} on mucous deposition in the fish

Anabas testudineus exposed to 5 mg/ L concentration of the nanomaterial showed a significant ($P<0.05$) increase in the mucous deposition after 96 h, and at all durations in long-term exposure groups when compared to the corresponding control groups (Fig. 2). In high sublethal concentration group (10 mg/ L), there was a significant ($P<0.05$) increase in mucous deposition after 72 h onwards in time-dependent manner than the respective control groups (Fig. 2).

However, the treatment withdrawal group showed no excess deposition of mucous (Fig. 2).

4.3 Effects of fullerene C_{60} on absolute and relative weights of ovary

There was no remarkable change in the absolute weight of ovary after both sublethal exposure of fullerene C_{60} in short-term exposure groups. But long-term exposure groups showed significant ($P<0.05$) reduction in the weight of ovary at both sublethal concentrations after 30 and 60 d (Fig. 3). Similarly, the gonadosomatic index or relative weight of ovary did not change in the short-term exposure groups. Long-term exposure to fullerene nanomaterial significantly ($P<0.05$) decreased the relative weights of ovary after 60 d at 5 mg/ L concentration, and after 30 and 60 d at 10 mg/ L concentration (Fig. 4). The treatment withdrawal group recovered the weight of ovary as observed in both absolute measure and in relation to the body weight of fish (Figs. 3 and 4).

4.4 Effects of fullerene C_{60} on absolute and relative weights of testis

At both sublethal concentrations, the actual or absolute as well as the relative weights of testis remained unchanged in all durations of short-term exposure when compared with the corresponding control groups (Figs. 5 and 6). In long-term exposure groups at both sublethal concentrations the absolute and relative weights of testis showed significant ($P<0.05$) reduction after 30 and 60 d than the respective control tissues (Figs. 5 and 6). In the treatment withdrawal group, the

absolute and relative weights of testis were found similar to that of corresponding control groups (Figs. 5 and 6).

4.5 Effects of fullerene C_{60} on the antioxidant status in ovary

In short-term exposure groups, fullerene C_{60} nanomaterial exposed at low sublethal concentration showed no notable changes in the activities of antioxidant enzymes such as superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase (Figs. 7-10). Similarly, the levels of hydrogen peroxide generation and lipid peroxidation was found similar to the respective control groups (Figs. 11 and 12). Fish exposed to high sublethal concentration of fullerene C_{60} for 96 h showed significant ($P<0.05$) decrease in the activities of all antioxidant enzymes with concomitant increase in the levels of hydrogen peroxide generation and lipid peroxidation (Figs. 7-12).

In long-term exposure groups, at both sublethal concentrations there was a significant ($P<0.05$) reduction in the activities of all antioxidant enzymes with associated significant ($P<0.05$) increase in the levels of hydrogen peroxide generation and lipid peroxidation (Figs. 7-12). The significant changes observed in ovarian tissue were prominent based on duration and concentration of fullerene C_{60} nanomaterial. In the treatment withdrawal group, all parameters evaluated for the antioxidant status showed similar changes like that of the long-term treatment groups (Figs. 7-12).

4.6 Effects of fullerene C_{60} on the antioxidant status in testis

The sublethal effects of fullerene C_{60} in testicular antioxidant status evaluated by the analysis of antioxidant enzymes such as

superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase showed significant ($P<0.05$) reduction after 7 d and 96 h at 5 mg/ L and 10 mg/ L concentrations of fullerene C_{60}, respectively (Figs. 13-16). However, the levels of hydrogen peroxide generation and lipid peroxidation increased significantly ($P<0.05$) after 7 d and 96 h onwards after low and high sublethal concentrations of fullerene C_{60} exposure, respectively which was duration-dependent (Figs. 17 and 18). After the treatment withdrawal, the antioxidant status of testis failed to recover to its normal state as evidenced by the similar observations like that of the long-term treatment groups (Figs. 13-18).

4.7 Effects of fullerene C_{60} on the histomorphology of ovary

The negative control and vehicle control tissues of ovary in the freshwater fish, *Anabas testudineus* showed normal histoarchitecture having mature vitellogenic and pre-vitellogenic oocytes (Fig. 19). Histological examination of ovary was performed in the last durations of short-term and long-term exposure groups, i.e., after 96 h and 60 d durations of both sublethal concentrations. Ovarian tissue exposed to 5 mg/ L concentration of fullerene C_{60} nanomaterial for 96 h showed atretic oocytes, whereas at high sublethal concentration showed both atretic and degenerated oocytes (Fig. 19).

In long-term exposure group, fullerene C_{60} exposed at low concentration showed severe degeneration of oocytes and atretic follicles, whereas at high sublethal concentration the severity of ovarian lesions were noted with degenerated oocytes and formation of melano-macrophage centers (Fig. 20). The histomorphological

alterations persisted in the treatment withdrawal groups after the recovery period of 60 d on low and high sublethal fullerene C_{60} treatment as indicated by degenerated oocytes, atretic follicles and vacuole formation (Fig. 20).

4.8 Effects of fullerene C_{60} on the histomorphology of testis

Histomorphology of negative control and vehicle control testis showed normal histoarchitecture predominantly occupied by mature spermatozoa (Fig. 21). In 96 h exposure group, the morphological changes such as reduction in spermatocyte number, vacuole formation, and empty seminiferous tubule was observed at low concentration. The same exposure group treated at high sublethal concentration of fullerene C_{60} showed break in seminiferous tubule, empty lobules, vacuolization, and reduction in spermatocyte (Fig. 21). The severity of testicular alterations increased on increase in duration to 60 d, which was observed with some common lesions like broken seminiferous tubule, vacuolization, empty seminiferous tubules with less number of spermatocytes and spermatozoa at both sublethal concentrations (Fig. 22). The testis of treatment withdrawal groups were found similar to fullerene C_{60} exposed groups thereby suggested that no recovery of tissue damage occurred in the testis (Fig. 22).

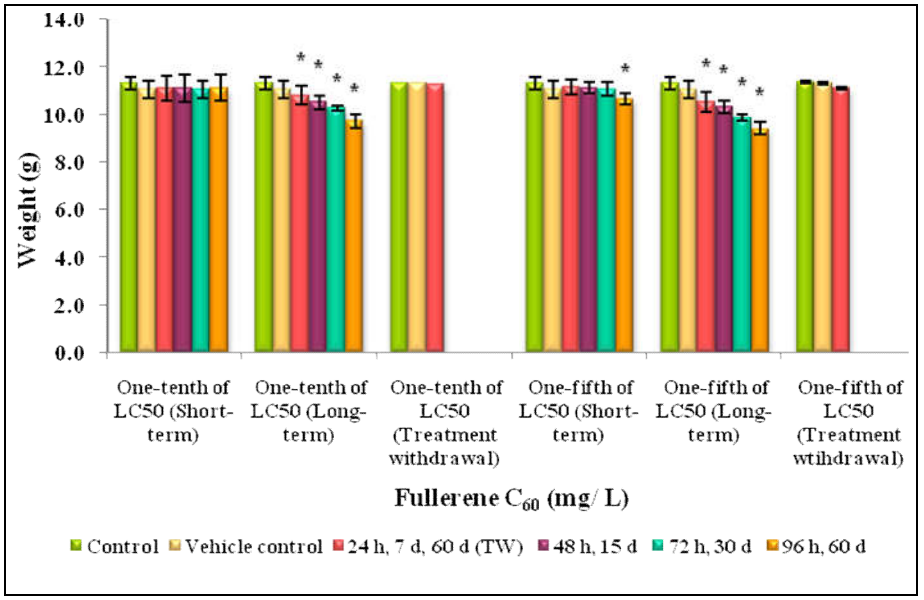

Fig. 1 Effect of C_{60} fullerene nanomaterial on the body weight of *Anabas testudineus* (n=10/ group; Mean ± SD; *P<0.05 against the control groups)

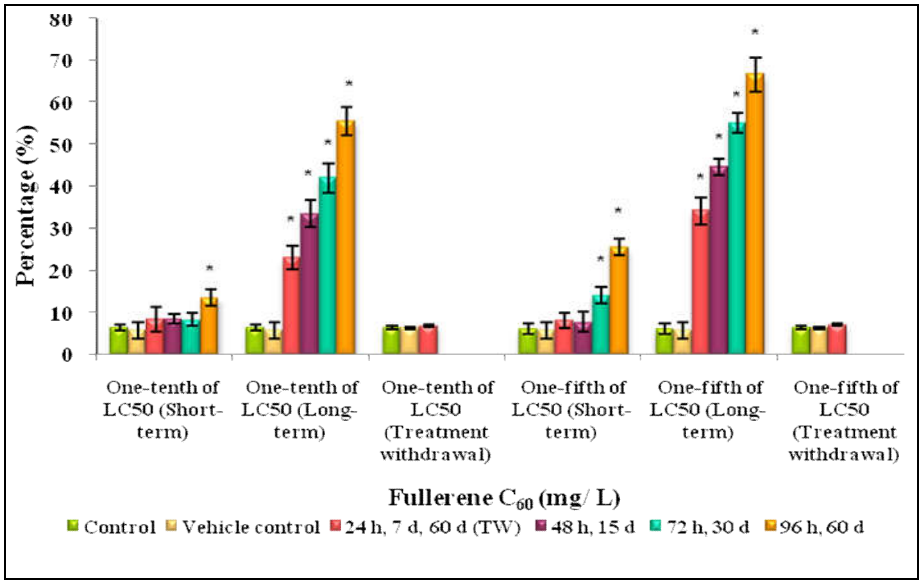

Fig. 2 Effect of C_{60} fullerene nanomaterial on mucous deposition in *Anabas testudineus* (n=10/ group; Mean ± SD; *P<0.05 against the control groups)

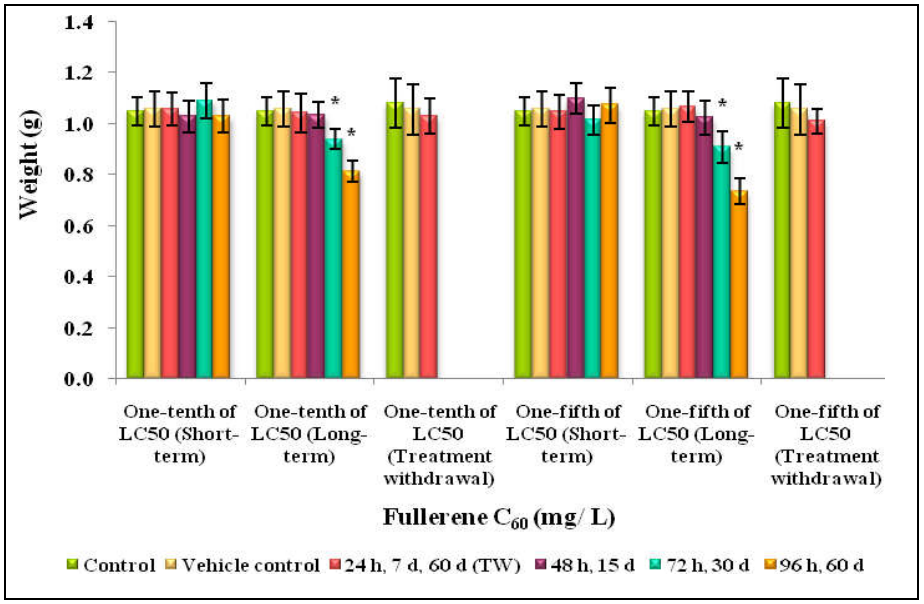

Fig. 3 Effect of C_{60} fullerene nanomaterial on the weight of ovary in *Anabas testudineus* (n=10/ group; Mean ± SD; *P<0.05 against the control groups)

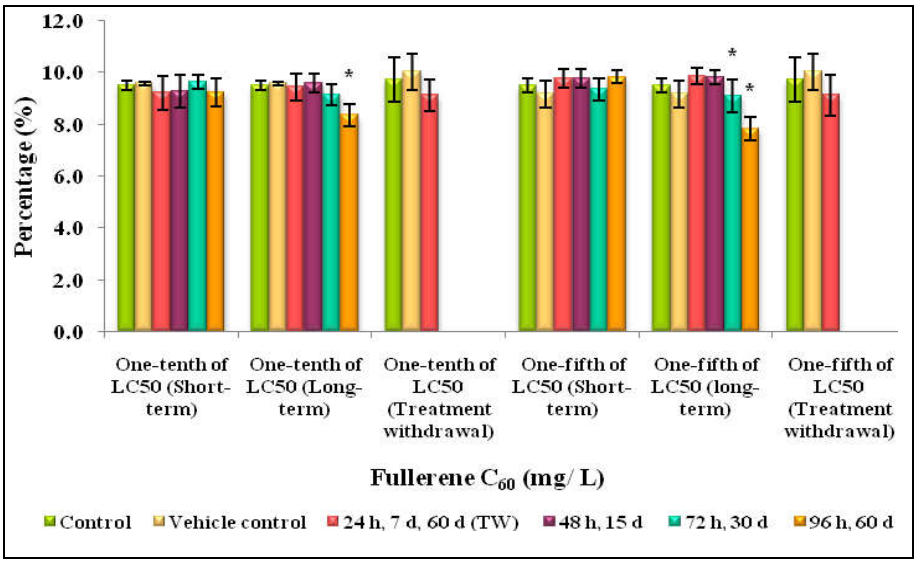

Fig. 4 Effect of C_{60} fullerene nanomaterial on the gonadosomatic index of ovary in *Anabas testudineus* (n=10/ group; Mean ± SD; *P<0.05 against the control groups)

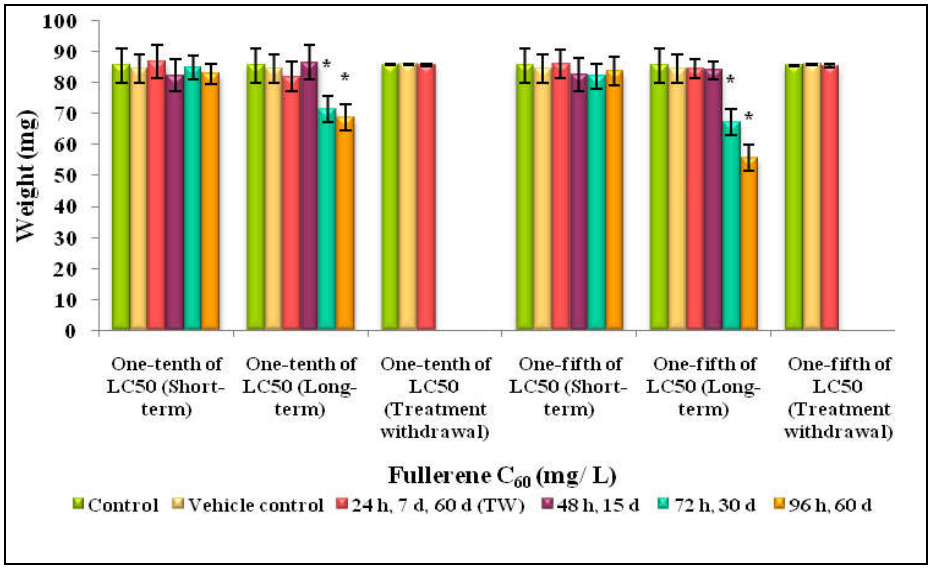

Fig. 5 Effect of C_{60} fullerene nanomaterial on the weight of testis in *Anabas testudineus* (n=10/ group; Mean ± SD; *P<0.05 against the control groups)

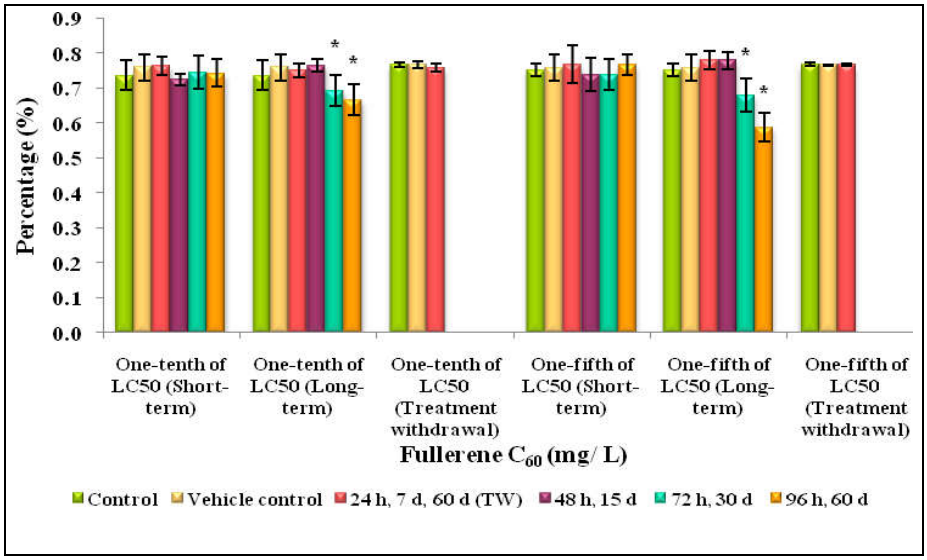

Fig. 6 Effect of C_{60} fullerene nanomaterial on the gonadosomatic index of testis in *Anabas testudineus* (n=10/ group; Mean ± SD; *P<0.05 against the control groups)

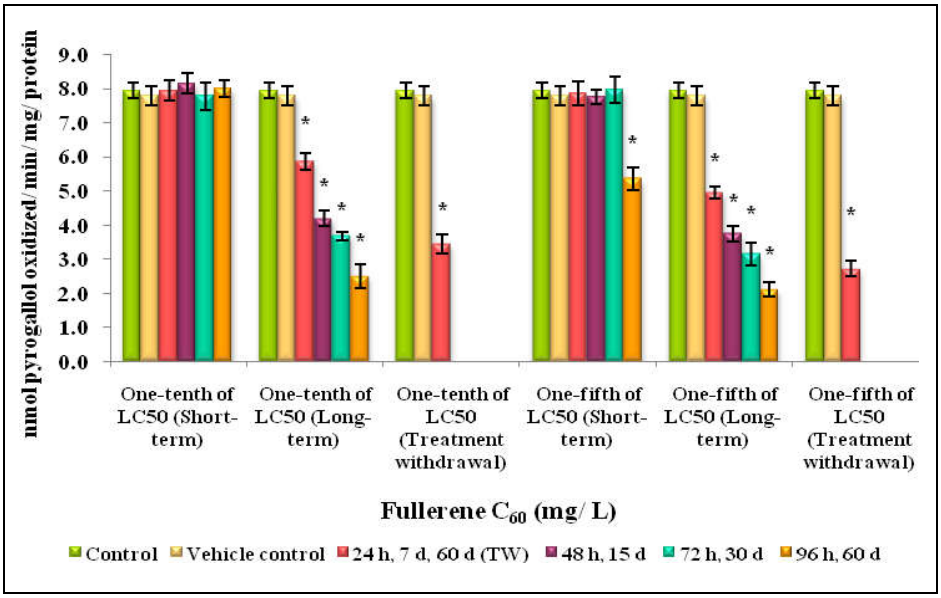

Fig.7 Effect of C_{60} fullerene nanomaterial on the activity of superoxide dismutase in ovary of *Anabas testudineus* (n=10/ group; Mean ± SD; *P<0.05 against the control groups)

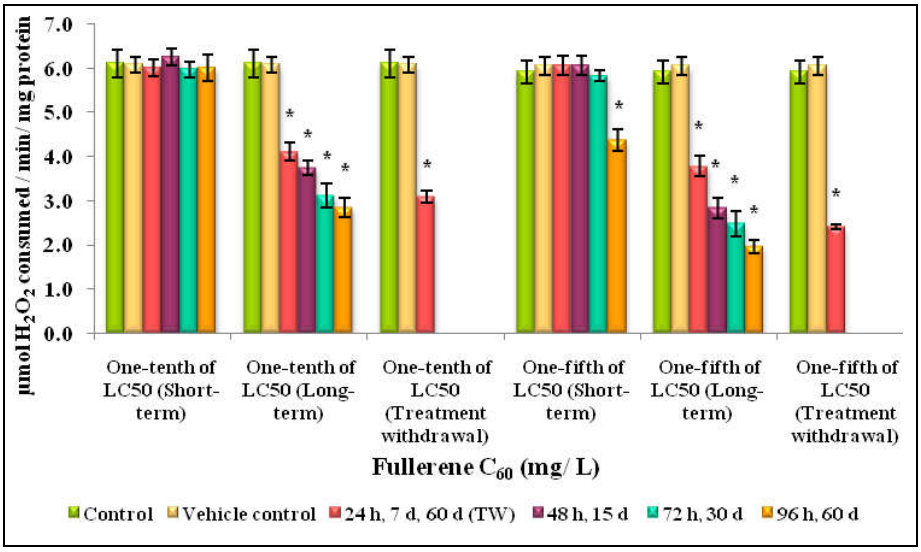

Fig. 8 Effect of C_{60} fullerene nanomaterial on the activity of catalase in ovary of *Anabas testudineus* (n=10/ group; Mean ± SD; *P<0.05 against the control groups)

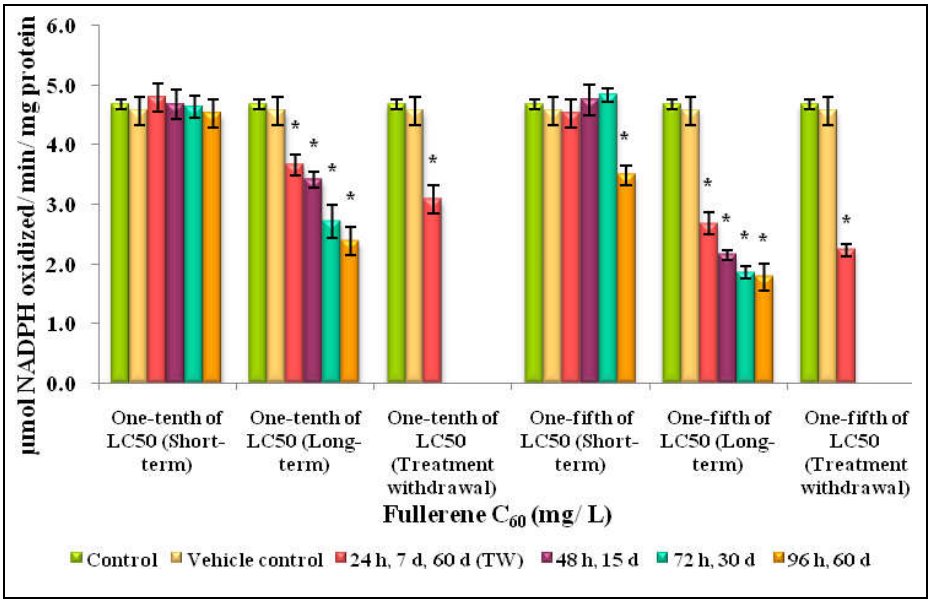

Fig. 9 Effect of C_{60} fullerene nanomaterial on the activity of glutathione reductase in ovary of *Anabas testudineus* (n=10/ group; Mean ± SD; *P<0.05 against the control groups)

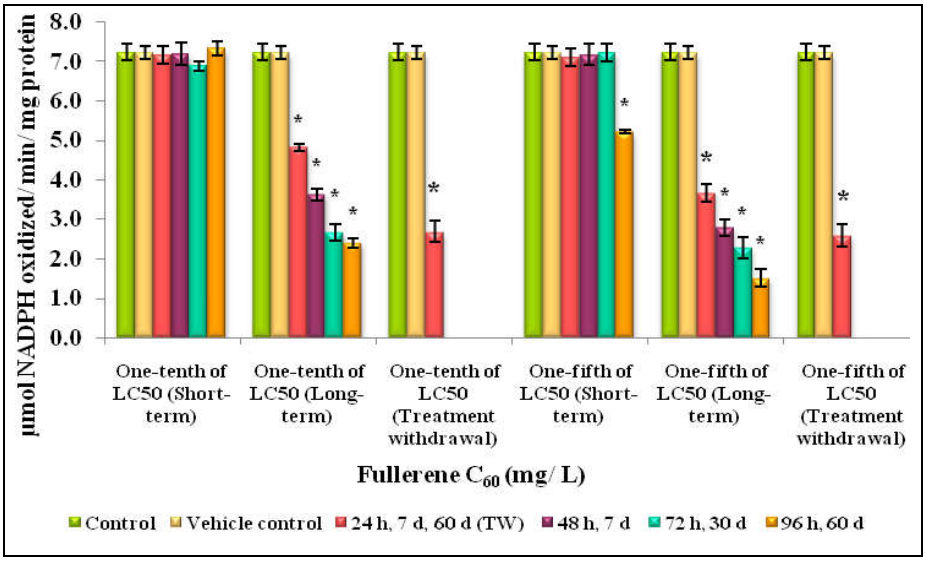

Fig. 10 Effect of C_{60} fullerene nanomaterial on the activity of glutathione peroxidase in ovary of *Anabas testudineus* (n=10/ group; Mean ± SD; *P<0.05 against the control groups)

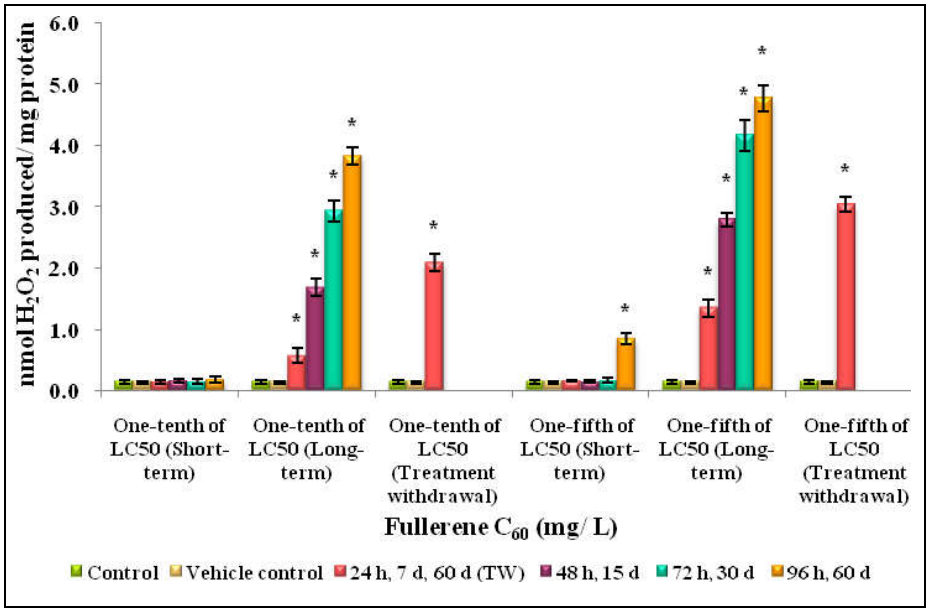

Fig. 11 Effect of C_{60} fullerene nanomaterial on the level of hydrogen peroxide generation in ovary of *Anabas testudineus* (n=10/ group; Mean ± SD; *P<0.05 against the control groups)

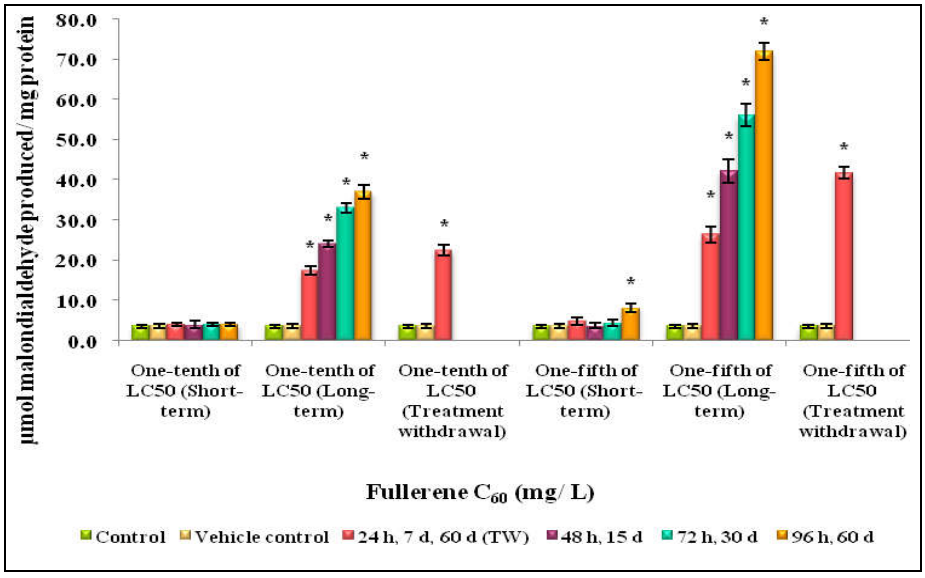

Fig. 12 Effect of C_{60} fullerene nanomaterial on the level of lipid peroxidation in ovary of *Anabas testudineus* (n=10/ group; Mean ± SD; *P<0.05 against the control groups)

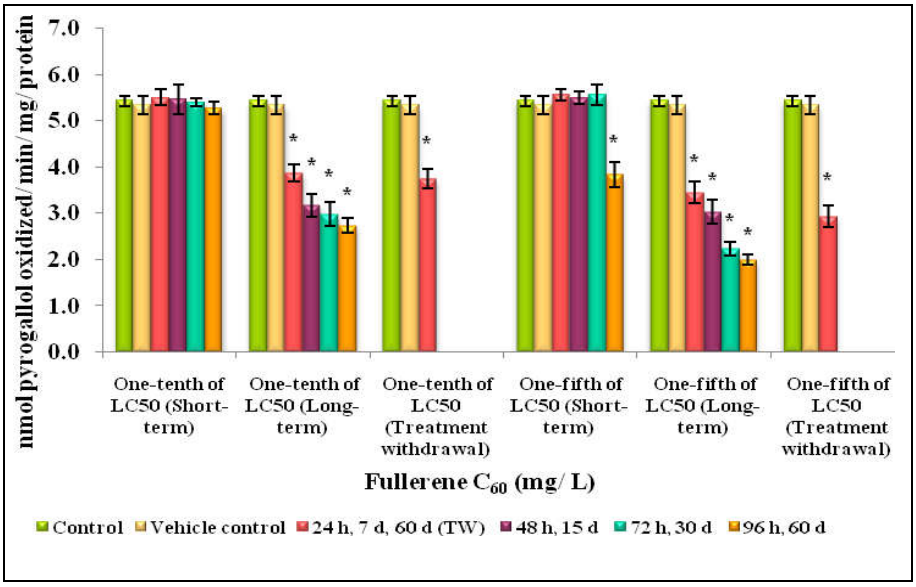

Fig. 13 Effect of C_{60} fullerene nanomaterial on the activity of superoxide dismutase in testis of *Anabas testudineus* (n=10/ group; Mean ± SD; *P<0.05 against the control groups)

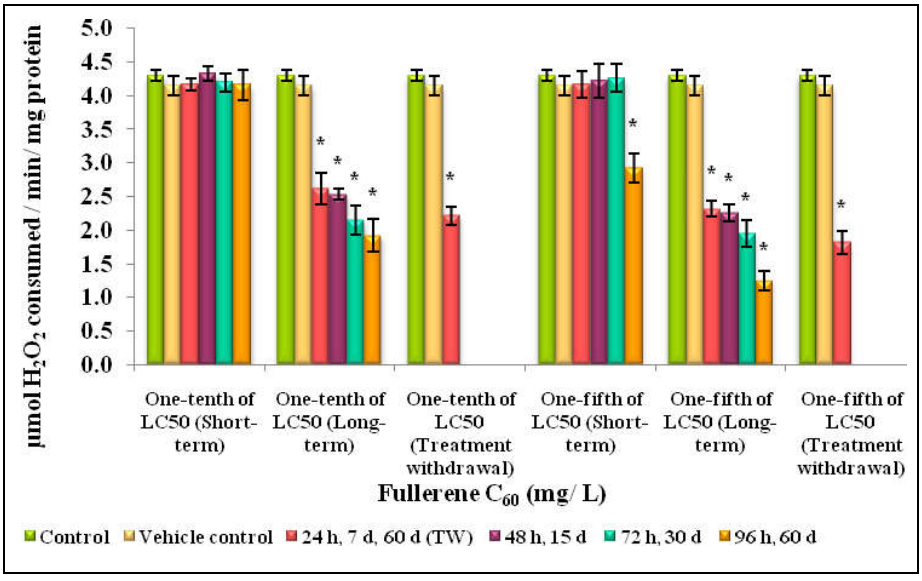

Fig. 14 Effect of C_{60} fullerene nanomaterial on the activity of catalase in testis of *Anabas testudineus* (n=10/ group; Mean ± SD; *P<0.05 against the control groups)

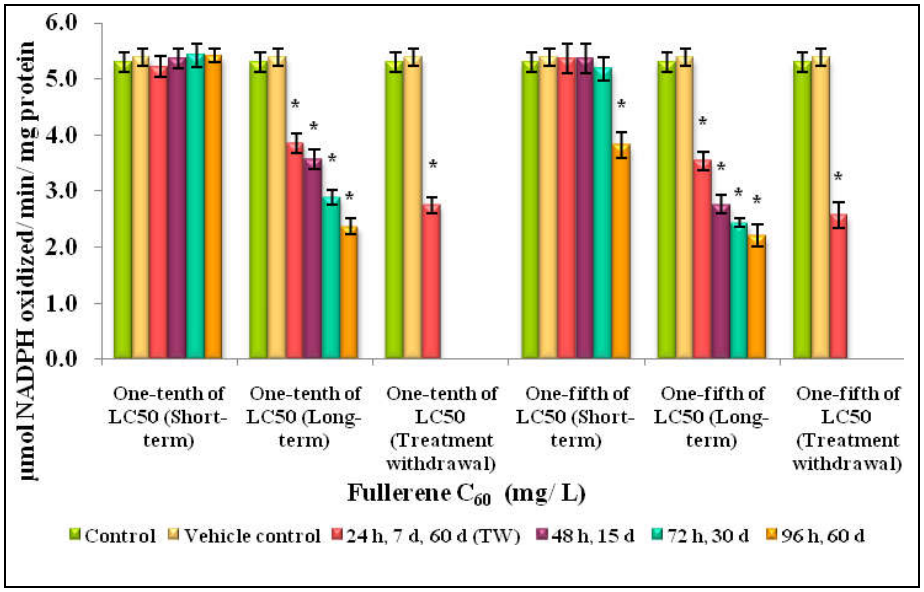

Fig. 15 Effect of C_{60} fullerene nanomaterial on the activity of glutathione reductase in testis of *Anabas testudineus* (n=10/ group; Mean ± SD; *P<0.05 against the control groups)

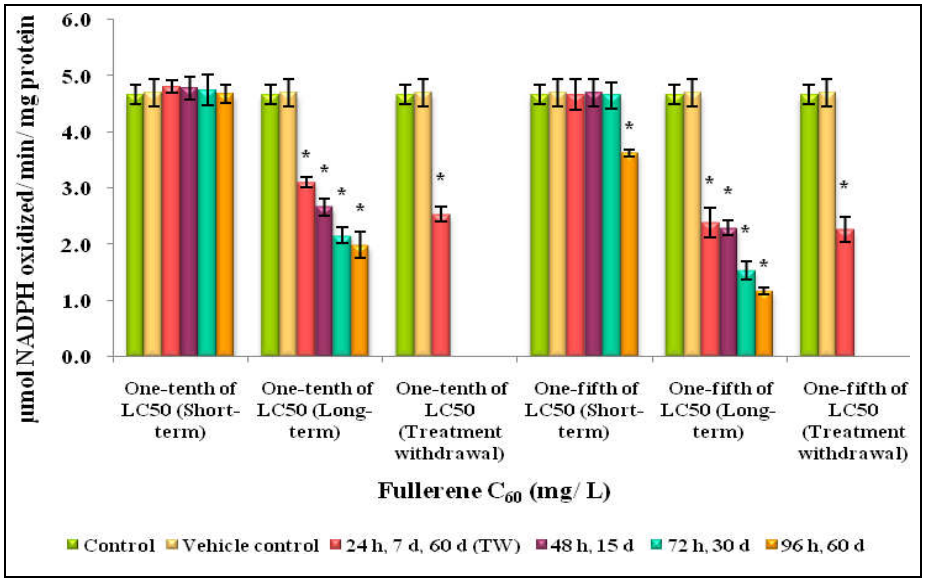

Fig. 16 Effect of C_{60} fullerene nanomaterial on the activity of glutathione peroxidase in testis of *Anabas testudineus* (n=10/ group; Mean ± SD; *P<0.05 against the control groups)

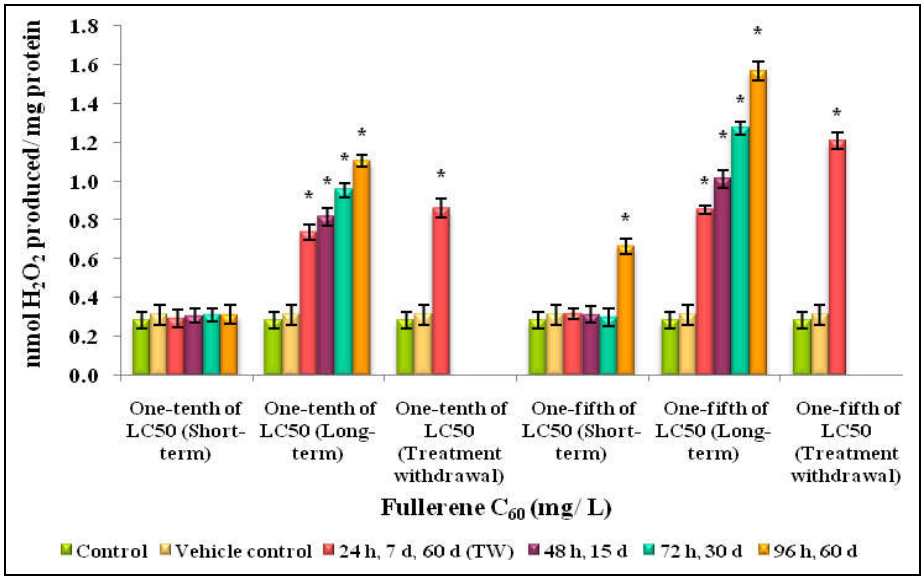

Fig. 17 Effect of C_{60} fullerene nanomaterial on the level of hydrogen peroxide generation in testis of *Anabas testudineus* (n=10/ group; Mean ± SD; *P<0.05 against the control groups)

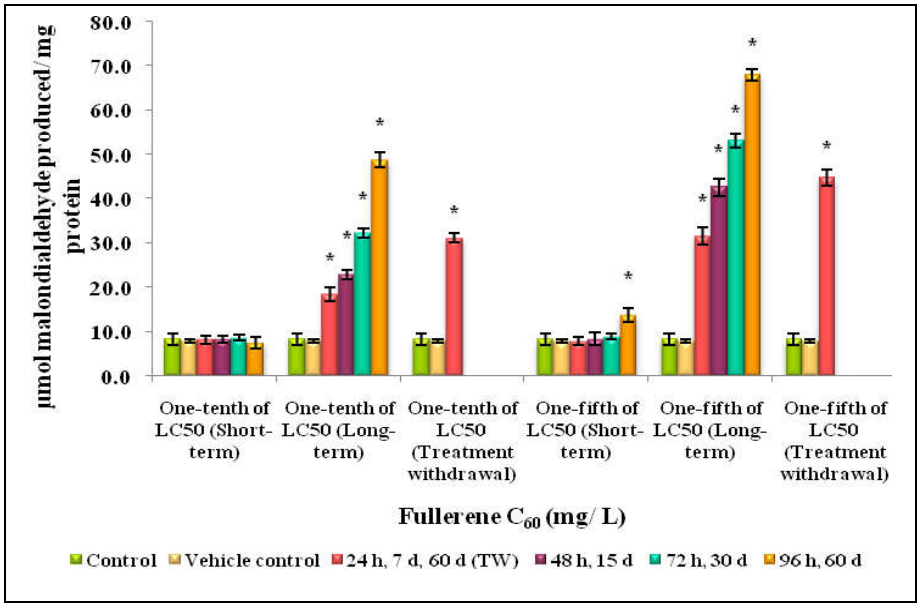

Fig. 18 Effect of C_{60} fullerene nanomaterial on the level of lipid peroxidation in testis of *Anabas testudineus* (n=10/ group; Mean ± SD; *P<0.05 against the control groups)

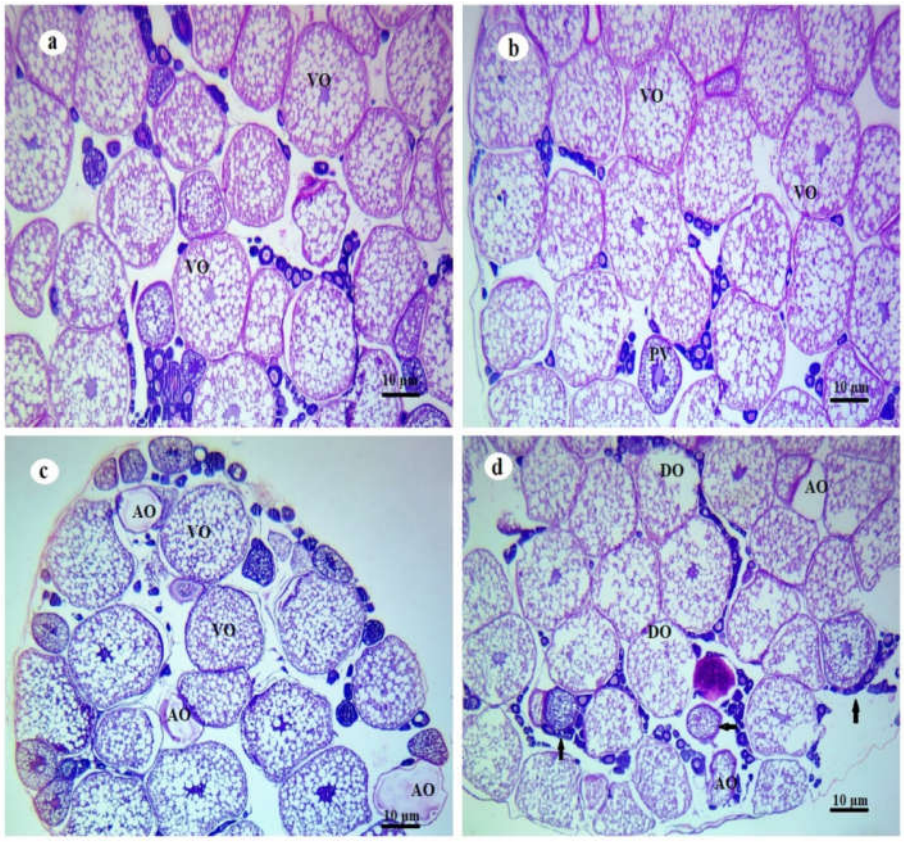

Fig. 19 Photomicrograph of ovary (T.S) of *Anabas testudineus* (a-d) (H & E). a-negative control ovary; b-vehicle control ovary (1% DMSO); c- Fullerene C_{60} (5 mg/ L) exposed for 96 h; d- Fullerene C_{60} (10 mg/ L) exposed for 96 h showing vitellogenic oocyte (VO), previtellogenic oocyte (PV), atretic oocyte (AO), thickening of oocyte membrane (←), degenerated oocyte (DO) and atretic follicle (↑)

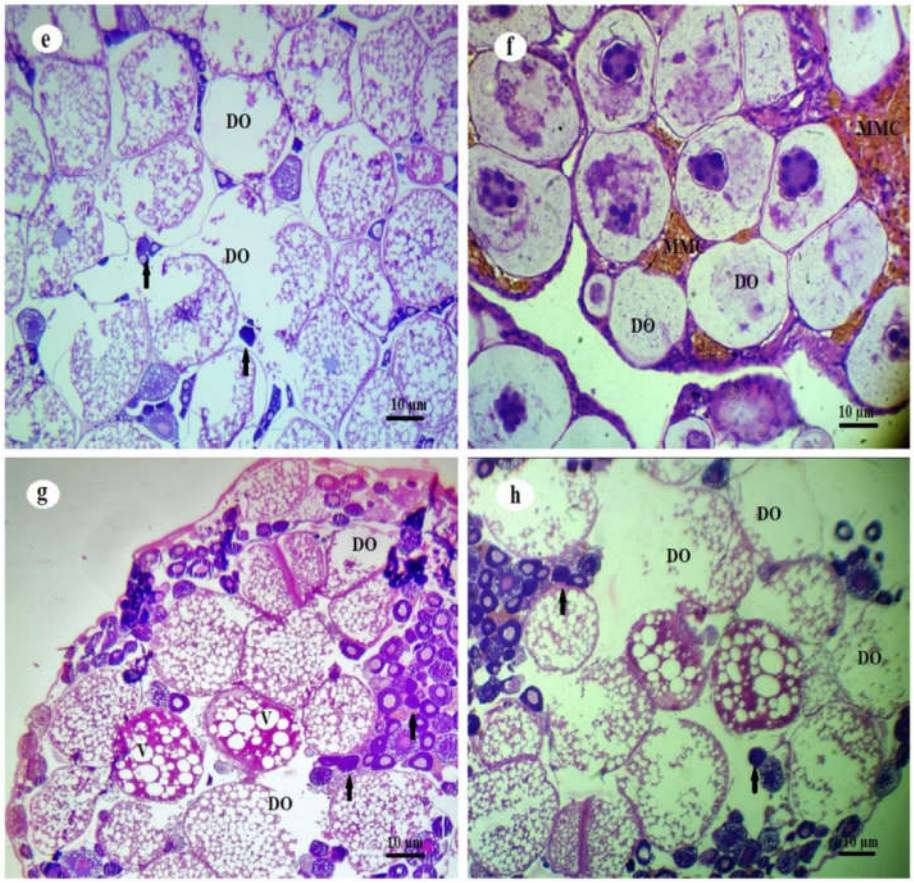

Fig. 20 Photomicrograph of ovary (T.S) of *Anabas testudineus* (e-h) (H & E). e- Fullerene C_{60} (5 mg/ L) exposed for 60 d; f- Fullerene C_{60} (10 mg/ L) exposed 60 d; g- Treatment withdrawal group after fullerene C_{60} (5 mg/ L) exposed for 60 d; h- Treatment withdrawal group after fullerene C_{60} (10 mg/ L) exposed for 60 d showing degenerated oocyte (DO), atretic follicle (↑), vacuoles (V) and melano-macrophage centers (MMC)

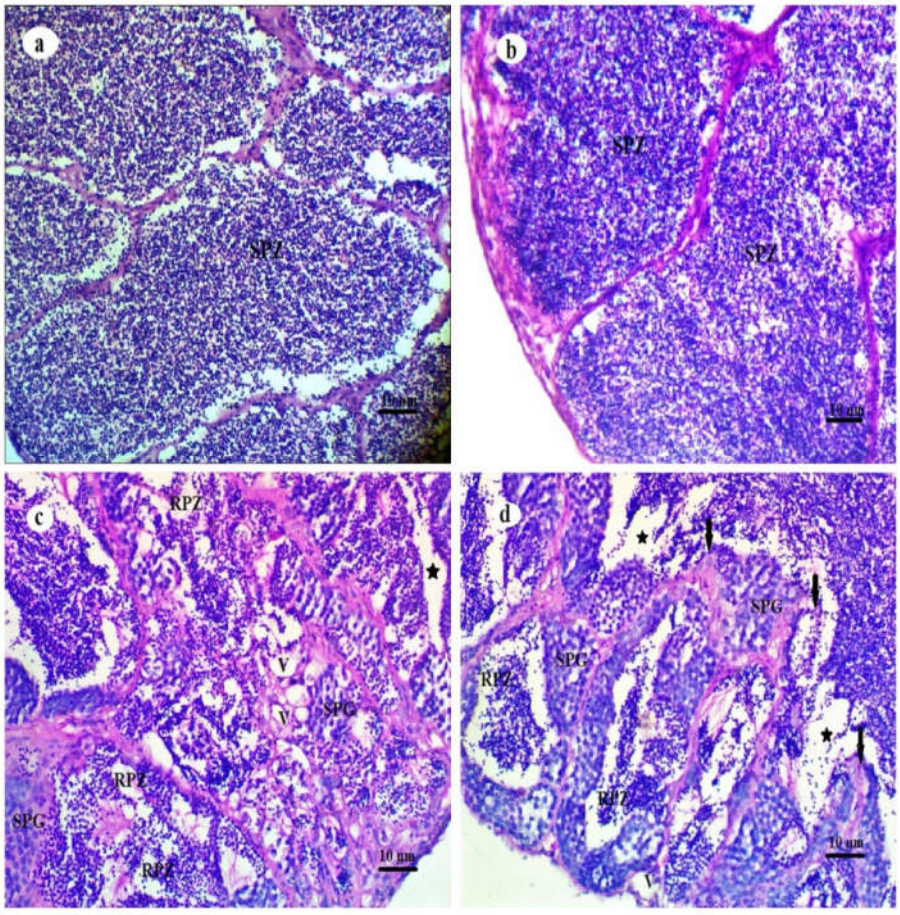

Fig. 21 Photomicrograph of testis (T.S) of *Anabas testudineus* (a-d) (H & E). a-negative control testis; b-vehicle control testis (1% DMSO); c- Fullerene C_{60} (5 mg/ L) exposed for 96 h; d- Fullerene C_{60} (10 mg/ L) exposed for 96 h showing densely packed spermatozoa (SPZ), reduced number of spermatozoa (RPZ), vacuoles (V), empty seminiferous lobule (*), spermatogonial cells (SPG) and broken seminiferous tubule (↓)

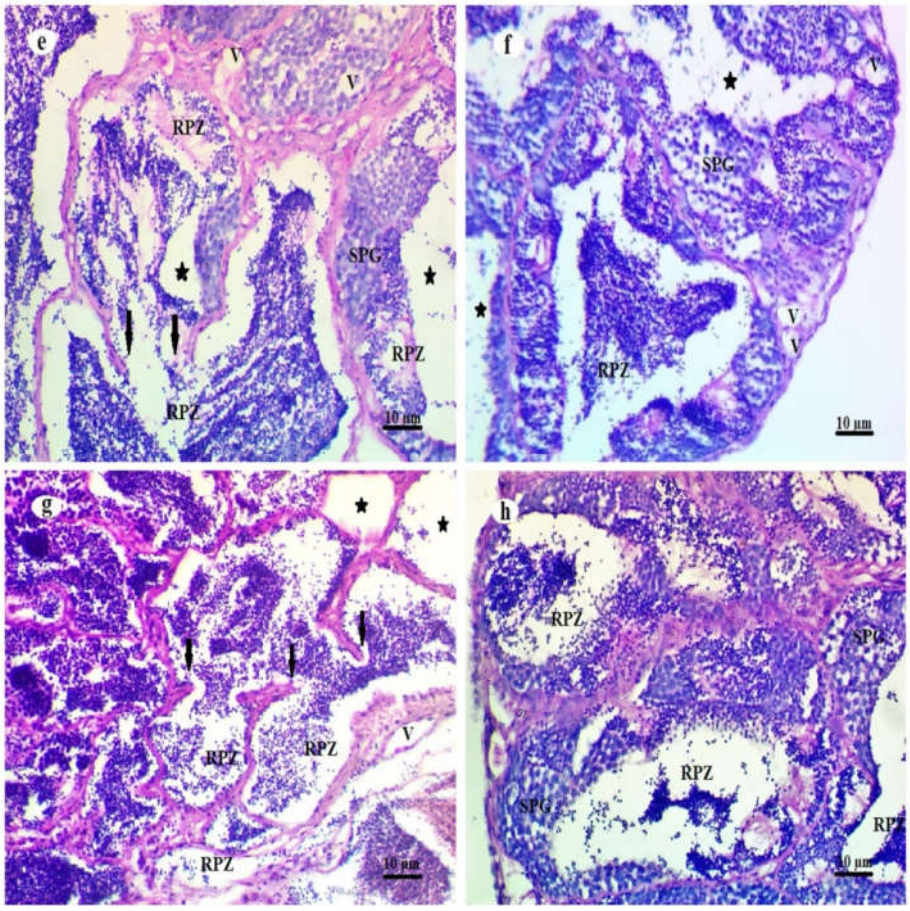

Fig. 22 Photomicrograph of testis (T.S) of *Anabas testudineus* (e-h) (H & E). e- Fullerene C_{60} (5 mg/ L) exposed for 60 d; f- Fullerene C_{60} (10 mg/ L) exposed 60 d; g- Treatment withdrawal group after fullerene C_{60} (5 mg/ L) exposed for 60 d; h- Treatment withdrawal group after fullerene C_{60} (10 mg/ L) exposed for 60 d showing reduced number of spermatozoa (RPZ), vacuoles (V), empty seminiferous lobule (*), spermatogonial cells (SPG) and broken seminiferous tubule (↓)

5.1 Sublethal effects of fullerene C_{60} nanomaterial on the body weight of the fish, *Anabas testudineus*

Exposure to fullerene C_{60} nanomaterial at two sublethal concentrations, such as 5 and 10 mg/ L, for short-term durations (24, 48, 72 and 96 h) did not alter the body weight of the fish, *Anabas testudineus,* except for 96 h at high sublethal concentration. The less pronounced effect on the body weight could be due to the short duration of exposure. However, the time-dependent reduction in the body weight of animal observed in long-term exposure groups such as 7, 15, 30 and 60 d indicated the long-term risk of the exposed nanomaterial. Therefore, the change in the magnitude of response over the period of exposure to fullerene nanomaterial can be used as an endpoint for the realistic view of long-term risk effects in the field. The body weight of animal observed regained after the treatment withdrawal for a period of 60 d, which suggested the attempt of the exposed fish to recover from the stress condition.

One of our previous findings also observed similar decline in the body weight of the freshwater fish, *Etroplus maculatus* after the dietary exposure of fullerene C_{60} nanomaterial (Sumi and Chitra, 2015). Chronic exposure of fullerene C_{60} aggregates to the freshwater fish, *Carassius auratus* also showed similar growth inhibition (Zhu *et al.,* 2008). On contrary, when fullerene C_{60} was administered orally to rats did not alter the body weight (Takahashi *et al*., 2012). Besides the carbon nanomaterial, other nanoparticles as titanium dioxide exposed to the goldfish, *Carassius auratus* showed significant reduction in the body weight (Ates *et al*., 2013). Aluminium oxide nanoparticles exposed for 30 and 60 d has been shown to reduce the body weight of

the fish, *Oreochromis mossambicus* with recovery in the weight loss after 60 d of treatment withdrawal (Vidya and Chitra, 2018a).

5.2 Effects of fullerene C_{60} on mucous deposition in the fish

The fish epidermis is a protective barrier against the waterborne toxicants as it possesses numerous goblet cells, which secrete thick mucous layer over the surface of body and around the gills (Coello and Khan, 1996). In uncontaminated environment, fish mucous functions to gather suspended nutrients around the water (Cone, 2009). In the present study, *Anabas testudineus* when exposed to fullerene C_{60} induced the secretion of mucous over the surface of body with a thickness of 3 to 7 folds, particularly at 10 mg/ L concentration, which indicated the stimulation of goblet cells to overcome the nanomaterial toxicity. The thick mucous deposition on the body surface, especially on the gill prevent the normal gas exchange, and result in reduced opercular ventilation rate, oxygen consumption and increase in surfacing behaviour, which was evident from our previous finding on another freshwater fish, *Pseudetroplus maculatus* exposed to fullerene C_{60} through diet (Sumi and Chitra, 2015).

Single-walled carbon nanotubes and titanium dioxide nanoparticles exposed to rainbow trout, *Oncorhynchus mykiss* showed increased mucous secretion on the gill surface (Smith *et al*., 2007; Federici *et al*., 2007). Similarly, copper nanoparticles exposed to zebrafish increased mucous deposition on the surface of gill (Griffitt *et al*., 2007). One of the studies reported that the size of the nanoparticles influences the secretion of mucous where silver nanoparticles of 10 nm sizes had shown remarkable increase in mucous secretion on gill surface than 35 nm and 600-1600 nm size nanoparticles in rainbow

trout thereby indicated more toxicity on the small size nanoparticles (Scown *et al*., 2010).

5.3 Effects of fullerene C_{60} on absolute and relative weights of gonads

The differences in the weights of gonads are considered as a good indicator to predict the adverse effects of toxicants in reproductive system (Bailey *et al*., 2004). Deposition of nanoparticles in gonads lead to several adverse effects including decreased weight and growth of gonadal tissues, and altered morphology of seminiferous tubules and oocytes (Wang *et al.,* 2018). In the present observation, sublethal treatment of fullerene C_{60} nanomaterial at both concentrations for 30 and 60 d caused significant reduction in the weights of ovary and testis, and this could be due to treatment-related tissue damage. The lesions in ovarian and testicular tissues after the nanomaterial exposure was further confirmed by histological analysis, and discussed in brief in the respective sections of this chapter.

Gonado-somatic index or the relative weights of gonads provide general indicator of normal growth and development in relation to the body weight of animal. Sublethal exposure of fullerene C_{60} nanomaterial at both concentrations for 30 and 60 d showed drastic decrease in the gonadosomatic index of ovary and testis, which revealed the retarded gonadal development. However, the treatment withdrawal group showed the gonadal weight similar to the control groups thereby indicated recovery of weight loss when not exposed to the nanomaterial, however the tissue damages persisted. The present observations was supported by another findings in which zinc sulphide nanoparticles exposed to the Asian striped catfish, *Mystus vittatus* showed reduction in the absolute weight and gonadosomatic index

with increasing nanoparticle concentrations, which ultimately disturbed the growth and gonadal maturity (Chatterjee *et al*., 2014).

5.4 Effects of fullerene C_{60} on the antioxidant status in gonads

Fish gonads are naturally equipped with well-defined antioxidant defense system to eradicate the free radical damage induced by any toxicants, however, continuous exposure of pollutants are suspected to imbalance the pooxidant/antioxidant status in the gonadal tissues. Besides, oxidative stress caused due to the imbalance in redox state lead to reproductive toxicity, which in turn affect gonadal growth, maturation, differentiation and its physiological functions leading to hormonal imbalance, histomorphological changes, downregulation of gene expression, and become reproductively unfit in the ecosystem thereby decline in the population rate. Therefore, the study of antioxidant status in reproductive system of fish is of high importance, and ecologically relevant topic to encourage towards the risk assessment and management of toxicants released into the aquatic ecosystems.

Several literatures reviewed in this chapter demonstrated the dual properties of fullerene C_{60} nanomaterial as prooxidant and antioxidant in various animal models. The results of the current study showed that on prolonged exposure i.e., for a period of 60 d, to the freshwater fish, *Anabas testudineus* at two sublethal concentrations such as 5 and 10 mg/ L decreased the activities of all antioxidant enzymes namely superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase in time-dependent manner. However, the levels of hydrogen peroxide generation and lipid peroxidation increased in all long-term exposure groups. These observations were

found similar in both ovary and testis of the exposed fish when compared to the respective control tissues.

It is well known fact that antioxidants protect cells or tissues from the endogenous prooxidants as well as from the exogenous environmental pollutants so as to maintain the normal oxido-reductive homeostasis. The present findings showed a time-dependent decrease in the activities of antioxidant enzymes, which suggested the failure of enzymatic antioxidants to scavenge the free radicals formed owing to the nanomaterial exposure. It was further confirmed by the elevated level of hydrogen peroxide generation, which was also duration-dependent. The elevated level of lipid peroxidation in gonads indicated that C_{60} exposure disturbed the membrane integrity and fluidity. Thus C_{60} fullerene elicited oxidative stress in gonads of the freshwater fish, *Anabas testudineus*.

Besides, the elevated lipid peroxidation also damages lipoproteins, protein aggregation and fragmentation, and also inhibit steroidogenic enzyme activities (Aitken *et al*., 2003). Fullerene C_{60} exposure decreased the activities of steroidogenic enzymes, namely 3β-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase in ovary and testis of the fish, which will be discussed in chapter 3. Thus the exogenous toxicant, fullerene nanomaterial, generated reactive oxygen species in ovary and testis by disrupting the antioxidant status. Such negative impact of fullerene C_{60} nanomaterial has been reported in gill, brain, liver and muscle tissues of the freshwater fish, *Pseudetroplus maculatus* (Sumi and Chitra, 2016; 2017a; 2017b; 2017c). The study was also in agreement with other findings on exposure of nanoscale zerovalent iron to the adult medaka fish, *Oryzias latipes* caused nano-specific reproductive toxicity by the

induction of oxidative stress in female gonads (Yang *et al*., 2019). In another study, chronic exposure of silver nanoparticles induced oxidative stress and germ cells apoptosis mediated through mitochondrial-dependent pathway, and ultimately impaired the reproduction in zebrafish (Ma *et al*., 2018).

In the treatment withdrawal study, the gonads failed to recover from the oxidative stress caused by the nanomaterial as evidenced by the reduction in the activities of all antioxidant enzymes with concomitant rise is the levels of hydrogen peroxide and lipid peroxidation, which indicated the accumulation and persistence of nanomaterial in gonadal tissue. Similar persistent toxic effects after the treatment withdrawal of nanomaterials such as aluminium oxide, silicon dioxide and titanium dioxide have been documented in the gill, liver and brain tissues of the freshwater fish, *Oreochromis mossambicus* (Vidya and Chitra, 2018a; 2018b; 2018c).

5.5 Effects of fullerene C_{60} on the histomorphology of ovary

Histomorphological analysis in tissues is an excellent biomarker for xenobiotic toxicity, which enables for the better understanding of deleterious effects of aquatic pollutants when compared to a single biochemical parameter (Poleksic *et al*., 2010). Ovarian tissue exposed to fullerene C_{60} nanomaterial at 5 mg/ L concentration for 96 h showed atretic oocytes, whereas at 10 mg/ L concentration showed atretic and degenerated oocytes. At 60 d exposure period, ovary showed severe degeneration of oocytes and atretic follicles at low concentration, and the severity of ovarian lesions increased at high concentration with the formation of melanomacrophage centers.

Follicular atresia is the degeneration or breakdown of ovarian follicles where it represents marked phagocytic activity with digestive vacuoles, myelin figures, and lipofuscin granules (Miranda *et al*., 1999). Atresia occurs during normal reproductive cycle as well as due to the exposure of toxicants, which affect the reproductive potential of females by reducing the healthy eggs that a female can spawn (Gonzalez-Kother *et al*., 2019). The current findings illustrated that the nanomaterial disrupted the reproductive ability of fish by the induction of atresia, which could be the reason for the reduction in absolute and relative weight of ovary as discussed earlier in this chapter. The study was in agreement when zinc sulphide nanoparticle exposed to the ovary of catfish, *Mystus tengara* (Chatterjee and Bhattacharjee, 2016).

Melanomacrophage centers observed in the ovary of fish exposed to high concentration of fullerene nanomaterial after long-term period indicated ovarian regression. Macrophage aggregates were found abundant only in regressing ovaries after spawning (Micale *et al*., 2019), however, the lesions found in the vitellogenic phase of ovary indicated premature tissue regression due to the nanomaterial exposure. Similarly in another study, zinc oxide nanoparticle along with a surfactant, perfluorooctane sulphonate has altered the normal histology of ovary with significant deformation such as cell swelling, damage in egg membrane and egg yolk (Du *et al*., 2014). The ovarian lesions persisted in the treatment withdrawal group showing degenerated oocytes, atretic follicles and vacuole formation, which indicated the persistent toxic effects of fullerene nanomaterial.

5.6 Effects of fullerene C_{60} on the histomorphology of testis

Fullerene C_{60} nanomaterial altered histology of testis showing reduction in spermatocyte number, vacuole formation, and empty

seminiferous tubule. The severity of tissue damage increased based on the duration of exposure and concentration of the nanomaterial. Testicular alterations such as vacuole formation, atresia, distortion of seminiferous epithelium, and reduction of spermatozoa could be the reasons for the decline in absolute and relative weights of testis as discussed in section 5.3 of this chapter. Similar observations were reported in zebrafish after exposure of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and bisphenol A (Baker *et al*., 2016; Lora *et al*., 2016). The present findings showed agreement with another study on titanium dioxide nanoparticles, which induced autophagy and necrosis in Sertoli cells, and consequently affected the number of spermatogenic cells and testicular morphology in zebrafish (Kotil *et al*., 2017). Besides, C_{60} also caused histopathological changes in various other tissues like gill, muscle, and liver in the freshwater fish, *Pseudetroplus maculatus* (Sumi and Chitra, 2017d).

The testis of treatment withdrawal groups did not show recovery of tissue damage thereby indicated the accumulation and persistence of fullerene C_{60} nanomaterial in the testis. Similarly, the other nanomaterials such as aluminium oxide, silicon dioxide, titanium dioxide and iron oxide nanoparticles induced irrecoverable persistent tissue damages in gill, liver and brain tissues of the freshwater fish, *Oreochromis mossambicus* (Vidya and Chitra, 2018d; 2018e; 2018f; 2019).

The present findings represent that sublethal exposure of fullerene C_{60} nanomaterial caused gonadal toxicity in the freshwater fish, *Anabas testudineus* mediated through the induction of oxidative stress, and the gonadal dysfunction was further proved by histology, as the standard biomarker of tissue damage.

1. Sublethal concentrations (5 mg/ L and 10 mg/ L) of fullerene C_{60} nanomaterial for long-term exposure caused growth inhibition in the freshwater fish, *Anabas testudineus* thereby revealed the risk effects of the toxicant.

2. Mucous deposition increased after fullerene C_{60} exposure as the primary defense of the fish to avoid the toxicant.

3. Gonadal toxicity of C_{60} fullerene was evident by the reduction in the absolute and relative weights of ovary and testis.

4. C_{60} nanomaterial induced reactive oxygen species generation in gonads as indicated by alteration in the antioxidant status in ovary and testis of the fish.

5. Sublethal exposure of fullerene caused prominent histomorphological changes in ovary and testis, which represented tissue-specific toxic effects of the nanomaterial.

6. Toxicity induced by fullerene C_{60} nanomaterial was not recovered within 60 d, since the oxidative imbalance and histomorphological changes persisted in gonads even after the treatment withdrawal.

7. To brief, fullerene C_{60} at sublethal concentrations caused oxidative stress, which in turn lead to gonadal dysfunction in the fish, *Anabas testudineus*.

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1.1 Reproductive system of fish

Teleost fish are the large and most diverse group of the class Actinopterygii that makes 96% of all fish species. Reproduction in teleost fish is usually by sexual mode, and only few are hermaphrodites where they use numerous strategies to care for the developing young ones. Most of them lay eggs, in which about 99% die or eaten up before they hatch. Reproduction is the most important process by which genetic material is transferred from one generation to the next, and the reproductive biology of teleost fishes have great diversity compared with the other vertebrates (Wootton and Smith, 2014). In teleost fishes, there are mixtures of gender systems, among which approximately 88% are gonochoristic, which means unisexualism or having atleast one distinct sex in an individual organism with clear specification of male and female characteristics. Only 2% of teleost fish species exhibit hermaphroditism as the gender system, which is exceptionally rare in other vertebrates (Patzner, 2008). Hermaphroditism are classified as simultaneous or sequential, the latter mode is further classified as protogynous, protandrous or bidirectional hermaphroditism, which is the most common one in teleost families (Avise and Mank, 2009). Parthenogenesis is rare among vertebrates, which is also found in few teleost fishes that transfer the female genome from one generation to another (Neaves and Baumann, 2011).

Most of the teleost fish displays external fertilization, in which the females release unfertilized planktonic eggs into the water and are shortly fertilized by the male fish. However, internal fertilization is

very rare where fish release the free-swimming larvae, or juveniles into the water bodies. Egg ripening and spawning are controlled by some ecological factors, level of hormones, and nutritional status of the female fish (Hempel, 1979). Maturation and spawning are not regulated by specific factors but by the combination of some endogenous and exogenous factors such as endocrine control, physiological and ecological factors, stress and the nutrition status of the female fish (Mule and Sarve, 2017). During development and maturation, the major portions of dietary energy reserves are utilized for the production of gametes, maintenance of reproductive bahaviours and for the development of secondary sexual characteristics (Wootton, 1998). Thus the biological success of an organism depends upon the number of offspring produced during the lifespan of an individual (Wootton, 1998). Moreover, fish adopt different strategies in the changeable environment to improve parental life expectancy, reproductive success and producing reproductively active offsprings (Saborido-Rey *et al*., 2010).

Fecundity of fish is positively related with the size of fish, nutritional status and spawning experience where the older and large fish spawn plentiful large sized eggs, which hatch into more viable and larger fry (Berkeley *et al*., 2004). Moreover, large fishes have longer spawning period, and the spawning frequency was higher in the batch spawner (Schaefer, 2001). Mature female fish with rich nutritional status shows early maturation, which produces more eggs, whereas fish living in poor feeding conditions have reduced oocyte growth and disrupted spawning (Lambert *et al*., 2000). Some environmental

factors such as photoperiod and temperature also influence in the reproductive success as well as growth, maturation, fecundity, and the quality of eggs in fish species (Lambert *et al*., 2003). Environmental and social cues also promote the reproductive behaviour, which is under the control of hypothalamo-pituitary-gonadal axis, by rise in the level of androgens in males and the levels of estrogens or progestins in females (Juntti and Fernald, 2016).

1.2 Stages of reproductive process

Fish comprises two important aspects for the successful production, growth, maturation and functions of gonads. The functions of two distinct gametes therefore depend upon the two processes namely gametogenesis and gonadal steroidogenesis. The germ cells of fish proliferate as a single gonadal primordium that splits into two bilateral primordial germ cells (Saito *et al*., 2007). Type I division is a self-renewal one, which divides into two daughter cells surrounded by the supporting cells, whereas type II division synchronously divide the cells resulting in the development of gametes (Saito *et al*., 2007). After the formation of oocyte and spermatocyte, the meiosis is arrested in prophase I leading into the differentiation process (Saborido-Rey, 2016). Based on the occurrence, gametogenesis is classified into two types namely oogenesis and spermatogenesis.

1.2.1 Oogenesis

Oogenesis is the dynamic process that occurs in ovary where oogonia are transformed into the mature oocytes (Lubzens *et al*., 2010). In all teleost fish species, the stages of oogenesis are relatively

constant, but the time required in each phase varies. In zebrafish and cyprinidae, oogenesis takes place within few days while in salmonids it require few months (Knoll-Gellida and Babin, 2007). During transition of oogonium into oocyte, the chromosome divides and produces two daughter chromatids that are linked with centromere, which form the primary oocyte enveloped by granulosa cells (Lubzens *et al*., 2010). The granulosa layer completely surrounds the oocyte to form ovarian follicle, which undergoes further growth and maturation (Grier *et al*., 2009).

During the primary growth of ovarian follicle along with protein synthesis for vitellogenin the appearance of cortical alveoli occurs, which play a significant role in ovum-sperm fertilization (Grier *et al*., 2009). In growth phase, the oocytes increase in volume covered with a proteinaceous structure called zona pellucida (Le Menn *et al*., 2007; Modig *et al*., 2007). The oocyte then enters into the secondary growth phase where the maturation occurs, which is essential for spawning (Tyler and Sumpter, 1996). At the end of meiosis II, several hydrolytic enzymes including serine proteases are involved in the breakdown of the basement lamina, and finally the mature oocyte is discharged into the ovarian lumen by ovulation process (Clelland and Peng, 2009).

1.2.2 Spermatogenesis

Spermatogenesis is a highly organized and co-ordinated process that occurs in testis leading to the formation of spermatozoa from spermatogonial cells (Schulz *et al*., 2010). The process consists

of various stages including differentiated spermatogonia, primary and secondary spermatocytes, spermatids and spermatozoa (Blum, 1986; Schulz *et al*., 2010). Type A and type B diploid spermatogonium are differentiated through first meiotic division to form primary spermatocyte, followed by two secondary spermatocytes having haploid number of chromosomes. The secondary spermatocyte then enters into second meiotic division to form four haploid spermatids (Rupik *et al*., 2011). Spermatid undergoes final differentiation process called spermiogenesis where they develop flagella, acrosome, compact small nucleus and DNA. In the last stage, disintegration of occluding junctions at the adluminal pole of Sertoli cells release the sperm into the tubular lumen by the process called spermiation (Schulz *et al*., 2010). Finally maturation of spermatozoa occurs in testis and gonoduct under the influence of hormones and external environment. Motility of sperm is acquired only in the osmotic environment with suitable pH and stable concentrations of K^+ or Ca^{2+} inorganic ions (Kinsey *et al*., 2007).

1.2.3 Gonadal steroidogenesis

Gonadal steroidogenesis includes ovarian and testicular steroidogenesis. Sex steroids are involved in the regulation of all aspects of vertebrate reproduction, including fish. The teleost fish produce various types of bioactive gonadal steroids including estrogens, androgens, progestogens and numerous other steroids from the specialized cells such as theca and granulosa cells found in ovarian follicle, and Leydig cells in testis (Young *et al*., 2005). The steroids produced at appropriate times play a major role in the direct

development of germ cells, organs and accessory glands, and behavioural modification to ensure the sexual reproduction (Young *et al*., 2005). The essential and prime substances required for steroid synthesis in gonads are cholesterol along with the steroidogenic enzymes of cytochrome P450 superfamily located in the inner mitochondrial membrane such as cytochrome P450 side-chain cleavage (P450scc). The enzyme system hydroxylates carbons 20 and 22 of cholesterol and removes isocaproaldehyde thereby generate the pregnenolone, which is the rate-limiting step (Stocco, 2001). The transport of cholesterol into the mitochondrial membrane was mediated by a sterol transporter protein named steroidogenic acute regulatory (StAR) protein (Stocco, 2002).

Pregnenolone serves as an immediate precursor for the formation of the androgens, dehydroepiandrosterone or androstenedione (Auchus and Miller, 1999). The shift in the steroidogenic pathway for the generation of androgens is regulated by lyase activity and the level of luteinizing hormone in males. Besides, several steroidogenic enzymes such as 3β-hydroxysteroid dehydrogenase, 17β-hydroxysteroid dehydrogenase are involved in the conversion of Δ5 and Δ4 pathway while the 20β-hydroxysteroid dehydrogenase are involved in the synthesis of 17, 20β-dihydroxy-4-pregnen-3-one, which is crucial for the maturation of gonads in several teleost fishes (Higashino *et al*., 2003).

Cytochrome P450 aromatase converts Δ4-androgens into estrogens, the potent female hormone. Estrogen also plays an important role in male reproduction by the proliferation of gonial stem

cells (Miura *et al*., 1999). Some of the enzymes such as reductases, hydroxylases, isomerases, and oxidoreductases convert the derivatives of androstene, pregnene, and estrene into other steroids. In teleost, gonads of male and female fish produce the potent androgen 11-ketotestosterone (11-KT), which is essential for the production of large number of germ cells, spermatogonial proliferation, and also for the successful fertilization of spawned eggs (Schulz and Miura, 2002).

1.3 Vitellogenin as the reproductive biomarker

Vitellogenin, the high molecular weight (300-640 kDa) glycophospholipoprotein is the main precursor molecule of egg yolk produced in liver under the influence of hormones (Mommsen and Korsgard, 2008). In liver, vitellogenin molecule undergoes post-translational modifications including phosphorylation, lipidation and glycosylation, and transported through the blood stream as a dimer to reach the surface of oocyte (Babin *et al*., 2007). The surface of oocyte consists of several vitellogenin receptor molecules to which the ligand binds (Mommsen and Korsgard, 2008). The density of vitellogenin receptors per unit area on the oolemma increases drastically during vitellogenesis, which helps in the high rate of vitellogenin transportation into the oocyte, and the density decreases shortly before ovulation (Tyler and Sumpter, 1996). The entry of vitellogenin into oocyte initiate the enzymatic cleavage by cathepsin D and other enzymes to generate yolk proteins such as lipovitelline I, lipovitelline II, phosvettes and phosvitin (Babin *et al*., 2007; Brion *et al*., 2000). The yolk proteins are then incorporated in the form of yolk globules or

yolk platelets as energy reserves essential for the development of oocyte (Brooks *et al*., 1997).

Male fish also possess vitellogenin genes, but are not significantly expressed due to low level of circulating estrogen (Flouriot *et al*., 1993). However, on exposure to exogenous estrogenic compounds induce males to express vitellogenin genes (Jobling and Sumpter, 1993). In females, downregulation of vitellogenin genes are used to assess the antiestrogenic activity of exogenous xenobiotics (Panter *et al*., 2002; Smeets *et al*., 1999). Thus the analysis and measurement of vitellogenin gene expression and its protein level in blood plasma, liver cytosol or in the whole body homogenate is widely used as a biomarker to evaluate the reproductive toxicity of environmental toxicants (Brion *et al*., 2002).

There are various techniques used for the analysis of vitellogenin level, which includes radioimmuno assay (Allner *et al*., 1999), enzyme-linked immunosorbent assay (Zerulla *et al*., 2002), Western blotting (Fossi *et al*., 2001) immunohistochemistry (Van der Ven *et al*., 2003), reverse transcriptase polymerase chain reaction (Ackermann *et al*., 2002), northern analysis (Buhler *et al*., 1997), ribonuclease protection assay (Korte *et al*., 2000), and hybridization protection assay (Thomas-Jones *et al*., 2003). However, the measurement of indirect endpoints such as total protein content, calcium concentration, phosphoprotein and alkaline-labile phosphoprotein (ALP) is the cost effective, rapid and widely used method (Bon *et al*., 1997).

1.4 Aromatase activity

Aromatase enzyme is the member of cytochrome-P450 superfamily that catalyzes the conversion of androgen into estrogen, which play crucial role in growth, development and reproduction (Simpson *et al*., 2002). In teleost fish, two isoforms of aromatase genes namely Cyp19a and Cyp19b, which differ in substrate affinity and catalytic properties, are identified (Kazeto *et al*., 2001). Genes of aromatase are expressed in various tissues of fish but brain shows 100-1000 fold higher activity than the ovarian tissue (Callard *et al*., 1978). The other tissues such as pituitary, kidney, retina, liver and spleen also express aromatase mRNA in trace amount, and the expression of P450aromA is higher in ovary than in testis (Devlin and Nagahama, 2002). The expression of aromatase also depends on various factors such as age, sex and reproductive status (Blazquez and Piferrer, 2004). Aromatase activity can be measured in various tissues using direct methods such as determination of total estrogens like estrone and estradiol (Tinwell *et al*., 2011), ELISA method (Satoh *et al*., 2008) and indirectly by titrated water release assay (Tilson-Mallett *et al*., 1983).

1.5 Role of hormones in reproduction

Hormones are the chemical messengers produced from endocrine tissues and are transported into the target organs through the blood stream. Like other vertebrates, the hypothalamo-pituitary-gonad axis of fish is responsible for the production of hormones. The granulosa cells of the ovarian follicle complex and theca cells are the target cells of hormones in ovary, while Leydig cells are targeted in testis (Wootton and Smith, 2014). Stimulation of hypothalamus secretes gonadotropin-releasing hormone (GnRH) into pituitary for the release of two hormones namely luteinizing hormone (LH) and

follicle-stimulating hormone (FSH) to act on gonads for the secretion of reproductive hormones (Chauvigne *et al*., 2010). The production of gonadal steroid hormones regulates oogenesis and spermatogenesis (Planas and Swanson, 2008). Generally, androgens in male and estrogens and progestagens in females are mainly involved in the regulation of normal reproduction.

In females, during the development of oocyte, meiosis is initiated by the influence of 17β-estradiol (E2) and 17α, 20β-progestagen (Miura *et al*., 2007). The process of vitellogenesis is also assisted by estradiol along with the expression of aromatase enzyme (Clelland and Peng, 2009). FSH stimulates the follicular theca cells to produce testosterone in the presence of aromatase enzyme. In addition, FSH and estradiol modulates the production of non-steroid insulin growth factors for the synthesis of steroids in follicular and theca cells (Lubzens *et al*., 2010). After vitellogenesis, the short-term surge of LH and fall of FSH induce maturation of oocytes (Munakata and Kobayashi, 2010). Later, the production of estradiol is replaced by maturation-inducing hormone (MIH), a progestagen, for further maturation of oocytes.

In males, the receptors of LH and FSH are found in Leydig cells of testis while FSH receptors are concentrated in Sertoli cells (Schulz *et al*., 2010). During spermatogenesis, the plasma level of FSH initiate to increase with transient peak during spermiation, which is declined just before the onset of spawning season, while the plasma level of LH is not elevated until close to the spawning season (Campbell *et al*., 2003). Estradiol level also increases at the beginning of male reproductive cycle for a short period of time in order to promote the proliferation of the undifferentiated spermatogonia. The

level of progestagen is found to increase especially in the transition of spermatogonia to spermatocytes, and also during spermiation and the spermatozoa maturation. The Leydig cells synthesize potent androgens namely testosterone and 11-ketotestosterone under the influence of FSH, which downregulate anti-mullarian hormone for the completion of spermatogenesis (Schulz *et al*., 2010). The elevated levels of progestagen and 11-ketotestosterone are essential for the rupture of spermatocyte walls and release of spermatozoa into the testis tubules (Schulz *et al*., 2010).

In teleost fish, cortisol, the major corticosteroid hormone through the activation of hypothalamo-pituitary-interrenal (HPI) axis is involved in reproduction (Bonga, 1997). Cortisol also alter the functioning of hypothalamo-pituitary-gonadal (HPG) axis by inhibiting estrogen receptor (Lethimonier *et al*., 2000), and reducing the gonadal size (Carragher and Sumpter, 1990). The exogenous administration of cortisol is known to lower estradiol level in circulation and also reduced estradiol-mediated vitellogenesis (Lethimonier *et al*., 2000). Cortisol play crucial role in oocyte development, oocyte hydration and ovulation (Faught and Vijayan, 2018) however, high level of cortisol leads to ovarian atresia (Sopinka *et al*., 2017).

1.6 Effects of nanomaterials on reproduction

Studies have reported that the exposure of nanomaterials cause critical reproductive toxicity in organisms either through direct or indirect mechanisms (Wang *et al*., 2018). The direct entry of nanoparticles into the target organs including the reproductive system occurs by absorption, translocation and deposition (Wang *et al*., 2018). Besides, the nanoparticles can cross all lipid-rich membranes, including blood-brain barrier and blood-testis barrier (Araujo *et al*.,

1999; Kim *et al*., 2006). The indirect mode of reproductive toxicity occurs by the induction of oxidative stress and inflammatory response in male and female reproductive systems (Wells *et al*., 2005). Nanoparticles enter into embryos or foetus directly and interact with the cellular and extracellular components thereby causes developmental and reproductive disorders mediated through oxidative stress and inflammation (Ema *et al*., 2015).

Nanoparticles target male reproductive system through various routes, which in turn lead to reduced body weight, relative weight of testis and accessory male sex organs (Morgan *et al*., 2017). Besides, some nanoparticles altered histology of testis, sperm DNA damage, decreased the testis weight, daily sperm production and sperm motility (Qin *et al*., 2019). Nanoparticles exert toxicity through hypothalamo-pituitary-testis axis by the impairment of spermatogenesis, steroidogenic enzymes, and alterations in male reproductive hormones (Qin *et al*., 2019). Nanoparticles also equally target female reproductive system either by direct action on the growth and development of oocytes, alteration in serum hormone and gene expression in ovary (Zhao *et al*., 2013). The indirect mode of action is mediated through the generation of free radicals or by the induction of inflammation (Kong *et al*., 2014).

The consequence of nanomaterials on reproduction may also increase the risk of decline in fish population. Thus the study was designed to evaluate the risk assessment of fullerene C_{60} nanomaterial in reproduction of the fish, *Anabas testudineus* by analyzing certain reproductive endpoints. Steroidogenic enzyme activities, reproductive hormones, aromatase and vitellogenin products were tested as the specific and sensitive parameters to evaluate the reproductive toxicity.

In toxicology, reproductive toxicity plays a crucial role because it adversely affects sexual functions and fertility of organisms. Moreover, reproduction and development comprises a series of complex physiological phenomena that are perceptive to change in the environment. But researches on the reproductive toxicity of nanomaterial are in the stage of infancy. Therefore, attention on the reproductive toxicity of nanomaterial has created great concern to the public and toxicologists in recent years. Relatively few studies, which is less than 3% of the total toxicological research has been documented on the nanomaterial-induced biological effects, particularly on reproduction of various organisms (Greish *et al*., 2012; Arora *et al*., 2012). Humans and other animals are continuously exposed to nanomaterials, and its related health risks are due to the intentional use in medicine and pharmacy, unintentional discharge into the environment and lack of adequate knowledge on the adverse effects (Qin *et al*., 2019). Thus the potential reproductive hazards of nanomaterials released from various sources in different organisms are reviewed in detail.

Incubation of fullerene C_{60} with various concentrations of polyvinylpyrrolidone (PVP) has been shown to inhibit cell differentiation and proliferation in midbrain of mouse, while intraperitoneal injection at 50 mg/ kg to pregnant mice on day 10 of gestation resulted in distribution of C_{60} into the yolk sac and embryos leading to mortality (Tsuchiya *et al*., 1996). Exposure of single-walled carbon nanotubes (SWCNTs) to estuarine meiobenthic copepod,

Amphiascus tenuiremis has been shown to reduce the rate of fertility along with elevated incidence of mortality and declined moulting success (Templeton *et al*., 2006). However, the reproductive and developmental toxicity of SWCNTs assessed in zebrafish embryos has been shown to cause delay in embryo hatching without affecting survival and development of embryos (Cheng *et al*., 2007).

Exposure of fullerenes C_{60}, C_{70} and fullerenol $C_{60}(OH)_{24}$ to embryonic zebrafish for 24 h has resulted in morphological and cellular abnormalities including embryo mortality and caudal fin malformations. Exposure to C_{60} showed increased necrotic and apoptotic cellular death throughout the embryo, while exposure of C_{60} and C_{70} increased malformations, pericardial edema, and mortality. However, exposure of $C_{60}(OH)_{24}$ fullerenol induced an increase in embryonic cellular death without the induction of apoptosis thereby indicated fullerenol as less toxic than C_{60} fullerene (Usenko *et al*., 2007). Similarly, the exposure of fullerol, $C_{60}(OH)_{16\text{-}18}$, nano aggregates of C_{60}, and combination of GSH and nano C_{60} for 96 h on zebrafish revealed significant developmental toxicity as described by delay in embryo and larval development, decreased hatching and survival rates, pericardial edema and alterations in the rate of heart beat (Zhu *et al*., 2007).

Oxidative potential of C_{60}, the effects of light, chemical supplementation and depletion of glutathione are the major factors that induced C_{60} toxicity as evidenced by increase in mortality rate,

pericardial edema and fin malformations in embryonic zebrafish (Usenko *et al*., 2008). Carbon nanomaterials have been proved as a reproductive toxicant in rodents where administration of carbon black nanoparticle, printex 90, printex 25, and flammruss 101 to adult male mice has been shown to cause alteration in spermatogenesis, histology of testis and partial vacuolization in seminiferous tubules (Yoshida *et al*., 2008). Toxicity of water-soluble multi-walled carbon nanotubes (MWCNTs) tested in zebrafish, *Danio rerio* caused significant phenotypic defects, apoptosis, delayed hatching, reduced blood circulation, formation of abnormal spinal chord, and increased mortality rate (Asharani *et al*., 2008). In another study, fluorescent-labeled MWCNTs when introduced into zebrafish embryos at one-cell stage and in 72 h post-fertilization through microinjection showed low survival rate in second generation, and also suggested negative effects on the reproductive potential (Cheng *et al*., 2009).

Administration of carbon-14 labeled C_{60} prepared in PVP to pregnant rats through tail vein injection has distributed nanomaterial in the reproductive tract, placenta and fetuses, and crossed through the placental cord and milk as detected by the radioactivity in the tissues of lactating rats (Sumner *et al*., 2010). Intratracheal instillation of carbon black nanoparticle in pregnant ICR mice and male offspring has been shown to reduce daily sperm production and histopathological alterations such as vacuolization in seminiferous tubules, and reduced seminiferous epithelial cellular adhesion (Yoshida *et al*., 2010). Repeated intravenous injection of water-soluble

MWCNTs on male mice caused reversible testis damage without affecting fertility. The testicular damage has been illustrated by partial damage in seminiferous tubules, reduced thickness in germ layers and decreased number of spermatogonia, which was mediated through the induction of oxidative stress, but the damage recovered after 60 and 90 d (Bai *et al*., 2010).

Mammalian embryotoxicity has been reported after intravenous injection of pristine and oxidized form of SWCNTs to pregnant mice at 0.01-30 mg/ mouse soon after implantation, which resulted in increased incidence of miscarriage, small-sized fetuses with vascular damage in labyrinth layer and malformations in head, abdominal wall, limb hypoplasia, and growth retardation (Pietroiusti *et al*., 2011). On contrary, oral administration of MWCNTs at 1000 mg/ kg/ day to pregnant rats showed no embryo-fetal toxicity and fetal weight but caused minimal maternal toxicity and reduced the weight of thymus (Lim *et al*., 2011). Exposure of MWCNTs prepared in nitric acid solution sonicated for long duration to a single-celled stage of zebrafish embryos resulted in the developmental toxicity as evidenced by the distribution in blastoderm cells leading to developmental arrest and embryo malformation (Cheng and Cheng, 2012).

Multiple injections of the conjugated SWCNTs with amino-functionalized polyethylene glycol in the female mouse on 5.5 of gestation day revealed fetal and placental abnormalities with delayed development, and deformity in head and paws (Campagnolo *et al*.,

2013). Injection of SWCNTs into chicken embryos showed 80% embryo mortality within 12 d of incubation period, along with inhibition of growth and angiogenesis, and downregulated expression of genes related to cell proliferation, survival, apoptosis, cell cycle and angiogenesis (Roman *et al*., 2013). Hydrocolloids of nanodiamond, a carbon nanoparticle injected in the air sac of fertilized chicken eggs has been shown to reduce the levels of serum triglycerides but upregulated expression of genes related to cell proliferation and differentiation thereby accelerated the development of chick embryo (Grodzik *et al*., 2013). Embryonic toxicity of carbon nanoparticles such as diamond and graphite has been evaluated in chicken embryo, which showed vascularization of chorioallantoic membrane and downregulated gene and protein expression of the proangiogenic basic fibroblast growth factor (Wierzbicki *et al*., 2013). Fertilized chicken eggs administered with pristine graphene have been shown to downregulate the expression of genes and proteins associated with brain development (Sawosz *et al*., 2014).

Oxidized-MWCNTs exposure on mice declined body weight gain, reduced level of vascular endothelial growth factor thereby caused placental dysfunction leading to reduced fetal growth, reduced serum progesterone level along with the induction of oxidative stress, increased abortion rate, increased serum estradiol level, and histology of placenta showed narrowed and reduced number of blood vessels (Qi *et al*., 2014). Zebrafish embryos at two hour post-fertilization exposed to MWCNTs, graphene oxide, and reduced graphene oxide at different

concentrations for 96 h has affected spontaneous movement of embryo, and altered heart rate, hatching rate, and length of larvae while reduced graphene oxide accumulated in chorion of embryo thereby reduced oxygen content leading to mortality and malformations in embryo (Liu *et al*., 2014). Maternal exposure to ultrafine carbon black in mice induced penetration through blood-brain barrier and contributed to developmental toxicity by the alteration in phenotypes of astrocyte and enlarged granules of perivascular macrophages in the brain of offspring (Onoda *et al*., 2014).

Carbon black nanoparticle exposed during middle and late gestation to male mice caused developmental immunotoxicity as evidenced by allergic or inflammatory effects such as increase in total thymocytes and splenocyte phenotypes with upregulated expression of genes related to peripheral tolerance (El-Sayed *et al*., 2015). Reproductive and developmental toxicity of carbon based nanomaterials such as fullerene C_{60}, carbon black and other metal oxide-based nanoparticles has been reported in mouse embryos, and the variation in nanoparticles toxicity was based on different characterization, route of exposure, doses administered and so on (Ema *et al*., 2015). Similarly, graphene oxide exposed to zebrafish embryo adhered to the chorion and blocked chorionic membrane, caused hypoxia, delayed hatching, damaged mitochondria, induced excessive generation of reactive oxygen species and increased oxidative stress, DNA damage and apoptosis, along with other developmental

malformation such as edema in eye, cardiac and yolk sac, tail flexure and reduced heart rate (Chen *et al*., 2016).

Maternal exposure of carbon black nanoparticle showed neurodevelopmental toxicity in male mouse offsprings, which was evidenced by elevated expression of glial fibrillary acidic protein in hippocampal astrocytes and cerebral cortex regions, and also caused enlargement of lysosomal granules in brain (Umezawa *et al*., 2018). Mature male NMRI mice exposed to four different carbonaceous nanomaterials such as graphene oxide, printex 90, flammruss 101 and SRM1650b weekly for seven consecutive weeks through intratracheal instillation has not affect semen parameters including sperm morphology, sperm viability, sperm motility, sperm DNA damage, epididymal sperm concentration, daily sperm production, and plasma testosterone concentration (Skovmand *et al*., 2018). Similarly, the maternal inhalation of carbon black nanoparticle, printex 90 at occupational concentration has not affected male-specific reproductive and fertility endpoints as well as the gestational and litter parameters in male offspring for four generations (Skovmand *et al*., 2019). It has been recently reported that the toxicity of carbon nanomaterials depends on its functional groups (Liu *et al*., 2019). Thus the reviewed literatures stated that several factors influenced reproductive and developmental toxicity of carbon-based nanomaterials in different animals. However, the gap of knowledge in the reproductive toxicity

of fullerene C_{60} in fish attracted attention to evaluate certain valuable parameters such as steroidogenesis, reproductive hormones, aromatase enzyme activity and indirect endpoints of vitellogenesis in gonads of the freshwater fish, *Anabas testudineus*.

3.1 Preparation of test chemical

The test chemical, fullerene C_{60} purchased from Sigma Aldrich Chemical Company was dispersed in dimethyl sulfoxide (100%) by sonication. Final test concentrations of 1% DMSO-C_{60} suspension (5 and 10 mg/ L) were used to study the C_{60}-mediated reproductive toxicity in *Anabas testudineus.*

3.2 Test animal

The acclimatized adult mature fish, *Anabas testudineus* (11 ± 1 g weight and 8 ±1 cm size) were used for the present study. *Anabas testudineus* breeds seasonally and the spawning period was from April to October (Jacob, 2005). Thus the present study was conducted between May and August so as to get the mature reproductive phase of gonads. There is no distinct sexual dimorphism in *Anabas testudineus* so sex were identified by pressing gently on the abdominal area. Abdomen of female fish was comparatively bulged than male due to the presence of mature eggs, which are extruded by applying gentle force whereas milt are released from male fish. After sex identification, male and female fishes were separated and placed in separate tanks for further experiment.

3.3 Experimental design

The selected sublethal concentrations of fullerene C_{60} (5 and 10 mg/ L) were exposed for 4, 7, 15, 30 and 60 d along with negative control (toxicant and solvent-free) and solvent control (1% DMSO) groups. Ten animals were maintained in each group and the experiments were carried out in replicates to reduce the statistical errors.

3.4 Preparation of samples

At the end of every treatment period, ten fish were captured from both male and female groups with least stress using a dip net. Fresh blood sample drawn from caudal vein were collected in the vial containing the anticoagulant, PBS-heparin. It was kept undisturbed at room temperature for 15-30 min, centrifuged at 1000 *g* for 10 min to obtain blood plasma, and was used for the analysis of alkali labile phosphates, calcium concentration and total protein. Remaining blood without adding anticoagulant was centrifuged at 1700 *g* for 10 min at 4 ºC, and the supernatant was used for hormone analysis. Both serum and plasma samples were then stored at -80 ºC until used. After the exposure periods, fish from both control and treatment groups (10 males and 10 females) were sacrificed, and the tissues such as gonads, brain and liver were dissected out, stored at -20 ºC for the biochemical analysis of steroidogenic enzymes, levels of alkali labile phosphates, calcium concentration, total protein, and aromatase enzyme activity.

3.5 Activity of hydroxysteroid dehydrogenase enzymes in gonads

Gonads (ovary and testis; 1% w/ v) were prepared in ice-cold sodium pyrophosphate buffer (pH 9.0) using a teflon homogenizer. The crude homogenates were centrifuged at 800 *g* for 15 min at 4 ºC, and supernatants were collected in separate microcentrifuge tubes for the enzyme analysis. The activities of 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD) were estimated according to the method of Bergmeyer (1974).

The reaction mixture of 3β-hydroxysteroid dehydrogenase contained pyrophosphate buffer (100 mM), NAD (0.5 mM) and dehydroisoandrosterone (0.1 mM). The absorbance was measured in a spectrophotometer at 340 nm for 5 min at 20 sec intervals against the reagent blank. Activity of enzyme was expressed as µmol of NAD reduced/ min/ mg protein.

The reaction mixture of 17β-hydroxysteroid dehydrogenase contained pyrophosphate buffer (100 mM), 1,4-androstenedine-3,17-dione (0.8 mM), and NADPH (0.5 mM). The absorbance was read at 340 nm immediately after the addition of sample in a spectrophotometer at 340 nm for 5 min at 30 sec intervals against the reagent blank. Activity of the enzyme was expressed as µmol of NADP formed/ min/ mg protein. The total soluble protein was evaluated by Lowry *et al* (1951).

3.6 Serum hormone analysis

The serum collected from both control and treatment groups of male and female fish were used for the analysis of hormones such as testosterone, estradiol and cortisol. The commercial ELISA kits for fish testosterone (Catalog Number: CSB-E17554Fh), estradiol (Catalog Number: CSB-E13017Fh) and cortisol (Catalog Number: CSB-E08487f) were purchased from Cusabio Biotech Co., Ltd., China, and the estimations were strictly performed according to the procedure explained in the kits.

Testosterone

Briefly, samples and standard were added to the antibody-coated microplate, then 50 µl of horse radish peroxidase (HRP)-

conjugate solution was added, incubated for 1 h at 37 °C. After incubation, wells were aspirated and washed twice with wash buffer. Then substrate A and B (50 µl each) were added, incubated for 15 min at 37 °C in dark. Stop solution was added to end the reaction, and finally the optical density was measured in a microplate reader (Synergy HT, BioTek) at 450 nm against the reagent blank within 10 min. The values are expressed as ng/ ml.

Estradiol

The antibody pre-coated microplate was added with samples and standard followed by HRP-conjugated solution (50 µl), and incubated for 1 h at 37 °C. Microplate wells were aspirated and washed twice with wash buffer. Then 50 µl of substrate A and substrate B were added, incubated for 15 min at 37 °C in dark. The reaction was stopped by the addition of 50 µl of stop solution and mixed well. The colour developed was measured within 10 min in a microplate reader (Synergy HT, BioTek) at 450 nm against the reagent blank. The values are expressed as ng/ ml.

Cortisol

Samples and standard were added into microplate wells then 50 µl of antibody (1X) was added and mixed gently for 60 sec. The plate was covered and incubated for 40 min at 37 °C. After the incubation, the plate was washed twice with wash buffer and kept again for 2 min for complete removal of buffer. Then 100 µl HRP-conjugate (1X) was added to each wells and incubated for 30 min at 37 °C. The aspiration and washing process was repeated to remove the buffer completely. Then the substrate, 3,3',5,5'-tetramethylbenzidine (90 µl) was added to

each well, incubated for 20 min at 37 °C and kept in dark. The reaction was stopped by the addition of 50 µl of stop solution, mixed gently. Then the optical density was measured in a microplate reader (Synergy HT, BioTek) at 450 nm within 5 min against the reagent blank. The values are expressed as ng/ ml.

3.7 Levels of alkali labile phosphates, calcium concentration and total protein

Gonads and liver tissues were dissected out from both male and female fish and homogenized in ice-cold homogenizing buffer (Tris-acetate, pH 8.0 containing EDTA, dithiothreitol). The crude tissue homogenate of 1% w/ v were centrifuged at 4 °C for 15 min at 800 *g***,** the supernatants were collected and stored in -80 °C until used. The supernatants of gonads and liver, along with blood plasma from both male and female fish were used for the estimation of alkali labile phosphates (ALP), total protein, and calcium concentration. ALP was measured in high molecular weight proteins after fractionating with acetone according to the method of Gagne (2014). The total protein was measured according to Lowry *et al*. (1951). The results of ALP were expressed as µg or ng phosphates/ ml for blood plasma and µg phosphates/ mg proteins for tissue samples.

The calcium concentration was estimated according to Zettner and Seligson (1964) using the atomic absorption spectrophotometry (Spectra-AA240FS, Varian). Briefly, the supernatants of the tissues (ovary, testis and liver) and plasma samples of both male and female fish were diluted (40 fold) with 0.1% of lanthanum chloride, mixed well and centrifuged for 10 min at 1000 *g*. The collected samples were read in atomic absorption spectrophotometer. The results were expressed as µg/ ml.

3.8 Activity of aromatase enzyme

The gonads (ovary and testis) and brain dissected from both control and experimental groups of male and female fish were homogenized in ice-cold phosphate buffered saline (1X-PBS) to make 1% w /v tissue homogenates. The homogenates were centrifuged at 800 *g* for 15 min at 4 °C, and the supernatants obtained were used for the analysis of aromatase enzyme. The analysis was performed using the fish aromatase ELISA kit (Catalog Number: CSB-EL006394F) purchased from Cusabio Biotech Co., Ltd., China.

Briefly, the samples and standard (50 µl) were added into antibody pre-coated microplate wells, and then HRP-conjugate solution was added, incubated for 1 h at 37 °C. Wells were aspirated, washed twice with wash buffer then 50 µl of substrate A and substrate B were added, incubated at 37 °C for 15 min in dark. Finally 50 µl of stop solution was added, mixed well and the optical density was measured in a microplate reader (Synergy HT, BioTek) within 10 min at 450 nm. The total protein was measured by Lowry *et al*. (1951) and the values were expressed as ng/ mg protein.

3.9 Statistical analyses

Statistical analysis was performed by using the SPSS V-21.0, a statistical package. One-way analysis of variance (ANOVA) followed by the Duncan's multiple range as Post-hoc test was performed. The data were represented as Mean ± SD in graphs for ten fish per group, and the significant differences were denoted as *$P<0.05$ against the control groups. All the experiments were performed in replicates so as to minimize the statistical errors.

4.1 Effect of fullerene C_{60} on the activities of gonadal hydroxysteroid dehydrogenase enzymes

Exposure of fullerene C_{60} at sublethal concentrations (5 mg/ L and 10 mg/ L) showed significant ($P<0.05$) decrease in the activities of 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD) after 15 d in testis and ovary of fish in time-dependent manner than the respective control groups (Figs. 1-4).

4.2 Effect of fullerene C_{60} on the levels of serum hormones

In male fish, the level of serum estradiol remained unchanged at both sublethal concentrations (Fig. 5), while in female, the hormone level declined significantly ($P<0.05$) after 60 d (Fig. 6). C_{60} fullerene exposure at both sublethal concentrations decreased the level of serum testosterone after 60 d in male fish (Fig. 7), whereas no significant changes were observed in the female fish (Fig. 8). The level of cortisol increased significantly ($P<0.05$) after 30 and 60 d of both sublethal exposures in male and female fish when compared to the corresponding control groups (Figs. 9 and 10).

4.3 Effect of fullerene C_{60} on the levels of alkali labile phosphates, calcium and total protein

Gonads

In testis, the levels of alkali-labile phosphate, and calcium remained unchanged while the concentration of total protein decreased significantly ($P<0.05$) in time-dependent manner after 15 d in both sublethal concentrations of fullerene C_{60} treatment (Figs. 11-13). In ovary, there was a significant ($P<0.05$) reduction in the levels of alkali-labile phosphate, total protein and calcium after 30 and 60 d of fullerene C_{60} exposure than the corresponding control groups (Figs. 14-16).

Liver

In liver of male fish, there was no significant changes in the levels of alkali-labile phosphate and calcium, however, the concentration of total protein decreased significantly ($P<0.05$) after 15 d onwards in time-dependent manner (Figs. 17-19). In female liver tissue, the levels of alkali-labile phosphate, total protein and calcium showed significant ($P<0.05$) reduction after 30 and 60 d at both sublethal concentrations (Figs. 20-22).

Blood plasma

In the blood plasma of male fish, the levels of alkali-labile phosphate and calcium remained unchanged while the total protein concentration decreased significantly ($P<0.05$) at both sublethal concentration after 15 d than that of the corresponding control groups (Figs. 23-25). In female fish, the plasma levels of alkali-labile phosphate and calcium showed significant ($P<0.05$) reduction after 30 and 60 d whereas the concentration of total protein decreased significantly ($P<0.05$) after 15 d onwards in time-dependent manner (Figs. 26-28).

4.4 Effect of fullerene C_{60} on the activities of aromatase enzyme

In male fish, the activity of aromatase enzyme remained unchanged in testis, and decreased significantly ($P<0.05$) after 60 d at 10 mg/ L concentration fullerene C_{60} in brain tissue (Figs. 29 and 30). In female fish, the activity of aromatase decreased significantly ($P<0.05$) after 30 and 60 d in both sublethal groups of ovary (Fig. 31) while the enzyme activity decreased significantly ($P<0.05$) only after 60 d of both sublethal exposure groups in brain tissue (Fig. 32).

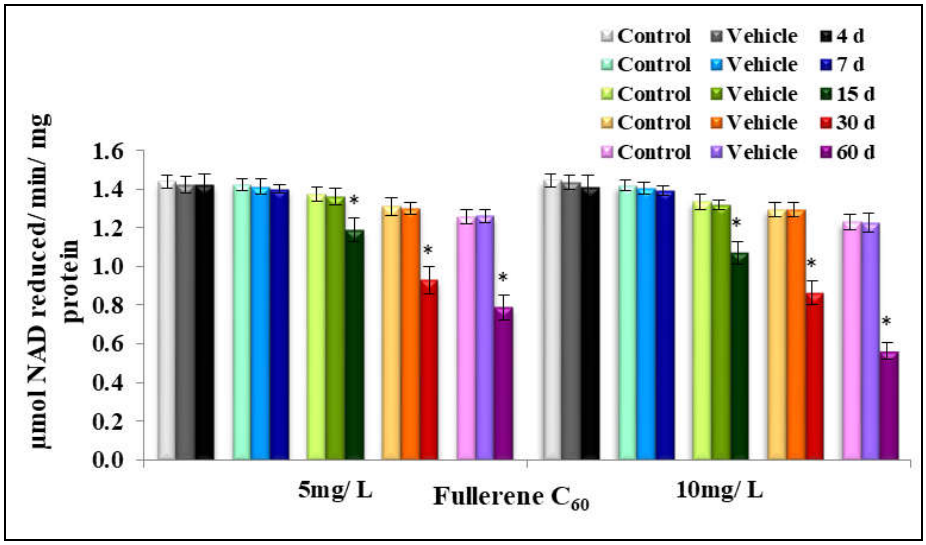

Fig. 1 Effect of fullerene C_{60} on the activity of 3β-hydroxysteroid dehydrogenase in testis of the fish, *Anabas testudineus* (n=10/ group; Mean ± SD; *P<0.05 against the control groups)

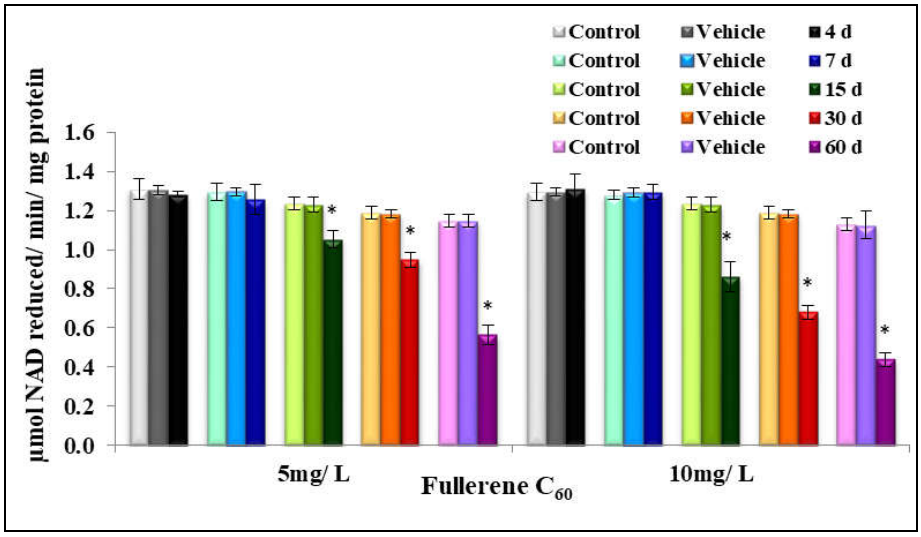

Fig. 2 Effect of fullerene C_{60} on the activity of 3β-hydroxysteroid dehydrogenase in ovary of the fish, *Anabas testudineus* (n=10/ group; Mean ± SD; *P<0.05 against the control groups)

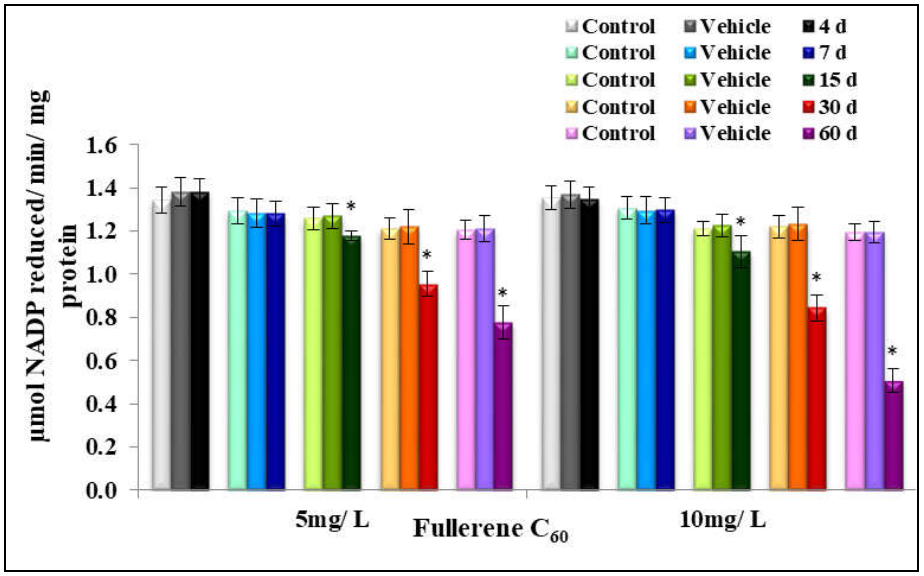

Fig. 3 Effect of fullerene C_{60} on the activity of 17β-hydroxysteroid dehydrogenase in testis of the fish, *Anabas testudineus* (n=10/ group; Mean ± SD; *P<0.05 against the control groups)

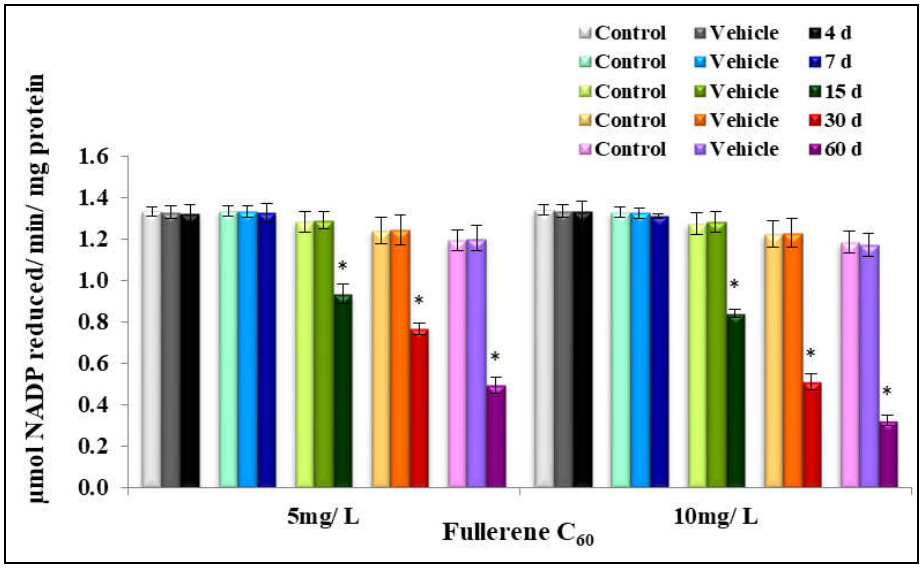

Fig. 4 Effect of fullerene C_{60} on the activity of 17β-hydroxysteroid dehydrogenase in ovary of the fish, *Anabas testudineus* (n=10/ group; Mean ± SD; *P<0.05 against the control groups)

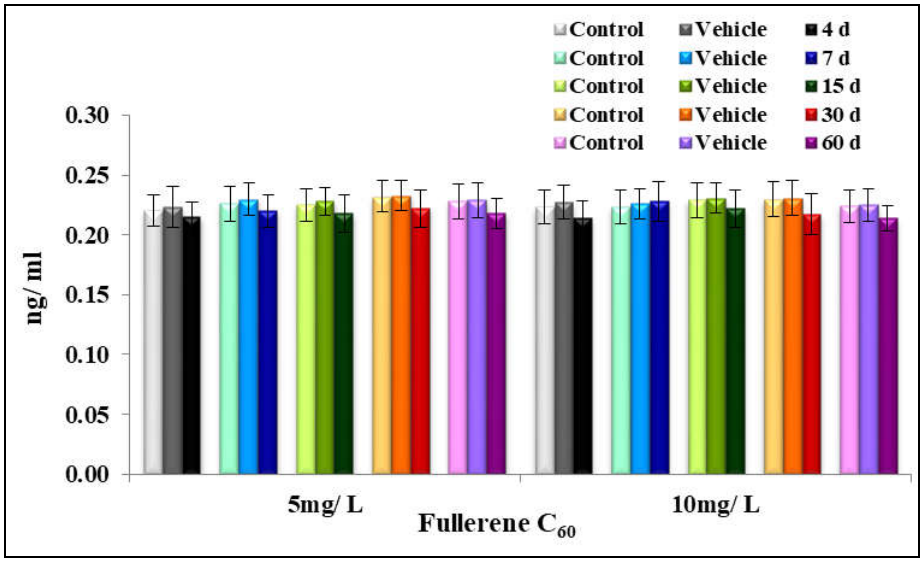

Fig. 5 Effect of fullerene C_{60} on the level of serum estradiol in the male fish, *Anabas testudineus* (n=10/ group; Mean ± SD)

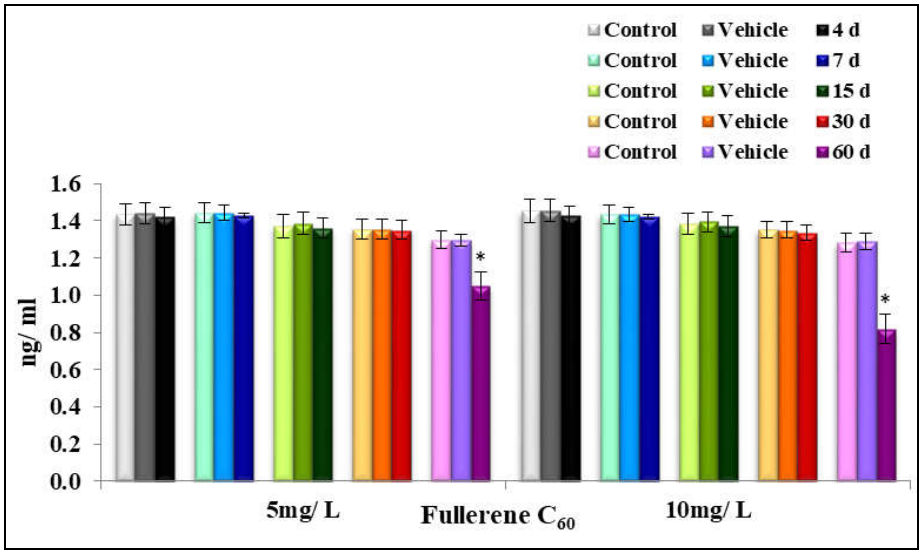

Fig. 6 Effect of fullerene C_{60} on the level of serum estradiol in the female fish, *Anabas testudineus* (n=10/ group; Mean ± SD; *P<0.05 against the control groups)

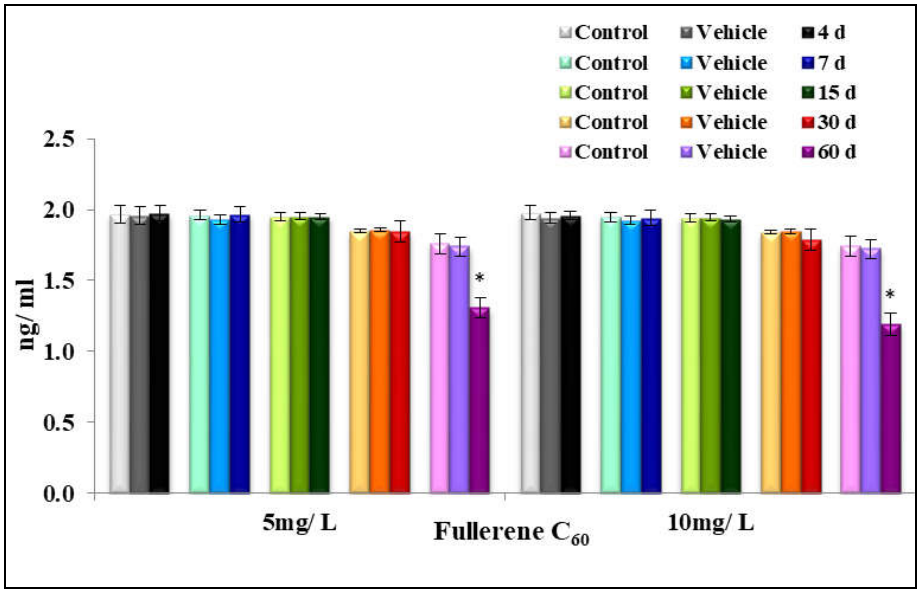

Fig. 7 Effect of fullerene C_{60} on the level of serum testosterone in the male fish, *Anabas testudineus* (n=10/ group; Mean ± SD; *P<0.05 against the control groups)

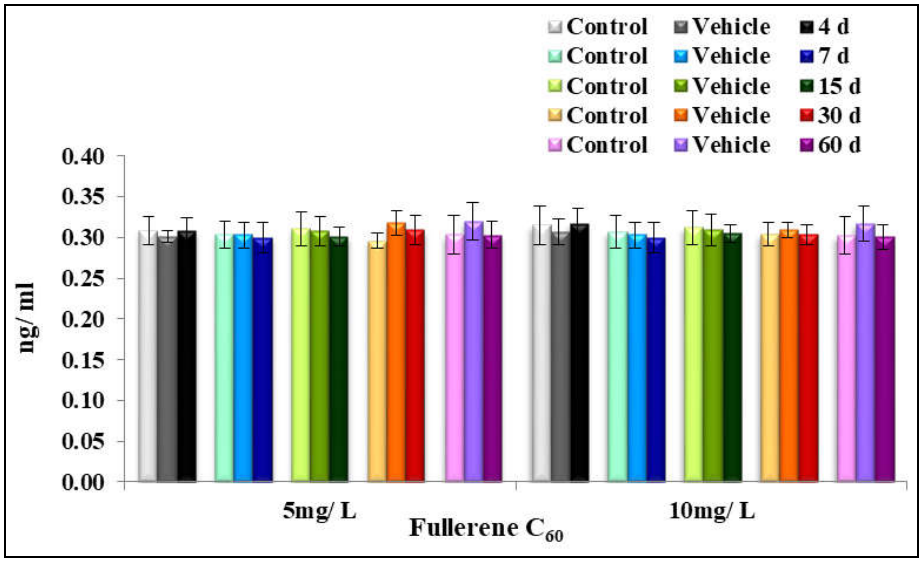

Fig. 8 Effect of fullerene C_{60} on the level of serum testosterone in the female fish, *Anabas testudineus* (n=10/ group; Mean ± SD)

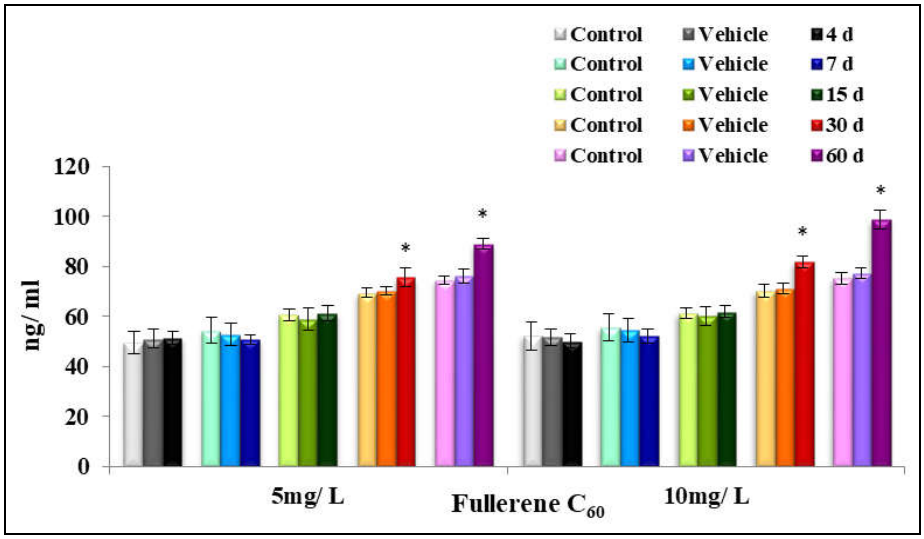

Fig. 9 Effect of fullerene C_{60} on the level of serum cortisol in the male fish, *Anabas testudineus* (n=10/ group; Mean ± SD; *P<0.05 against the control groups)

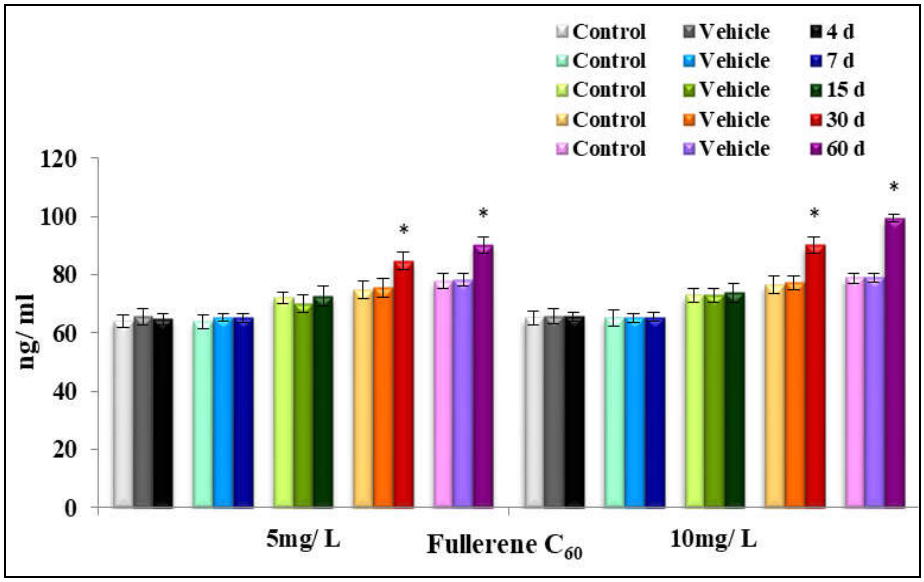

Fig. 10 Effect of fullerene C_{60} on the level of serum cortisol in the female fish, *Anabas testudineus* (n=10/ group; Mean ± SD; *P<0.05 against the control groups)

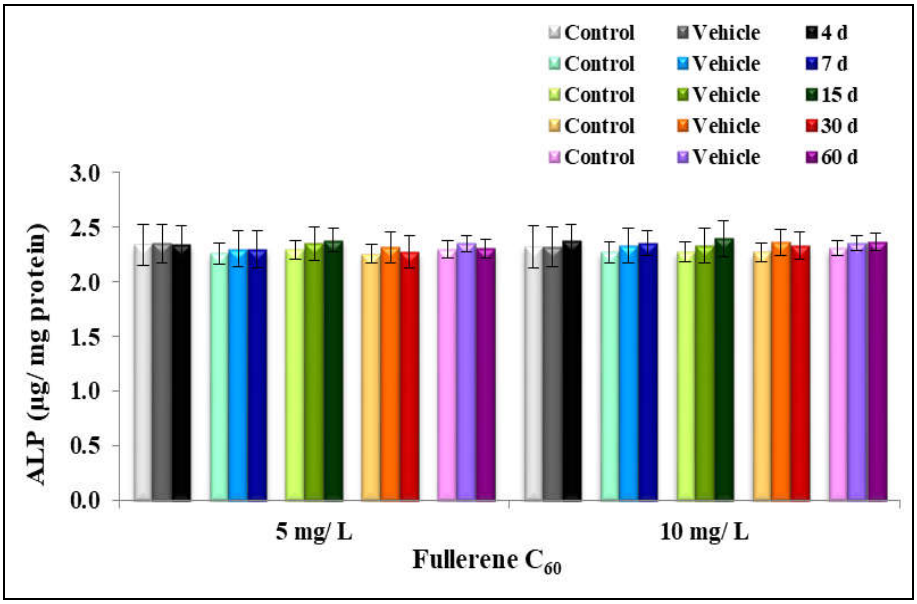

Fig. 11 Effect of fullerene C_{60} on the level of alkali-labile phosphates (ALP) in the testis of the fish, *Anabas testudineus* (n=10/ group; Mean ± SD)

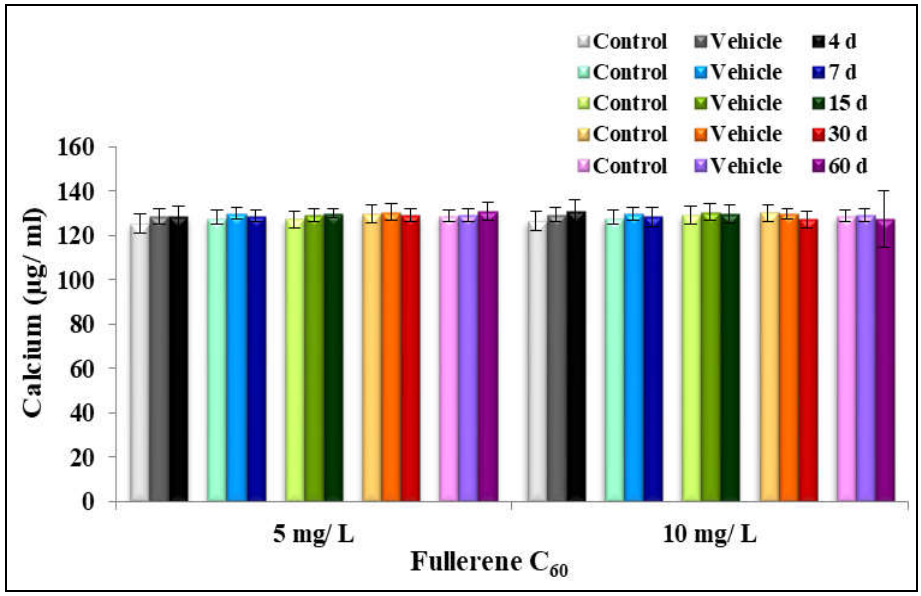

Fig. 12 Effect of fullerene C_{60} on the level of calcium in the testis of fish, *Anabas testudineus* (n=10/ group; Mean ± SD)

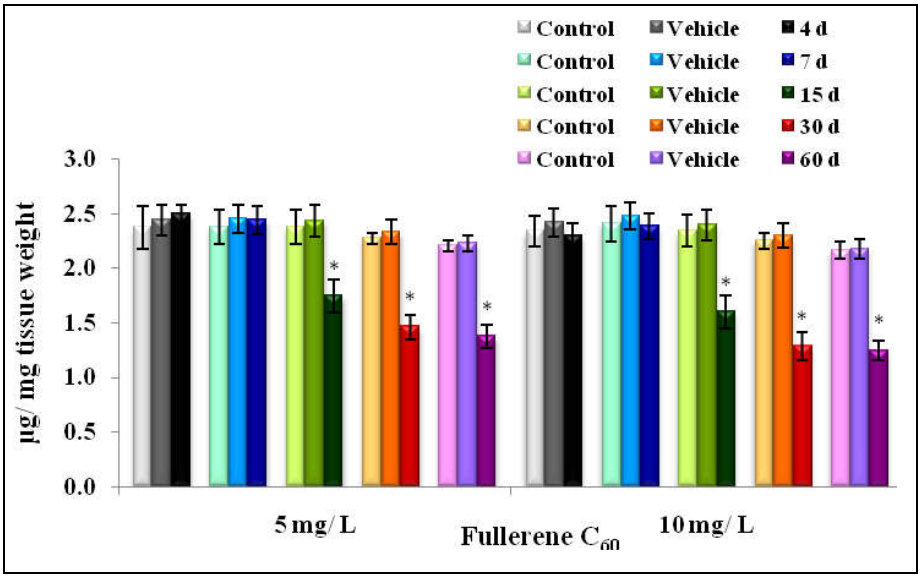

Fig. 13 Effect of fullerene C_{60} on the level of total protein in the testis of the fish, *Anabas testudineus* (n=10/ group; Mean ± SD; *P<0.05 against the control groups)

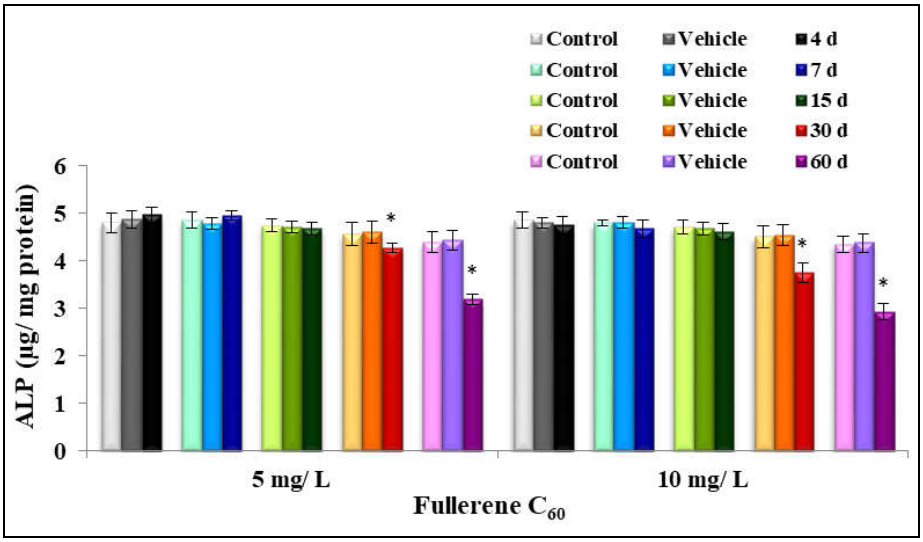

Fig. 14 Effect of fullerene C_{60} on the level of alkali-labile phosphates (ALP) in the ovary of fish, *Anabas testudineus* (n=10/ group; Mean ± SD; *P<0.05 against the control groups)

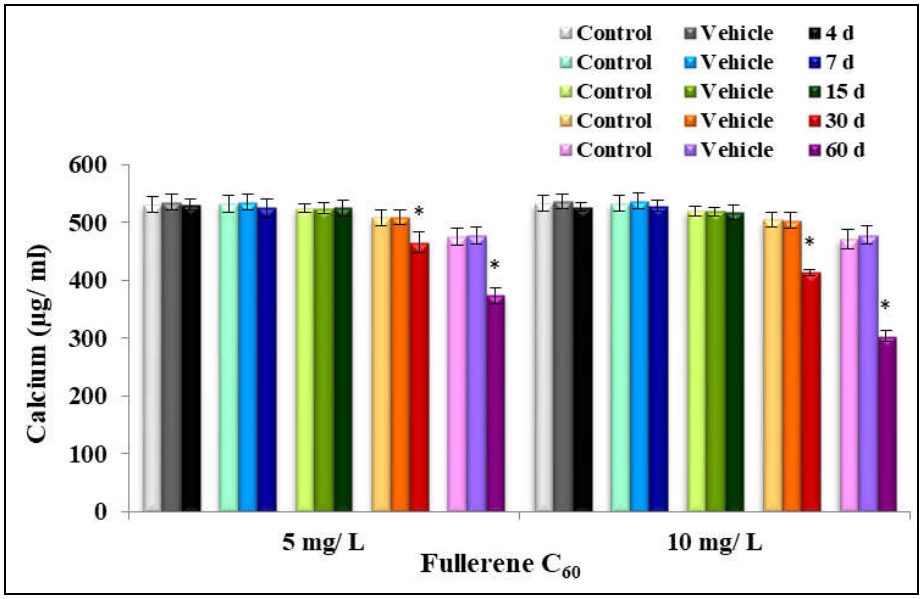

Fig. 15 Effect of fullerene C_{60} on the level of calcium in the ovary of fish, *Anabas testudineus* (n=10/ group; Mean ± SD; *P<0.05 against the control groups)

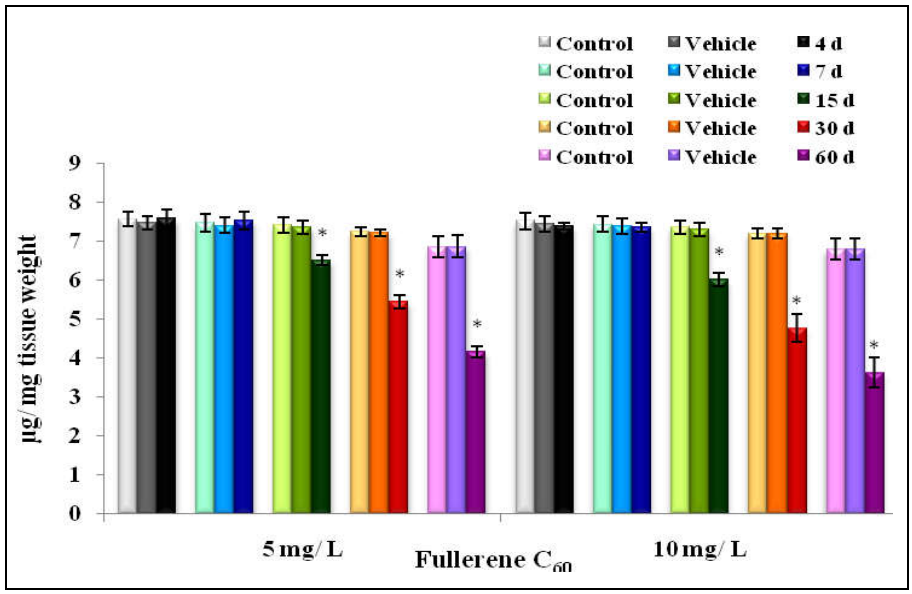

Fig. 16 Effect of fullerene C_{60} on the level of total protein in the ovary of fish, *Anabas testudineus* (n=10/ group; Mean ± SD; *P<0.05 against the control groups)

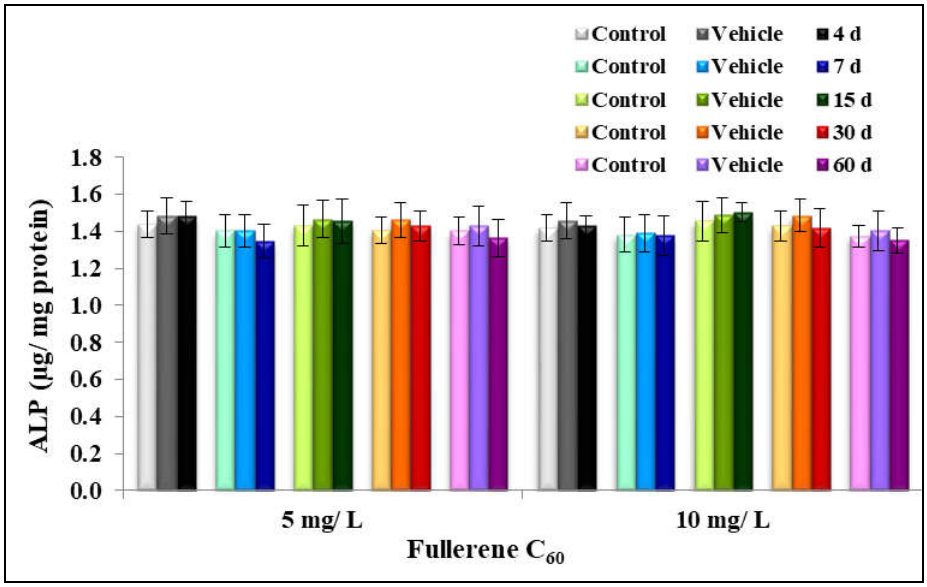

Fig. 17 Effect of fullerene C_{60} on the level of alkali-labile phosphates (ALP) in the liver of male fish, *Anabas testudineus* (n=10/ group; Mean ± SD)

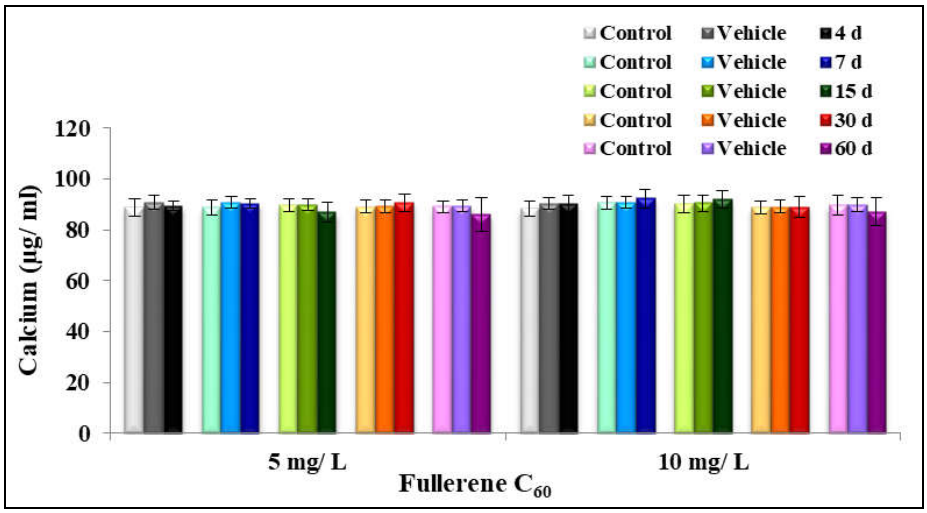

Fig. 18 Effect of fullerene C_{60} on the level of calcium in the liver of male fish, *Anabas testudineus* (n=10/ group; Mean ± SD)

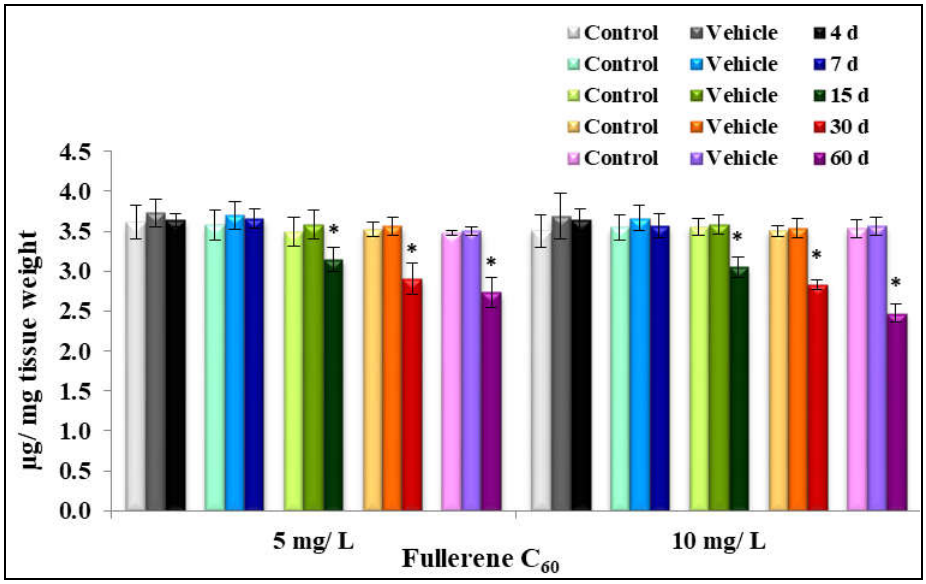

Fig. 19 Effect of fullerene C_{60} on the level of total protein in the liver of male fish, *Anabas testudineus* (n=10/ group; Mean ± SD; *P<0.05 against the control groups)

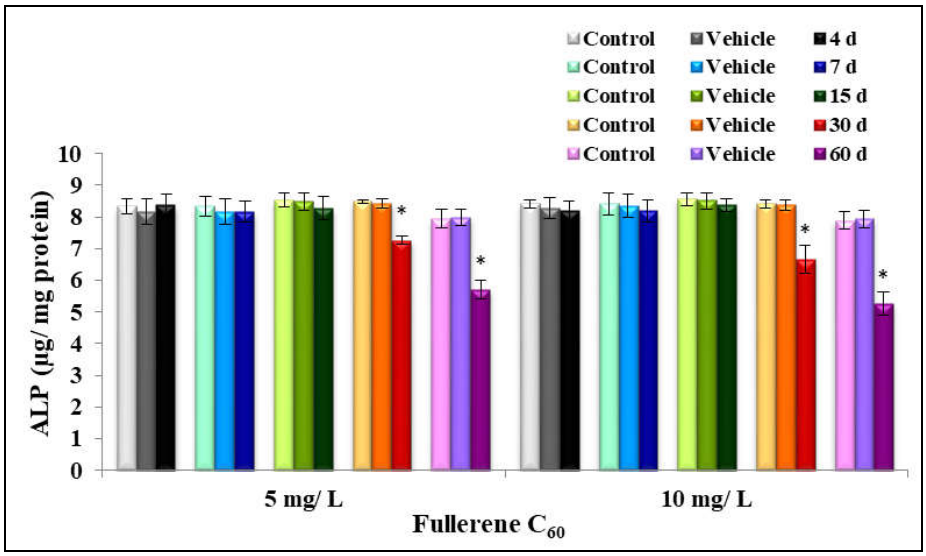

Fig. 20 Effect of fullerene C_{60} on the level of alkali-labile phosphates (ALP) in the liver of female fish, *Anabas testudineus* (n=10/ group; Mean ± SD; *P<0.05 against the control groups)

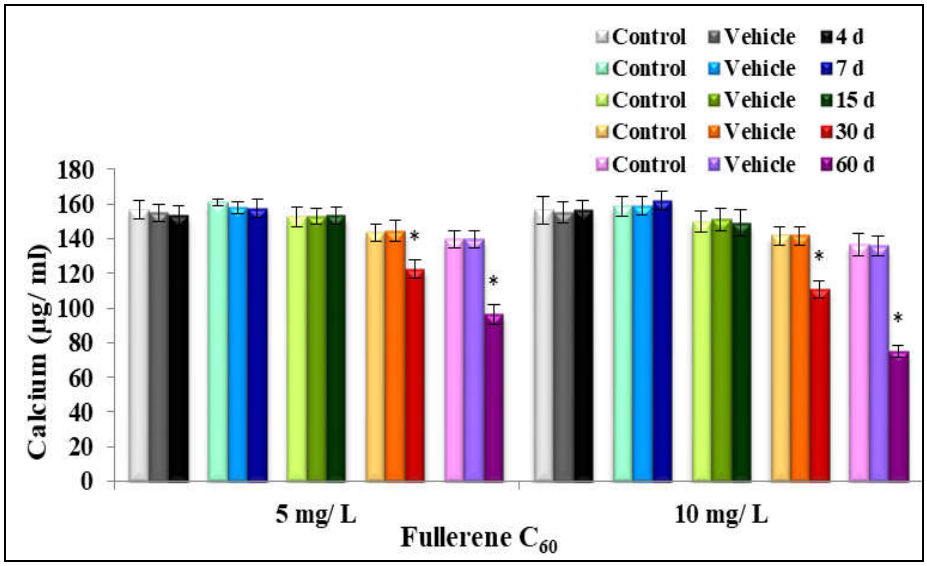

Fig. 21 Effect of fullerene C_{60} on the level of calcium in the liver of female fish, *Anabas testudineus* (n=10/ group; Mean ± SD; *P<0.05 against the control groups)

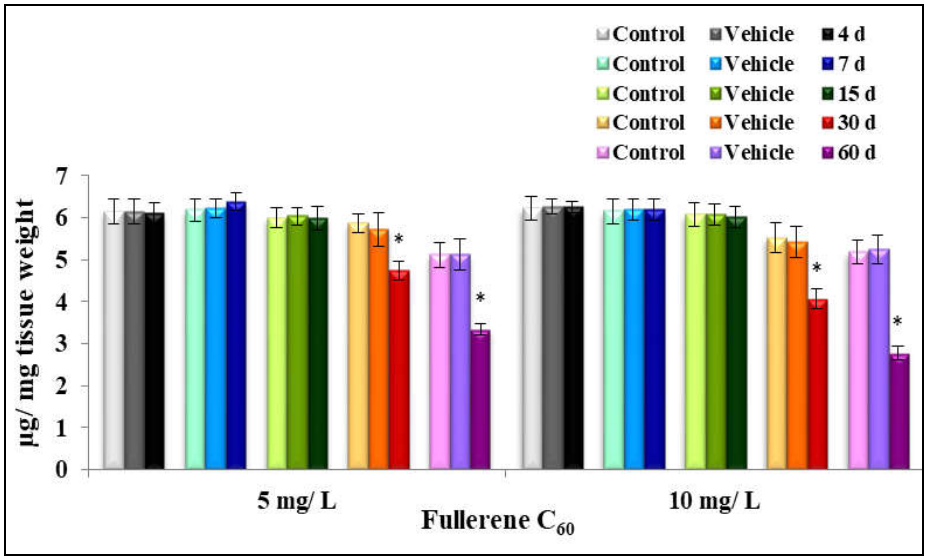

Fig. 22 Effect of fullerene C_{60} on the level of total protein in the liver of female fish, *Anabas testudineus* (n=10/ group; Mean ± SD; *P<0.05 against the control groups)

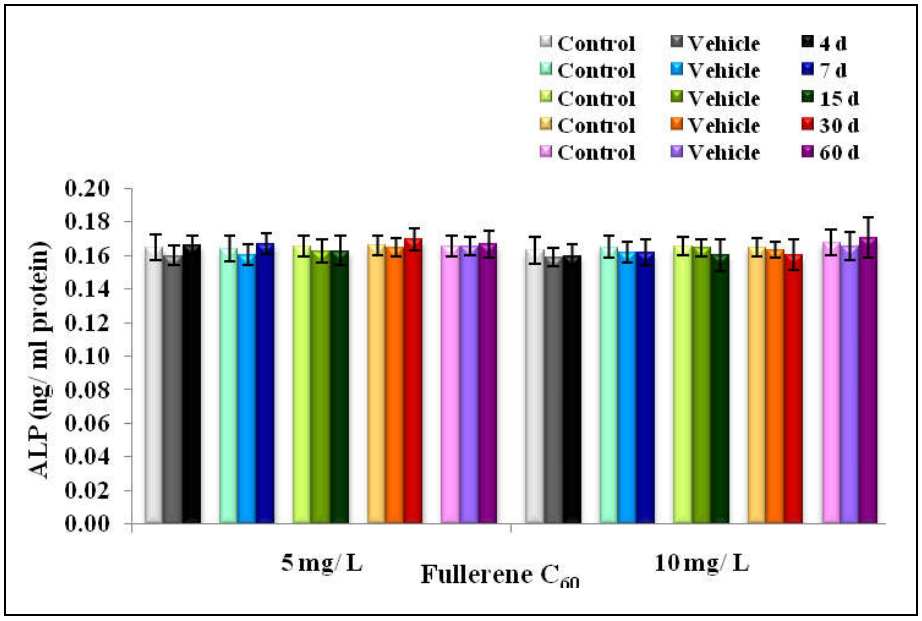

Fig. 23 Effect of fullerene C_{60} on the level of alkali-labile phosphates (ALP) in the plasma of male fish, *Anabas testudineus* (n=10/ group; Mean ± SD)

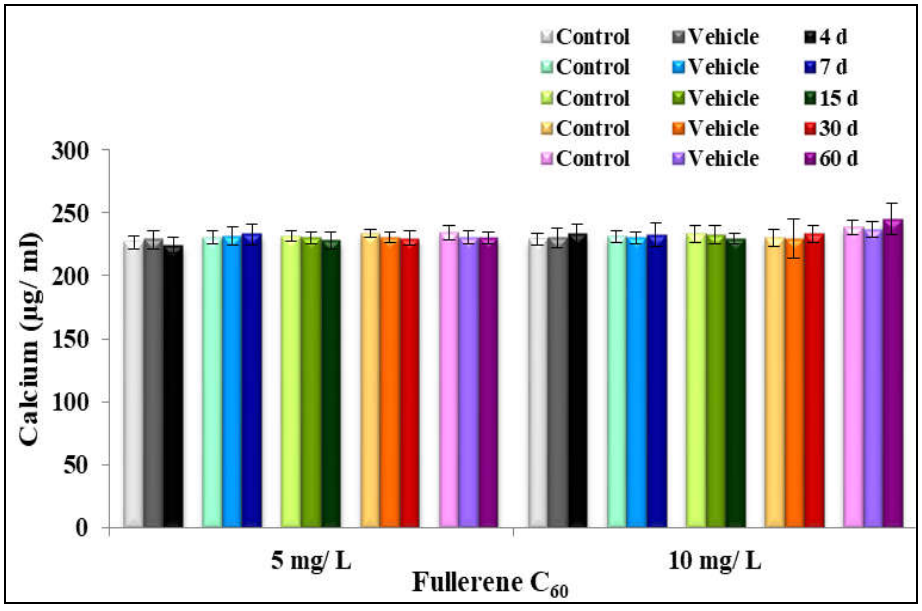

Fig. 24 Effect of fullerene C_{60} on the level of calcium in the plasma of male fish, *Anabas testudineus* (n=10/ group; Mean ± SD)

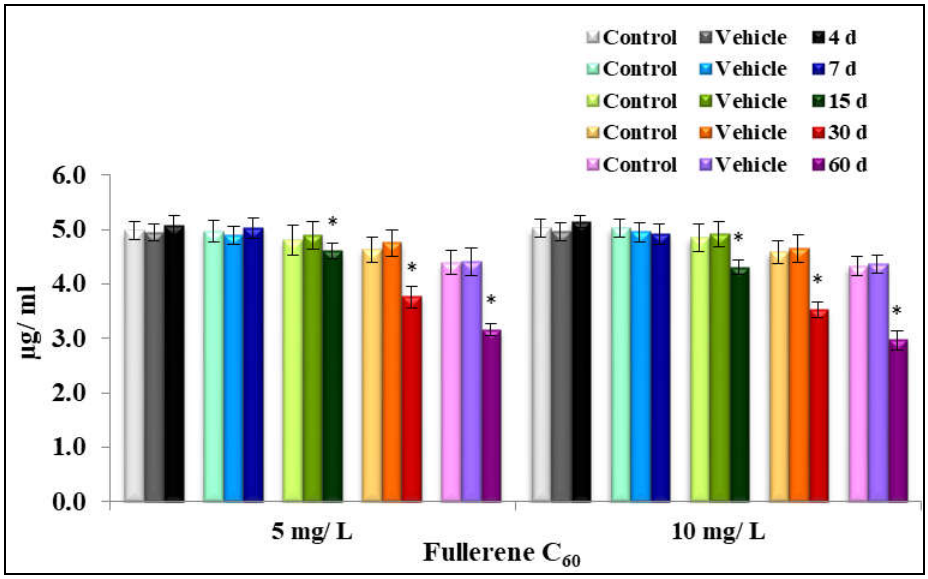

Fig. 25 Effect of fullerene C_{60} on the level of total protein in the plasma of male fish, *Anabas testudineus* (n=10/ group; Mean ± SD; *P<0.05 against the control groups)

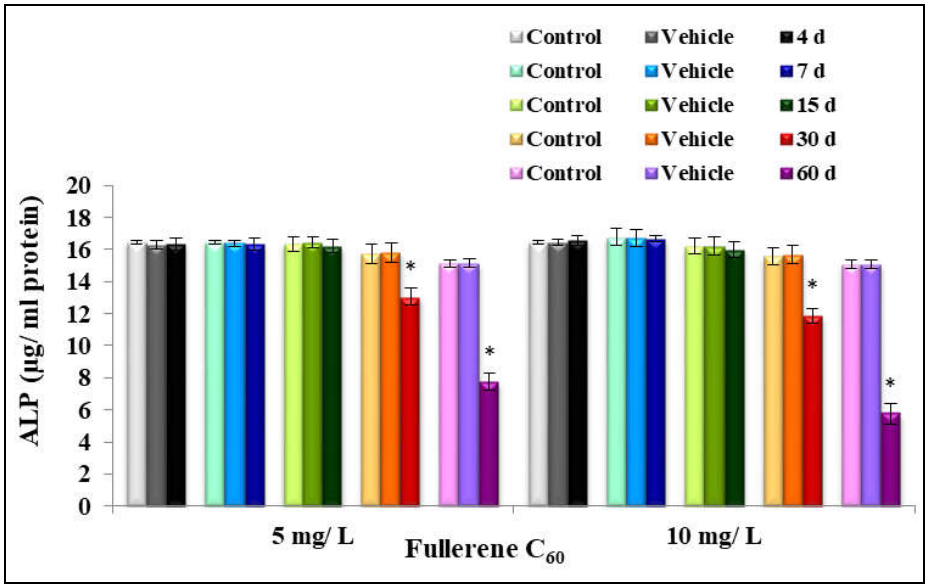

Fig. 26 Effect of fullerene C_{60} on the level of alkali-labile phosphates (ALP) in the plasma of female fish, *Anabas testudineus* (n=10/ group; Mean ± SD; *P<0.05 against the control groups)

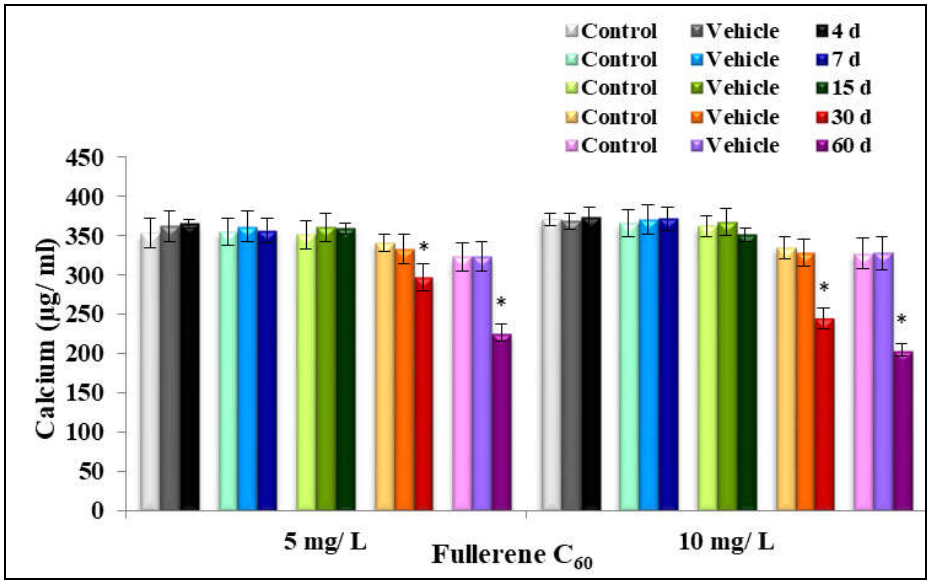

Fig. 27 Effect of fullerene C_{60} on the level of calcium in the plasma of female fish, *Anabas testudineus* (n=10/ group; Mean ± SD; *P<0.05 against the control groups)

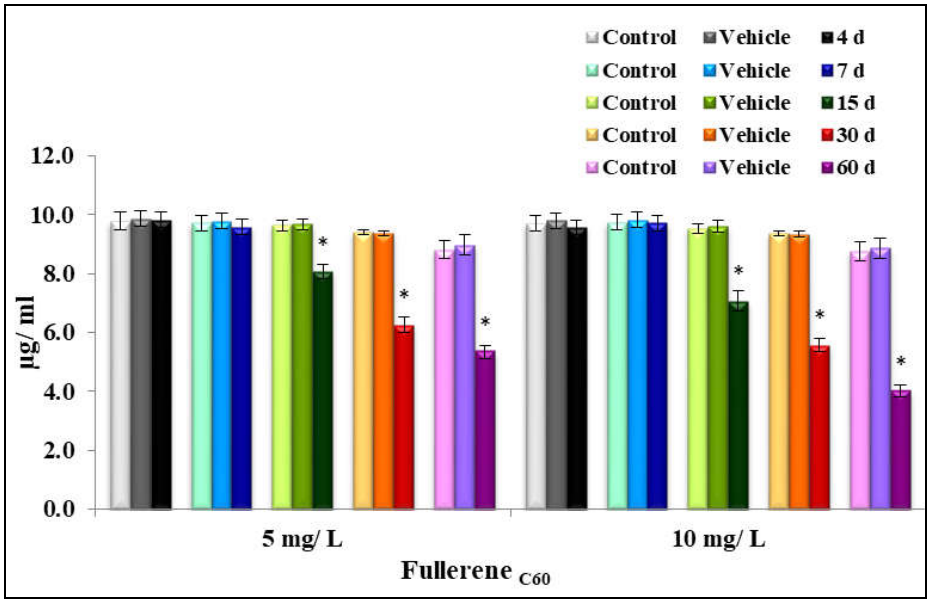

Fig. 28 Effect of fullerene C_{60} on the level of total protein in the plasma of female fish, *Anabas testudineus* (n=10/ group; Mean ± SD; *P<0.05 against the control groups)

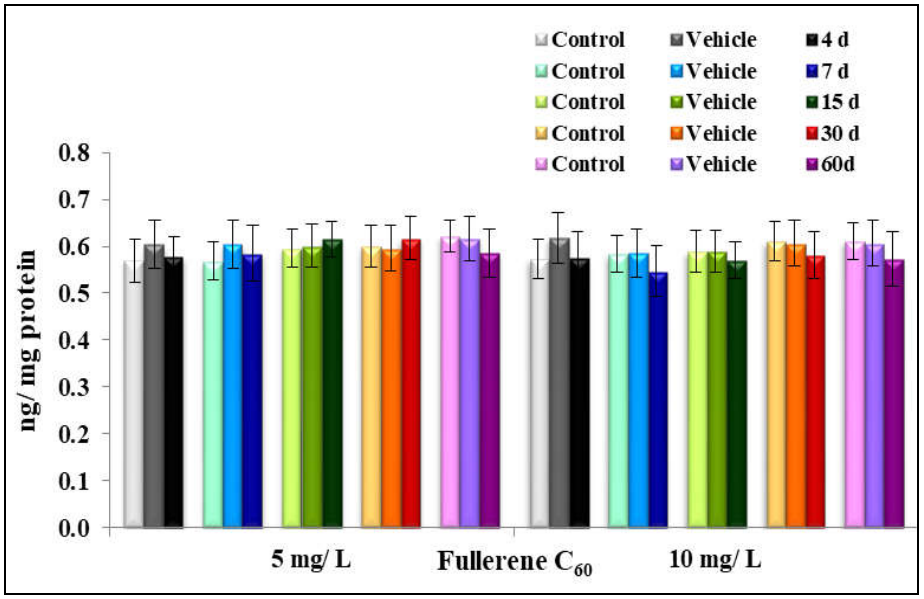

Fig. 29 Effect of fullerene C_{60} on the activity of aromatase in the testis of fish, *Anabas testudineus* (n=10/ group; Mean ± SD)

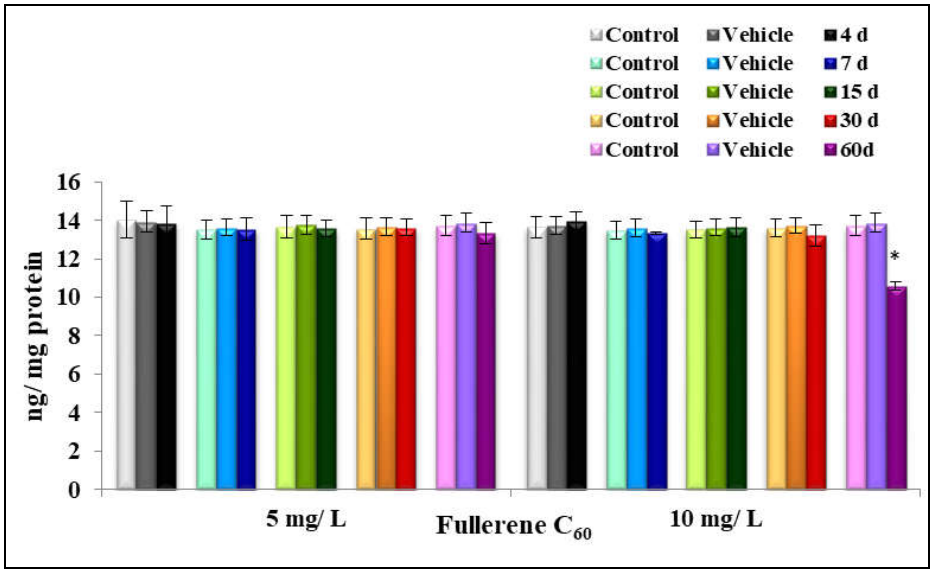

Fig. 30 Effect of fullerene C_{60} on the activity of aromatase in the brain of male fish, *Anabas testudineus* (n=10/ group; Mean ± SD; *P<0.05 against the control groups)

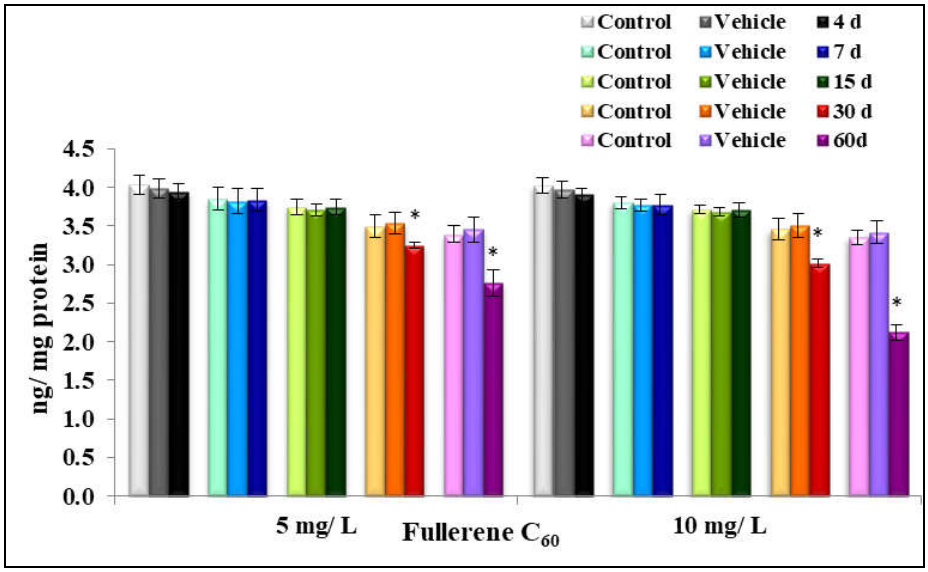

Fig. 31 Effect of fullerene C_{60} on the activity of aromatase in the ovary of fish, *Anabas testudineus* (n=10/ group; Mean ± SD; *P<0.05 against the control groups)

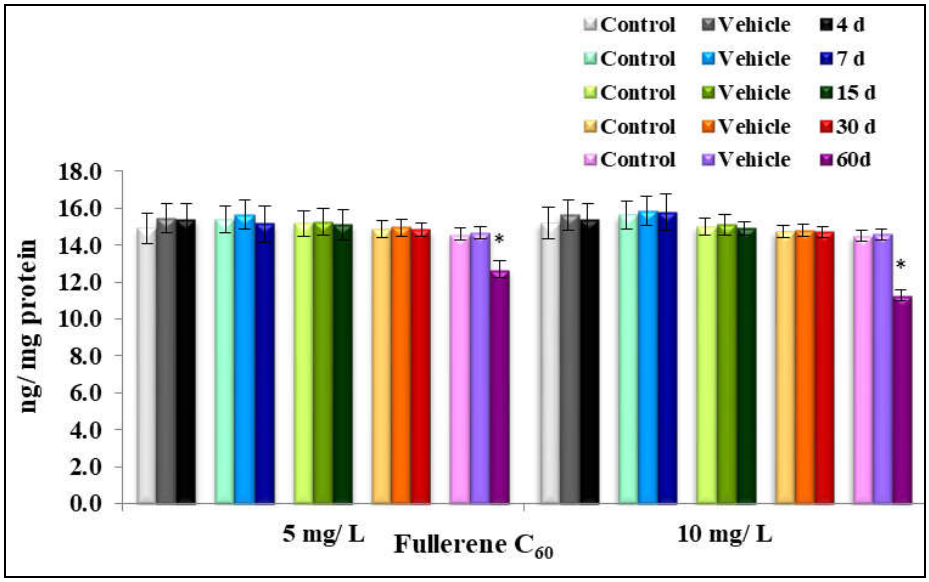

Fig. 32 Effect of fullerene C_{60} on the activity of aromatase in the brain of female fish, *Anabas testudineus* (n=10/ group; Mean ± SD; *P<0.05 against the control groups)

5.1 Effect of fullerene C_{60} on the activities of gonadal hydroxysteroid dehydrogenase enzymes

Steroid biosynthetic pathway is very crucial for the growth, development, maturation and normal functioning of theca and granulosa cells of ovary, and Leydig and Sertoli cells of testis (Svechnikov *et al*., 2010; Craig *et al*., 2011). Sublethal exposure of fullerene C_{60} at 5 and 10 mg/ L concentrations decreased the activities of 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD) after 15 d in testis and ovary of fish. Similarly, in another study exposure of oleic acid-coated silver nanoparticles showed downregulation of specific genes regulating steroidogenesis in ovarian primary cells obtained from marine medaka, *Oryzias melastigma* (Degger *et al*., 2015).

On contrary, copper nanoparticles exposed to *Clarias batrachus* through pelleted fish feed for 21 d upregulated several steroidogenic enzymes and transcription factor genes involved in steroidogenesis and testis development (Murugananthkumar *et al*., 2016). However, reduction in the activities of hydroxysteroid dehydrogenase enzymes noted in the current study after fullerene C_{60} exposure suggested endocrine disrupting effect of the nanomaterial that prevented from the normal functioning of steroidogenic pathway, which in turn affected normal hormone synthesis and disrupted gonadal histology. Thus the disruption of steroidogenic output may ultimately perturb the reproductive function of the fish, *Anabas testudineus* that was mediated through the induction of oxidative stress as discussed in chapter 2.

5.2 Effect of fullerene C_{60} on the levels of serum hormones

Hormones play critical role in reproduction of fish as it is involved in a propagative function like reproductive behaviour and reproductive processes (Yousefians and Mousavi, 2011). In the present study, fullerene nanomaterial did not alter the level of serum estradiol in male fish whereas estradiol level declined in female fish after 60 d, and this could be due to the gender-specific action of nanomaterial on the ovarian granulosa cells in *Anabas testudineus*. Similar observation has been reported in zebrafish on exposure to fullerene C_{60} nanomaterial (Park *et al*., 2011). The elevated levels of oxidative stress also can directly or indirectly affect the hypothalamus-pituitary-gonadal (HPG) axis, and the crosstalk with hormonal production (Hardy *et al*., 2005; Spiers *et al*., 2014).

The level of serum testosterone declined after fullerene C_{60} exposure in male fish without significant changes in the female fish, and this could be due to the impairment in steroidogenic pathway. A study on zebrafish showed similar reduction in the level of testosterone after titanium dioxide nanoparticles exposure by modifying the bioavailability and toxicity of bisphenol A to aquatic organisms (Fang *et al*., 2016). In another study, co-exposure of titanium dioxide nanoparticles with tris(1,3-dichloro-2-propyl) phosphate to zebrafish for 21 d resulted in gender-dependent reduction in the level of testosterone thereby suggested adverse reproductive outcomes (Ren *et al*., 2018). Testicular and ovarian tissue damages observed after fullerene C_{60} exposure (discussed in chapter 2) may be another

possible reason for the reduction in the levels of testosterone and estradiol.

Level of cortisol is widely evaluated as a stress-indicator in fish as it functions as a multifaceted hormone involved in several physiological and metabolic activities (Carrasco and van de Kar, 2003). The present data showed increase in the level of cortisol after 30 and 60 d of fullerene exposure in both sexes thereby suggested stress response of fish due to nanoparticles exposure. The results observed were in accordance with another study on exposure of fullerene C_{60} to zebrafish for 12 d resulted in the elevated level of cortisol (Sarasamma *et al*., 2018). Increase in cortisol level has been correlated with elevated blood glucose level, which in turn impair glucose metabolism, and this was evidenced after fullerene C_{60} exposure in *Anabas testudienus* as discussed in chapter 1. Similar increase in the level of cortisol has been observed after subacute exposure to silver nanoparticles in silver carp, *Hypophthalmichthys molitrix* (Shaluei *et al*., 2013).

Similarly, other nanoparticles such as nano-gold in gilthead sea bream (Teles *et al*., 2017), aluminium oxide and titanium dioxide in *Oreochromis niloticus* (Canli *et al*., 2018), copper oxide in *Cyprinus carpio* (Miri and Rahdari, 2015), and nano-iron oxide in zebrafish (Malhotra *et al*., 2019) also showed elevated level of cortisol. Thus the results demonstrated that the nanoparticles exposure induced stress in fish, which stimulated the release of cortisol by the activation of hypothalamo-pituitary-interrenal axis.

5.3 Effect of fullerene C_{60} on the levels of alkali labile phosphates, calcium and total protein

At the time of reproduction, certain components like calcium, protein and alkali labile phosphates (ALP) increase in female fish as a result of active vitellogenesis under the influence of the female hormone, estradiol (Linares-Casenave *et al*., 2003). Thus estradiol-dependent elevation in the levels of ALP, total protein and calcium were observed only in female fish, and are widely used as the indirect endpoint for the evaluation of vitellogenin in blood plasma. The current study observed decline in the concentrations of ALP, calcium and total protein in ovary, liver and blood plasma of female fish without remarkable changes in male fish. The results indicated that exposure of fullerene C_{60} nanomaterial affected vitellogenin synthesis either by the reduction in the circulating level of estradiol or the nanomaterial could have functioned as an inhibitor to circulating estradiol by preventing the binding of hormone to its receptor thereby vitellogenin is not synthesized. The present findings coincided with another study on exposure of silver nanoparticles to the rainbow trout, *Oncorhynchus mykiss*, downregulated the gene expression of vitellogenin-like proteins (Gagne *et al*., 2012). Moreover, phosphatidylcholine-based C_{60} fullerene has been shown to alter the content of vitellogenin in the embryo of *Danio rerio* (Kuznetsova *et al*., 2014). Proteomic analysis also revealed gender-specific differences in vitellogenin protein followed by the incubation of

polyvinylpyrrolidone-coated silver nanoparticles in the plasma of smallmouth bass, *Micropterus dolomieu* (Gao *et al*., 2017).

5.4 Effect of fullerene C_{60} on the activities of aromatase enzyme

Cytochrome P450 aromatase is a critical enzyme widely expressed in brain and gonads, which converts androstenedione and testosterone into estradiol, estrogen and estrone (Lephart, 1996). Aromatase A or cyp19a1a is the gonadal aromatase whereas aromatase B or cyp19a1b is the brain aromatase enzymes (Blazquez and Piferrer, 2004). In the present study, fullerene C_{60} at both sublethal concentrations decreased the activity of the ovarian and brain aromatase enzymes in female fish, while no remarkable changes were observed in testis, but brain aromatase declined in male fish. The present observations indicated that C_{60} fullerene inhibited aromatase A enzyme in female and aromatase B enzyme in both sexes, which suggested the failure of conversion of testosterone into estradiol. The results also positively correlated with the reduction in estradiol level in female fish. Aromatase is the key enzyme involved in sex differentiation and maintenance of secondary sexual characteristics of the fish, and the present findings illustrated downregulation of female-specific pathway genes. Inhibition of aromatase enzymes has been associated with sex reversal in the cichlid fish (Goppert *et al*., 2016) however, in the present study no sex reversal was observed after C_{60} fullerene exposure for 60 d. The present results showed similarity with another observation on exposure of carbon black nanoparticles

inhibited aromatase expression and estradiol secretion in human granulosa cells through the ERK1/2 pathway (Simon *et al*., 2017). The inhibition in the aromatase enzyme activity associated with the reduction in serum estradiol level observed in the current study reflected that fullerene exposure affected sexual behaviour and development in the fish, *Anabas testudineus*. Thus the findings clearly demonstrated gonadal nanotoxicity to the exposed fish, and contribute to the knowledge required for the risk assessment on the potential reproductive toxic effects.

1. Sublethal exposures of fullerene C_{60} in *Anabas testudineus* disrupted normal functioning of steroidogenic pathway as evidenced by reduction in the activities of gonadal steroidogenic enzymes.

2. Nanomaterial altered hypothalamo-pituitary-gonadal axis by decline in the serum levels of estradiol and testosterone in female and male fish, respectively.

3. Induction of stress and impairment of glucose metabolism was known by the elevated level of serum cortisol regulated through hypothalamo-pituitary-interrenal axis.

4. Estradiol-dependent reduction in the levels of alkali labile phosphates, total protein and calcium in ovary, liver and blood plasma revealed gender-specific effect on vitellogenin synthesis.

5. Inhibition in the activities of ovarian aromatase, and brain aromatase in both sexes indicated gonadal nanotoxicity.

6. The overall results suggested the potential reproductive toxicity of fullerene C_{60} in the freshwater fish, *Anabas testudineus*.

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1.1 Genotoxicity

In the past few decades, toxicological studies have attained significant attention in various fields as mutagenicity, teratogenicity, and carcinogenicity (Nagarathna *et al*., 2013). Genotoxicity refers to the study of chemicals that have the ability to change the structural integrity of the genetic material leading to mutations and heritable changes, or sometimes leading to non-mutagenic changes in the body of organisms (Guengerich, 1993). Genetic damage arise either at gene or chromosome level, where the point mutation, deletion or insertion are the reasons behind the DNA damage at gene level (Herceg and Hainaut, 2007). Sometimes the organism fail to eliminate the genotoxin from the body so that alteration in the repair mechanisms occur (Guengerich, 2003), and the unrepaired DNA damages result in mutations, apoptosis, progression of cancer, and the process of cellular senescence (Cooke *et al*., 2003).

The somatic cell mutations not only lead to carcinogenesis but also play potential role in several chronic degenerative diseases including heart diseases and atherosclerosis, and eventually leading to death (De Flora and Izzotti, 2007; da Silva *et al*., 2008). Mutation in germ cells are of great concern as the genetic damages are transferred to future generations thereby alter the gene pool at population level or reduce the reproductive fitness by high rate of mutation transfer by the offsprings and decline the normal population. Thus genotoxicity plays dual role in safety assessment programme, in which the primary role is to evaluate the adverse effects of genotoxins in the environment, and secondly to detect and eradicate the carcinogenicity of chemicals by

application of modern methodologies (Bhattacharya *et al*., 2011). Genotoxins are widely identified in the form of carcinogens, mutagens or teratogens.

1.1.1 Carcinogens

Carcinogens are the most diverse group of chemicals that are widely classified as genotoxic and non-genotoxic compounds (Hayashi, 1992). Genotoxic carcinogens like benzo[a]pyrene and aflatoxin B1 bind with the cellular DNA and induce tumors through mutations and DNA damage. Phenobarbital, diethylstilbestrol and carbon tetrachloride are the non-genotoxic carcinogens that induce tumors through different mechanisms like cytotoxicity, cell proliferation and hormonal effects but do not cause direct DNA damage (MacGregor *et al*., 2015). The genotoxic carcinogens exert carcinogenicity in organisms through direct or indirect mechanism. The genotoxins that cause direct toxicity include aryl and alkyl epoxide, nitrosamides, nitrosoureas, certain sulfate and sulfonate esters, whereas the polycyclic aromatic hydrocarbon, alkylnitrosamines, and aromatic amines are the indirect acting genotoxins (Moschel, 2001). Besides, the epigenetic carcinogens are the other class of chemicals that induce tumor by metabolic activation and not through direct DNA damage (Kobets *et al*., 2019). The mechanism of epigenetic carcinogens are by the induction of activating enzymes involved in the metabolic reaction that converts the carcinogen into the DNA reactive forms, or by the inhibition of essential detoxifying reactions that regulate the conversion of pro-carcinogens into DNA-unreactive compounds (Moschel, 2001).

1.1.2 Mutagens

Mutagens are either chemical substances or any radiations in the form of UV and X-rays that causes mutation, the irreversible heritable changes in DNA (Schrader, 2003). Most of the mutagens are carcinogenic, however all carcinogens are not mutagenic. The biological magnitudes of mutation depend on various factors like size of mutation, phase of cell cycle, target loci, the pre-existing mutations and its compounding effects. Mutation is basically classified as silent and lethal mutations, in which silent mutations occur in nonfunctional areas of DNA, and lethal mutations occur in the actively transcribing regions of DNA so that the phenotype and the expressions of genes are affected thus leading to cell death (Schrader, 2003). Chromosomal mutations such as deletion, amplification, and duplication of genes results in reduced, increased, or abnormal expression of genes located in the silent parts of the genome (Schrader, 2003).

1.1.3 Teratogens

Teratogens are chemicals that cause physical or functional abnormalities in embryo or fetus of organisms (Haroun, 2017). Teratogens are of different types which include physical agents as excessive heat and ionizing radiations, protozoal and spirochetal infections, pharmacological drugs like thalidomide, corticosteroids, excessive vitamin A, antimalarial, antiepileptic, antihypertensive and antileishmaniasis agents, industrial pollutants as cadmium and toluene, smoking and alcohol abuse, and the narcotics (Haroun, 2017).

Several factors such as the amount of teratogens, duration of exposure and stage of development determine the teratogenic potential of a chemical. However, exposure of teratogens, especially in early pregnancy affects physical malformations, behavioural and emotional development, decrease intellectual quotient and stillbirths in embryo and foetus, and sometimes leads to preterm labors, miscarriages or spontaneous abortions. Teratogens also affect aquatic animals and ecosystems where aquatic hypoxia affects the fish as known by delayed embryonic development, altered sex steroid hormones and sexual development (Shang and Wu, 2004).

1.2 Genotoxicity in aquatic ecosystems

Analysis of chemicals possessing the properties of genotoxic and carcinogenic potential provides an early warning signal for its long-term adverse effects on the aquatic animals (Rybakovas *et al*., 2009). The genetic damages are evaluated in the form of DNA adducts formation, DNA strand lesions, sister chromatid exchanges and unscheduled DNA synthesis (Dearfield *et al*., 2002). The unrepaired DNA damages initiates cascade of biological effects at the morphological, cellular, or whole animal and eventually at the community as well as the population level (Lee and Steinert, 2003). The prolonged exposure of genotoxins at very low concentrations cause severe adverse effects in population, which include altered fertilization, embryonic growth and development, and survival of organisms (Mcgregor, 2000; Lee and Steinert, 2003). Several genetic disorders are found associated with the interaction of genotoxicants with the genome thereby leading to heritable irreversible damages

(Jha, 2004). Thus the evaluation of genotoxic potential of toxicants in aquatic ecosystem plays major role in the environmental monitoring programme.

1.3 Genotoxicity as the biomarker

Organisms respond to the toxicants by alteration in the physiological, biochemical, genetical, morphological and behavioural modifications (Magalhaes and Ferrao-Filho, 2008). Several biomarkers are used to examine the effects of xenobiotics in cells, tissues, body fluids and organs or tissues of the organism (Vidal-Linan and Bellas, 2013). Pollutants also affect the genetic makeup of the organism either through mutations, genetic adaptations or genetic drift, in which ecogenotoxicology, the new branch of toxicology analyze the effects of genotoxins in the ecosystems (Belfiore and Anderson, 1998). Among the widely established methodologies used to analyze the interactions of toxicants with the DNA, micronucleus and comet assays are the most sensitive and reliable methods used to measure the level of genotoxicity in aquatic organisms.

1.3.1 Micronucleus assay

Micronucleus assay determines the numerical and structural chromosomal alterations in various *in vivo* and *in vitro* systems. Micronuclei are the masses of cytoplasmic chromatin that are not incorporated into daughter cells and remain in the cytoplasm after cell division. The formation of micronuclei occur either from the lagged whole chromosomes or acentric chromosome fragments during metaphase/ anaphase transition mediated by spindle dysfunctions, or

by the clastogens, respectively. The assay is performed in fish erythrocytes as well as in various cell types including kidney, gill, liver and fins (Bolognesi and Hayashi, 2011).

In addition to the micronucleus formation, other morphological nuclear abnormalities including blebbed, lobbed, notched and binucleated cells are described in erythrocytes of fish as the possible indicators of genotoxicity (Carrasco *et al*., 1990). Presence of micronucleus and nuclear abnormalities aided to evaluate the water quality and health status of fish species (Talapatra and Banerjee, 2007). Gill and liver cells undergo active proliferation, and thus ideal for the assessment of genotoxicity by the formation of micronuclei. Cell suspension obtained from these tissues is heterogeneous comprising large epithelial cells, well-spread nuclear chromatin cells having large cytoplasm and nucleus ratio (Bolognesi and Fenech, 2012).

1.3.2 Comet assay

Comet assay or single-cell gel electrophoresis (SCGE) is a rapid and sensitive method used to detect breaks in DNA strand, and measure DNA migration from individual immobilized cell nuclei (Fairbairn *et al*., 1995). The main advantage of comet assay is it mainly focuses on the DNA damage at single cell level. Comet assay is widely performed in both mammalian and non-mammalian species, mainly in erythrocytes as well as in germ cells, hepatopancreas and embryonic cells (Kim and Lee, 2004; Kuzmick *et al*., 2006). In fish,

the cells obtained from gill, liver and kidney tissues are widely used to assess the DNA damage.

1.4 Fish as genotoxic model

Fish are considered as an excellent model in the ecogenotoxicological studies as they exhibit a direct contact with the pollutant for longer durations (Kushwaha *et al*., 2012). Moreover, the quality of water in the environment is reflected by assessing the health status of fish because fish is more sensitive against the toxic response (Al-Sabti and Metcalfe, 1995). Fish respond to the mutagens even at the low concentrations and exert the toxic response either by bioaccumulation or through the biotransformation mediated by means of cytochrome P450-dependent oxidative metabolism (Helbling *et al*., 1999). Fish also retains the poikilothermic behaviour, which has been found to be more prone to the genetic damages and decline in the population (Kapour and Nagpure, 2005).

Several fish species have been used for the analysis of clastogenic, mutagenic and teratogenic effects of the environmental pollutants (Kushwaha *et al*., 2012). Several field and laboratory experiments proved that exposure of various chemicals have been found to increase the frequency of micronucleus formation in fish (Al-Sabti and Metcalfe, 1995; Kirschbaum *et al*., 2009). Genetic damages are evidenced by high micronucleus frequency and percent tail DNA damage in fish population inhabiting in the polluted aquatic ecosystem. Genotoxicity tests conducted in the fishes collected from the river with frequent discharge of tannery effluents showed high micronuclei

frequency (Cavas and Ergene-Gozubara, 2003a) and DNA damage (Blasiak and Kowalik, 2000). The freshwater fishes collected from River Ganga and Gomti showed genetic damages with elevated number of micronucleus and comet length (Kushwaha *et al*., 2012; Nagpure *et al*., 2016). A recent review has reported the use of different freshwater and marine fish species as the most studied organism for the genotoxicity assessment because of the economic and commercial importance in the food web and environmental biomonitoring (Gajski *et al*., 2019).

1.5 Genotoxicity of nanoparticles

The small size of the nanoparticles enables to cross the nuclear membrane and interact with the DNA to induce genotoxicity in organisms. There are two possible mechanisms through which the nanoparticles induce genetic damage, and they are referred as primary and secondary genotoxicity (Evans *et al*., 2017). The primary genotoxicity refers to the direct involvement of nanoparticles on DNA to induce damage whereas the secondary genotoxicity or indirect mechanism is mediated through reactive oxygen species (ROS) generation and inflammation (Schins and Knaapen, 2007).

The direct genotoxicity of nanoparticles, either by the penetration through nuclear pores or during mitosis, cause frameshift mutations, error-prone repair and physical strand breakages leading to DNA lesions and mutations (Magdolenova *et al*., 2013). The direct interaction of nanoparticle with DNA is the specific mechanism where the size less than 30 nm enter into the nucleus through the nuclear pore

complexes, and above 30 nm size are transported only after tagging with the nuclear localization sequence (Nabiev *et al*., 2007). Green quantum dots of 2.1nm enter into the nucleus of THP-1 cells through nuclear pore complexes while red quantum dots of 3.4 nm size failed to enter into the nucleus (Nabiev *et al*., 2007).

Silver nanoparticles of 60 nm (Kim *et al*., 2011), copper oxide with 50-100 nm (Wang *et al.*, 2012), and silicon dioxide having 40-80 nm size (Chen and Vonmikecz, 2005) were transported into the nucleus during division and interact with the DNA, which later influence the replication and transcription mechanisms. Nanoparticles with a size ranged from 3-46 nm shows high affinity for DNA and strongly inhibit DNA replication (Li *et al*., 2013). Use of reporter cells and formation of stalled replication forks is another novel mechanism to study the direct interaction of nanoparticles to DNA (Akerlund *et al*., 2017).

The indirect genotoxicity of nanoparticles occur by the induction of oxidative stress either by the surface reactions of nanoparticles or by the removal of redox-active transition ions including Fe^{2+}, Ni^{2+}, Cu^{+}, Ag^{+}, and Mn^{2+} ions through the Fenton-type reaction (Kruszewski *et al*., 2011). The generated ROS interact with the mitochondria and affect the electron transport chain through the induction of P450 enzymes. The nanoparticles-mediated oxidative stress was documented after fullerene C_{60} exposure and was explained in detail in chapter 2.

The nanoparticles also affect the proteins in DNA repair mechanism and antioxidant defense system thereby leading to indirect DNA damage (Magdolenova *et al*., 2013). Nanoparticles that interact with the centrioles, mitotic spindle apparatus and the associated proteins cause aneugenic effects including gain or loss of chromosomes in daughter cells (Siegrist *et al*., 2014). Nanoparticles also induce genetic damages through the intercellular signalling across certain channels and junctions like hemi-channels, pannexin channels, connexin gap junctions, and during the transmission of purine nucleotides (Bhabra *et al*., 2009). Reactive oxygen or nitrogen species generated through secondary mechanism activate phagocytes like neutrophils and macrophages for the nanoparticles-elicited inflammation (Bartek *et al*., 2010). The inflammatory responses due to nanoparticles exposure vary due to the surface reactivity, particle solubility and the threshold limit (Borm *et al*., 2011).

1.6 Cytotoxicity of nanomaterials

Nanoparticles on entering into the cell, decline the rate of cell proliferation while increases necrosis and apoptosis thus induce cytotoxicity. This was evidenced after exposure of several nanoparticles like gold, copper, silver, fullerenes and carbon nanotubes in various *in vivo* and *in vitro* studies. Cytotoxicity of nanoparticles mainly depends on the physicochemical properties including chemical composition, aggregation, crystalline structure, and the size (Jedrzejczak-Silicka and Mijowska, 2018). The composition of nanomaterial governs the chemical interaction, cellular uptake and the

internal localization, and resulted in oxidative stress (Jedrzejczak-Silicka and Mijowska, 2018). Despite the oxidative stress, the other mechanisms involved in the nanoparticle-induced cytotoxicity includes mitochondrial dysfunction, direct damage of cell membranes, metal ion release, DNA damage, actin cytoskeleton disruption, and the lysosomal dysfunction. It has been reported that the nanoparticles damage the membrane integrity either through the direct action on the membrane components (Lin *et al*., 2010) or through ROS generation and consequent oxidation of membrane lipids (Voinov *et al*., 2011). Nanoparticles such as silicon and zinc oxide have the ability to interact with the membrane surface receptors to induce cytotoxicity (Sun *et al*., 2011; Shi *et al*., 2012).

The simplest method used to assess cytotoxicity is dye uptake assay where either by visual inspection of cells through a bright-field microscopy or spectrophotonic measurements was employed for scoring the changes in the nuclear and cellular morphology (Fiorito *et al*., 2006). Neutral red and trypan blue dye exclusion methods are widely used for *in vitro* cytotoxicity tests (Goodman *et al*., 2004; Monteiro-Riviere and Inman, 2006). Lactate dehydrogenase (LDH) assay is another method used to measure the cytotoxicity where the concentration of LDH released from the damaged cell is directly proportional to the amount of cells damaged or lysed (Haslam *et al*., 2000). Another most widely used cytotoxicity method is the MTT viability assay, which produce a dark-blue formazan product in live cells to differentiate from the dead cells (Monteiro-Riviere and Inman,

2006). In the present chapter, the genotoxic and cytotoxic potential of fullerene C_{60} was evaluated in erythrocytes as well as in the cells of gill and liver tissues of the freshwater fish, *Anabas testudineus*.

DNA damage is considered as one of the major causes of genetic instability leading to the development of cancer. Nanoparticles have the ability to interact with the biomolecules like DNA, proteins, lipids, enzymes, and other components of cells (Mahmoudi *et al*., 2011). Nanoparticles on comparison with other well-known genotoxic compounds cause DNA damage due to its unique physicochemical characteristics and behaviour rather than the size of the particles (Knaapen *et al*., 2004). However, it has been considered that smaller nanoparticles possess the ability to interact with DNA through nuclear penetration than the particles of more than 4 nm size (Nabiev *et al*., 2007). The other indirect mechanism of nanoparticles-induced DNA damage has been related with the generation of reactive oxidative substances (Sharma *et al*., 2009; Vidya and Chitra, 2018). Thus there are several reasons for the initiation of DNA damage due to nanoparticles exposure that could promote carcinogenesis. Therefore, it is essential to consider the genotoxicity of nanoparticles while assessing its biocompatibility in medical, pharmaceutical and other applications.

Mutagenicity of fullerene C_{60} has been reported in few strains of *Salmonella* such as TA102, TA104 and YG3003, and the mechanism of DNA damage was mediated through the indirect action through the generation of singlet oxygen due to lipid peroxidation (Sera *et al*., 1996). Nano-C_{60} has been proved as cytotoxic to human neuronal astrocytes, dermal fibroblasts and liver carcinoma cells after 48 h exposure, and the damage to cell membrane was mediated through oxidative damage (Sayes *et al*., 2005). Cytotoxic action of pure fullerene suspension or nano-C_{60} differed from the water-soluble polyhydroxylated fullerene (C_{60}(OH)n), where nano-C_{60} exerted strong

pro-oxidant capacity responsible for the rapid necrotic cell death whereas polyhydroxylated C_{60} exerted mainly antioxidant/ cytoprotective action (Isakovic *et al*., 2006). The genotoxic and cytotoxic effects of single-walled carbon nanotubes examined using three different test systems such as the comet assay and micronucleus test in a lung fibroblast-V79 cell line, and Salmonella gene mutation assay in strains YG1024/ YG1029 has demonstrated the induction of DNA damage thus promoted the inflammatory action and formation of granulomas (Kisin *et al*., 2007). Similarly, C_{60} fullerene and single-walled carbon nanotubes exposed to FE1-Mutatrade markMouse lung epithelial cell line at 200 µg/ ml concentration caused mutagenicity evidenced by elevated levels of FPG sensitive sites/ oxidized purines thereby proved as cytotoxic and genotoxic to lung cell lines (Jacobsen *et al*., 2008).

Four typical nanomaterials with comparable properties such as carbon black, single-walled carbon nanotube, silicon dioxide and zinc oxide nanoparticles when tested for genotoxicity has evidenced that the primary role in the cytotoxicity of different nanoparticles were played by the composition of particles however, the shape of the particles attributed to the potential genotoxicity (Yang *et al*., 2009). On comparison of five different nanoparticles for the inflammatory and DNA damaging effects, it has been suggested that quantum dots had greatest effects followed by carbon black, SWCNT where fullerene C_{60} and gold nanoparticles as least genotoxic (Jacobsen *et al*., 2009). Several engineered nanoparticles such as quantum dots, metal and metal-oxide nanoparticles, fibrous nanomaterials and fullerenes has been known to damage or interact with DNA, and found to cause genetic damages such as point mutations, chromosomal fragmentation,

DNA strand breakages, oxidative DNA adducts and alterations in the profiles of gene expression (Singh *et al*., 2009).

Oral exposure to low doses of C_{60} fullerenes and SWCNT has been found associated with elevated levels of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) in the liver and lung, which is likely to be caused by a direct genotoxic ability rather than an inhibition of the DNA repair system (Folkmann *et al*., 2009). Water-soluble supramolecular inclusion complexes of alpha-, beta-, and gamma-cyclodextrin-bicapped C_{60} has been shown to cleave double-stranded DNA under visible light irradiation in the presence of NADH thereby attributed to cytotoxic properties (Wang *et al*., 2009). Poly-hydroxylated C_{60} fullerenols treated to Chinese hamster ovary cells has decreased the frequency of micronuclei and chromosome aberrations and has been proved as antigenotoxic agent (Mrdanovic *et al*., 2009).

Particles from combustion processes, silicate, titanium dioxide and nanoparticles such as C_{60} fullerenes and carbon nanotubes has been shown to elevate the levels of lipid peroxidation products and induced oxidative DNA damage in humans and various animal models (Moller *et al*., 2010). Exposure of multi-walled carbon nanotubes (MWCNTs) on human umbilical vein endothelial cells has been shown to induce cytotoxic and genotoxic effects, probably through oxidative damage pathways (Guo *et al*., 2011). A study has reported that colloidal fullerene C_{60} has DNA-damaging potential, and that the DNA damage was not influenced due to covalent DNA adduct formation but by the indirect mechanism of C_{60} itself (Matsuda *et al*., 2011). Polyethylene glycol-fullerene C_{60} suspension exposed to human connective tissue-derived fibrosarcoma cells HT1080 has been observed to induce intracellular DNA fragmentation according to TUNEL assay, and produced reactive oxygen species such as

hydroperoxides and peroxyl radicals or superoxide anion radicals in HT1080 cells as demonstrated by 2′,7′-dichlorodihydrofluorescein diacetate (CDCFH-DA) assay or nitroblue tetrazolium assay, respectively (Liao *et al*., 2011).

Size-dependent DNA damage has been detected on incubation of carboxylated polystyrene beads with macrophage cell line RAW 264.7 cells, and the changes was known to affect the balance between lesion incidence and repair mechanism differently (Zhang *et al*., 2011). Nanomaterials, such as quantum dots, the water-soluble fullerene derivative C_{60}(OH)n and titanium dioxide nanoparticles induced DNA damage even under irradiation by A-band UV (λmax = 365 nm), and showed more severe DNA damage than quantum dots under similar conditions (Yamazaki *et al*., 2011). Buckminsterfullerene (C_{60}) has been reported to enter into the bacterial cells and bind to DNA molecules thereby affected the thermal stability and enzymatic digestion of DNA molecules, which in turn induced DNA mutations (An and Jin, 2011). Combined exposure to C_{60} and fluoranthene has been found to additively enhance the levels of DNA strand breaks along with a two-fold increase in the total glutathione content thereby resulted in synergistic genetic damage in marine mussels (Al Subiai *et al*., 2012).

Carbon nanomaterials such as carbon nanotubes, graphene, and fullerenes C_{60} examined in *in vivo* and *in vitro* mammalian models showed that insoluble nanomaterials have caused polyploidy by blocking cytokinesis rather than by damaging DNA, probably due to non-DNA interacting mechanisms (Honma *et al*., 2012). A large-scale association study for C_{60} nanoparticle explored the mechanisms of nanotoxicity, in which the nanoparticle has been found to bind with the

minor grooves of double-stranded DNA and triggered unwinding and disruption of the DNA helix, which indicated that C_{60} inhibited DNA replication and induced DNA/ RNA damage (Xu *et al*., 2012). Gastrointestinal exposure to several nanoparticles such as single-walled carbon nanotubes, fullerenes C_{60}, carbon black, titanium dioxide and diesel exhaust particles has been shown to generate oxidized DNA base lesions in organs like bone marrow, liver and lung, which was found associated with increased level of lipid peroxidation derived exocyclic DNA adducts in the liver, suggesting multiple pathways of oxidative stress for particle-generated damage to DNA (Moller *et al*., 2012). On contrary, an *in vivo* study on the genotoxicity of fullerene C_{60} nanoparticles evaluated with comet assays using the lung cells of rats showed no increase in percent tail DNA (Ema *et al*., 2012).

Multi-walled carbon nano-onions at 0.2, 1 and 5 μg/ ml concentrations has been found to induce DNA damage and apoptosis in human umbilical vein endothelial cells, which was mediated through the generation of reactive oxygen species (Xu *et al*., 2013). In a generation to generation study, it has been reported that the larger sized multi-walled carbon nanotubes penetrated across the blood-placenta barrier, which resulted in the reduction of fetal development and induced brain deformity in mice, whereas single-walled and smaller sized multi-walled carbon nanotubes did not show fetotoxicity. The same study further confirmed by a molecular mechanism that multi-walled carbon nanotubes directly triggered p53-dependent apoptosis and cell cycle arrest in response to DNA damage (Huang *et al*., 2014). In another study, two different types of nanomaterials, such as carbon nanotubes and tungsten trioxide nanoplates, simply mixed

with naked DNA plasmid in dry or wet contact modes for varied time periods and analyzed using gel electrophoresis and fluoro-spectrometry found certain types and degrees of DNA damages like single and double strand break, and bacterial mutation, which was more prominent in wet contact condition thereby provided caution in DNA delivery (Thongkumkoon *et al*., 2014).

Xenopus laevis tadpoles exposed to multi-walled carbon nanotubes showed DNA damages in erythrocytes when measured using comet assay, and it was found associated with the induction of oxidative stress (Saria *et al*., 2014). Similarly, genotoxicity of metal oxide nanoparticles such as copper oxide, nickel oxide and zinc oxide, as well as quartz nanoparticles in various cell lines occurred mainly through oxidative stress pathway rather than the direct DNA binding with subsequent replication stress (Karlsson *et al*., 2014). In a comparative and mechanistic genotoxicity assessment of four engineered nanomaterials namely fullerene, titanium dioxide, carbon black and single-walled carbon nanotubes in *Escherichia coli*, yeast, and human cells has revealed that eukaryotes, especially mammalian cells, are likely more susceptible to genotoxicity than prokaryotes in the ecosystem (Lan *et al*., 2014).

Beside genotoxicity, some nanoparticles as fullerene and carbon nanotubes have been found to induce cytotoxicity in human T-leukemia cells and cancer cells (De Gianni *et al*., 2015; Zhang *et al*., 2015). DNA damage and cytotoxicity of hydroxylated fullerene has been demonstrated in freshly isolated rat hepatocytes as evidenced by DNA fragmentation, decrease in cellular ATP levels, formation of cell surface blebs, induction of reactive oxygen species and loss of mitochondrial membrane potential (Nakagawa *et al*., 2015). Likewise

in male human peripheral blood lymphocytes, administration of multiwall carbon nanotubes exhibited an increase in the level of intracellular reactive oxygen species, which lead to cell damage and death, proliferation inhibition, DNA damage, and an inflammatory response (Kim *et al*., 2016). Assessment of carbon- and metal-based nanoparticles in the induction of DNA damage using different microfluidic electrophoretic separation technology revealed that the physico-chemical properties of nanoparticles contributed for the DNA fragmentation in human lung macrophages (Schrand *et al*., 2015). An *in vitro* study on diploid human embryonic lung fibroblasts incubated with water-soluble fullerene derivative has increased DNA breaks along with an increase in the expression levels of TGF-β, RHOA, RHOC, ROCK1, and SMAD2 thereby revealed pulmonary fibrosis (Ershova *et al*., 2016).

Fischer-344, Long-Evans and Wistar strains of rats treated to multi-walled carbon nanotubes through oral gavage has increased the level of DNA lesions in lymphocytes proving Fisher-344 rats as the most sensitive and Wistar rats as the most tolerant to the genotoxic effects of nanotubes (Gerencser *et al*., 2016). A toxicological assessment of ten engineered nanomaterials in three human epithelial cell lines as A549, HK-2 and HepG2 has found to induce oxidant generation, DNA damage and cytotoxicity, which proved as toxicant to relevant target organs as lung, kidney and liver (Thongkam *et al*., 2017). In a study conducted in the human lung epithelial cell line, A549, both cytotoxic and genotoxic properties has been exerted by the uncoated titanium dioxide nanoparticles in the presence of citrate, and also exerted epigenotoxic effects as evaluated by the marked reduction in the levels of LINE-1 methylation (Stoccoro *et al*., 2017). Investigation of carbon black, carbon nanotubes and three titanium

dioxide nanoparticles on the pulmonary inflammation and DNA strand breaks has revealed that the use of different dispersion medium influenced the intensity of nanotoxicity (Hadrup *et al*., 2017). In microarray analysis of human primary endothelial cells, graphene intervention has been shown to induce DNA damage as measured using comet assay (Basheer *et al*., 2018). A comparative study on single-walled and multi-walled carbon nanotubes, it has been reported that single-walled nanotubes exposure showed hypermethylation on functionally important genes, such as SKI proto-oncogene, glutathione S-transferase pi 1 (GTSP1) and shroom family member 2 (SHROOM2) and neurofibromatosis type I (NF1), which were then hypermethylated and downregulated (Oner *et al*., 2018). The exposure of multi-walled carbon nanotubes and its pristine Mitsui-7 form represented a dose-dependent localization in the cell nuclei and caused elevated mitotic aberrations, especially in multi- and monopolar spindle structures thereby resulted in centrosomal fragmentation and chromosomal translocation (Siegrist *et al*., 2019). In a recent study on *Drosophila melanogaster* exposed to the water-soluble pristine C_{60} at 20 µg/ ml and 40 µg/ ml concentrations has induced the activation of mus209 gene and elevated the levels of DNA damage (Yasinskyi *et al*., 2019).

Although there are several researches that focused on the cytotoxicity and genotoxicity of nanoparticles in different systems, most of the studies expended considerable attention on *in vitro* models. There is lack of information regarding the DNA damaging effect of fullerene C_{60} on animal models; particularly on fish therefore the current study was conducted to evaluate the genotoxic and cytotoxic potential of fullerene C_{60} nanomaterial in the freshwater fish, *Anabas testudineus*.

3.1 Maintenance of test animal

The freshwater fish**,** *Anabas testudineus* (11 ± 1 g and 8 ±1 cm) collected from Pulimugham Hatcheries, Alappuzha district, Kerala, India were acclimatized in 50 L glass tanks for two weeks. Fish maintained in dechlorinated water was provided with standard fish pellets and continuously monitored for normal health conditions. All physicochemical parameters such as temperature (28 ± 2 ºC), pH (6.5-7.5) and oxygen saturation (70-100%) of the tap water was analyzed in accordance with APHA guidelines (1998).

3.2 Study design

Crystalline C_{60} fullerene with particle size of 34.95 nm dispersed in dimethyl sulfoxide (DMSO) by sonication was used. Two sublethal concentrations of fullerene C_{60}-DMSO suspension such as 5 mg/ L (one-tenth of LC_{50}) and 10 mg/ L (one-fifth of LC_{50}) were exposed to fish for 4, 7, 15, 30 and 60 d. Each group containing ten specimens was maintained in replicates along with negative- and solvent-control groups.

3.3 Sample collection and analysis

After every designated time period of fullerene exposure, fish blood was collected from caudal vein using heparinised syringes without killing the animal. The collected fresh blood was then transferred into microcentrifuge tubes for micronucleus and comet analysis. After the collection of blood samples, fish were sacrificed, and gill and liver tissues were dissected out, washed with cold Hank's

Balanced Salt Solution (HBSS) to remove blood clots and debris. Then the tissues were minced into small pieces, and the obtained cell suspensions were used to for micronucleus and comet assays.

3.4 Micronucleus assay

The micronucleus assay was performed in fish erythrocytes (Heddle, 1973; Schmid, 1975) by mixing a drop of fresh blood with equal volume of foetal calf serum to prepare a thin smear on a clean glass slide. The slides were thoroughly air dried and fixed in absolute methanol for 10 min, dried again and stained with 5% Giemsa for 30 min.

Micronucleus assay done in gill and liver cells were according to the standard method as described by Cavas *et al*. (2005) and Levorni *et al*. (2016). Gill and liver tissues fixed in Carnoy's fixative for 15 min were macerated with 20% and 45% acetic acid for 15 min, respectively. After the chemical maceration, gill tissue was place on a clean glass slide and then the epithelial cells were scrapped off from the tissue using a tweezer. The slides were air-dried, fixed in absolute methanol for 15 min, and then stained with 10% Giemsa for 10 min. Similarly, the minced liver tissue was microfuged and filtered to get a cell suspension. A few drops of the cell suspension was smeared on a clean glass slide, air-dried and stained with 5% Giemsa for 8 min. Three slides were prepared from each tissue and blood samples, and 1000 cells were scored from each slide under the Carl Zeiss Axioscope-2 plus Trinocular Research Microscope with 1000X magnification. Photographs for the frequencies of micronucleus,

cytoplasmic and nuclear abnormalities were captured using a canon shot camera.

3.5 Classification and scoring of nuclear and cytoplasmic abnormalities

The anomalies observed in cells were classified as cytoplasmic and nuclear abnormalities (Carrasco *et al*., 1990), which include:

(i) Anisochromatic erythrocytes: Outer pigmented and normal cytoplasm with inner central colourless nucleus.

(ii) Echinocytes or burr-like erythrocytes: Evenly spaced short and blunt projections throughout the cytoplasmic membrane.

(iii) Acnathocytes erythrocytes: Abnormal cytoplasmic membrane with horny projections.

The other cytoplasmic abnormalities in fish erythrocytes include the vacuolated, deformed and degenerated cytoplasm as well as sticky cells.

The nuclear abnormalities of fish erythrocyte include micronucleus, nuclear bud, irregular nucleus, vacuolated, notched, serrated nucleus and binucleated cell. Micronucleus was scored following the criteria as follows:

(a) Micronucleus should be smaller or one-third of the size of the main nucleus

(b) Micronucleus must be separated from the main nucleus

(c) Micronucleus must be on the same plain of focus having same stain colour when compared to the main nucleus.

Micronucleus was then scored by following the formula:

$$\text{Micronucleus (\%)} = \frac{\text{Number of cells containing micronucleus}}{\text{Total number of cells counted}} \text{ x } 100$$

3.6 Comet assay

The comet assay was done according to the standard protocol (Singh *et al*., 1988), with slight modification. The glass slides were dipped in absolute methanol and burned in blue flame to remove the dust and other particles. The cleaned glass slides coated with 1% normal melting point agarose (NMA) was dried at 4 ºC until use. Then, whole blood (10 µl) was mixed with low melting point agarose (LMPA) (0.5 %), and a uniform layer on the pre-coated agarose slide was made using a coverslip. After solidification for 5-10 min, the coverslip was removed to make a third agarose layer, coverslip was replaced and kept for 5-10 min. After proper solidification, the coverslip was removed and the slides were dipped in a freshly prepared ice-cold lysing solution (2.5 M NaCl; 100 mM Na_2EDTA; 10 mM Tris buffer, 1% SDS, 10% DMSO and 1% Triton-X 100; pH 10 at 4 ºC) for 1 h for cellular lysis. After lysis, the slides were washed with phosphate buffered saline (PBS), and placed in electrophoresis buffer (pH 13 at 4 ºC) for 20 min to denature. After denaturation, the same

buffer was used for electrophoresis with 24 V (~ 0.74 V/ cm) and 300 milliamperes (mA) for 30 min.

The slides were washed with PBS thrice for 5 min in neutralization buffer (pH 7.0). The slides were stained in ethidium bromide and observed under Epifluorescent inverted microscope (Olympus CKX41) using green filter (Excitation filter BP480-550C) and photos captured using C-mount camera (Optika pro5 CCD camera). The images were used to measure the comet parameters like head DNA, head DNA percentage, tail length, tail DNA, tail DNA percentage, tail moment and olive moment by using the software Open Comet-Image J, version 1.3.1.

The percentage of tail DNA damages were categorized into 5 grades by counting 100 cells where the damages less than 5%, 5-20%, 20-40%, 40-95% and more than 95% are graded as grade zero, one, two, three and four, respectively (Collins, 2004). The total score was evaluated as follows:

Total score = (% of cells in grade 0 X 0) + (% of cells in grade 1 X 1) + (% of cells in grade 2 X 2) + (% of cells in grade 3 X 3) + (% of cells in grade 4 X 4)

3.7 Statistics

The data for micronucleus and comet assays were represented as Mean ± SD for ten replicated fish in each group. IBM SPSS 21.0 statistical software were used for testing one-way ANOVA followed

by Duncan's multiple range as post-hoc test. Data represented in asterisks (*) were found significant at $P<0.05$ against the respective control groups.

4.1 Effect of fullerene C_{60} on nuclear and cytoplasmic abnormalities in peripheral erythrocytes

Micronucleus test conducted in peripheral blood collected from negative-control and vehicle-control groups of *Anabas testudineus* showed normal erythrocytes with clear cytoplasm and distinct nucleus without any damages (Fig. 1). Exposure of fullerene C_{60} at sublethal concentrations (5 and 10 mg/ L) caused nuclear and cytoplasmic abnormalities in peripheral erythrocytes compared to the control groups. The nuclear abnormalities like micronucleus, binucleated, nuclear bud, and cells with irregular, vacuolated, and serrated nucleus were more prominent up to 15 d of fullerene exposure, which declined after 30 d onwards (Fig. 1; Table 1). In addition, the cytoplasmic abnormalities like echinocyte, acanthocyte, anisochromatic cells, sticky cells, vacuolated cytoplasm, cytoplasmic degeneration, and erythrocytes with irregular membrane were observed in fullerene C_{60} exposed groups (Fig. 2). The severity of cytoplasmic abnormalities increased with concentrations and durations.

4.2 Effect of fullerene C_{60} on nuclear abnormalities in gill and liver cells

Gill and liver cells showed nuclear abnormalities such as micronucleus, nuclear bud and binucleated cells in *Anabas testudineus* after sublethal exposure of fullerene C_{60} nanomaterial, which was concentration- and time dependent (Fig. 3; Table 2). However, control gill and liver cells showed no nuclear and cytoplasmic deformities (Fig. 3; Table 2).

4.3 Effect of fullerene C_{60} on DNA damage in erythrocytes using comet score

Grade 0 and grade 1 DNA damage were observed in negative-control and vehicle-control groups showing 1.1 and 1.3 total scores, respectively (Table 3). Grade 2 DNA damage was observed after 4, 7 and 15 d of fullerene exposure having total score of 2.5, 4 and 5.5, respectively at 5 mg/ L concentration, and total score of 3.9, 5.2 and 6.9, respectively at 10 mg/ L concentration (Table 3). Fullerene exposed for 30 and 60 d showed grade 3 DNA damage with a total score of 8.5 and 11.2 at 5 mg/ L concentration, and 11.2 and 12.5 as total score at 10 mg/ L concentration (Table 3). The comet components such as head DNA, percent head DNA, tail DNA, percent tail DNA, tail length, tail moment and olive moment showed significant changes in erythrocytes of *Anabas testudineus*. The severity of DNA damages were concentration- and time-dependent (Table 4).

4.4 Effect of fullerene C_{60} on DNA damage in gill cells using comet score

Comet score of negative-control and vehicle-control gill cells showed grade 0 and 1 DNA damage with total score of 1.9 and 2.2, respectively (Table 5). Similarly, fullerene C_{60} exposed group showed grade 0 DNA damage at 5 mg/ L concentration, however at high concentration grade 2 DNA damage was observed (Table 5). Grade 2 DNA damage was seen after 7 and 15 d of fullerene exposure at 5 mg/ L concentration, and only after 7 d at 10 mg/ L concentration (Table 5). DNA damage was increased to grade 3 after 30 and 60 d at low

sublethal concentration whereas grade 3 DNA damage was noted in high concentration after 15, 30 and 60 d (Table 5).

The comet parameters such as head DNA, percentage head DNA, tail DNA, percentage tail DNA, tail length, tail moment and olive moment showed time-dependent and concentration-dependent changes when compared to the control gill cells (Table 6).

4.5 Effect of fullerene C_{60} on DNA damage in liver cells using comet score

Liver cells obtained from negative-control and vehicle-control groups showed DNA damage of grade 0 and grade 1 with the total score of 2.4 and 2.8, respectively (Table 7). Grade 1 DNA damage was observed only after 4 d of fullerene exposure at 5 mg/ L concentration (Table 7) whereas fullerene exposed for 7 and 15 d at low sublethal concentration and after 4 and 7 d at high sublethal concentration showed grade 2 DNA damage (Table 7). Grade 3 DNA damage was observed after 30 and 60 d of fullerene C_{60} exposure at 5 mg/ L concentration, and after 15, 30 and 60 d at 10 mg/ L concentration groups (Table 7).

A significant increase in the comet parameters such as head DNA, percentage head DNA, tail DNA, percentage tail DNA, tail length, tail moment and olive moment was observed at both sublethal fullerene C_{60} exposure groups, and the severity of damages were concentration- and time-dependent (Table 8).

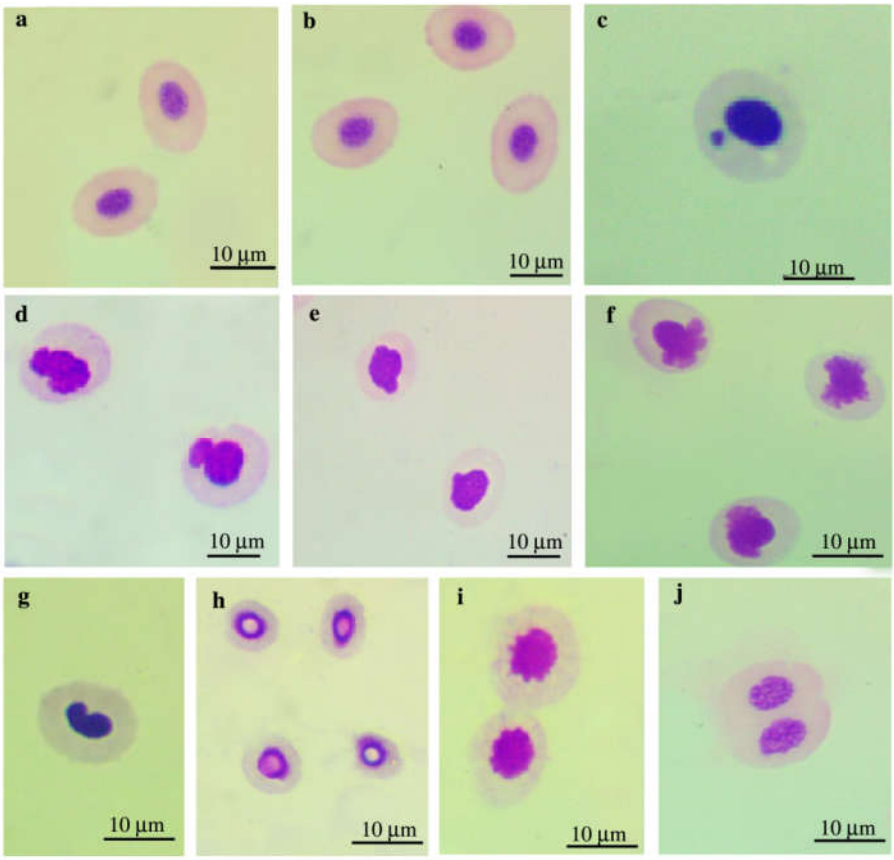

Fig. 1 Effect of fullerene C_{60} showing nuclear abnormalities in the peripheral erythrocytes of *Anabas testudineus*: (a) Control; (b) Vehicle control (DMSO); (c) Micronucleus; (d) Nuclear bud; (e & f) Irregular nucleus; (g) Notched nucleus; (h) Vacuolated nucleus; (i) Serrated nucleus; (j) Binucleated cells

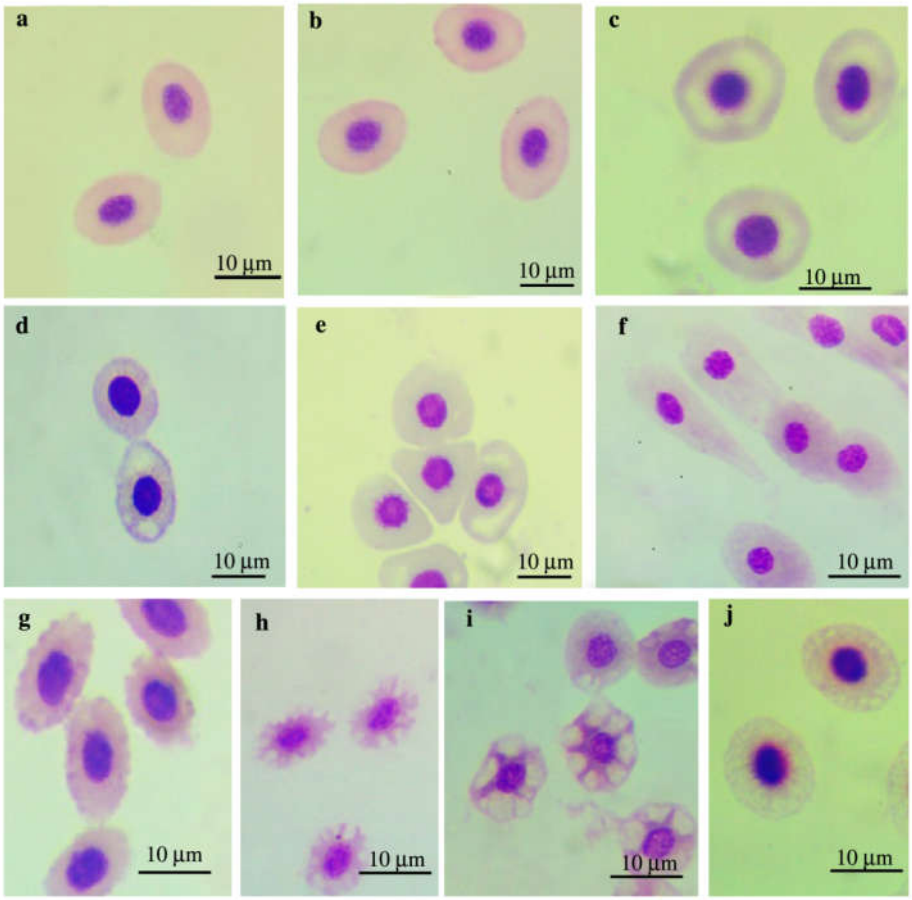

Fig. 2 Effect of fullerene C_{60} exposure showing cytoplasmic abnormalities in the peripheral erythrocytes of *Anabas testudineus* (a) Control; (b) Vehicle control (DMSO); (c) Anisochromatic cytoplasm; (d) Sticky cells; (e-f) Deformed cytoplasm; (g) Echinocytes; (h) Acanthocytes; (i) Vacuolated cytoplasm; (j) Degenerated cytoplasm

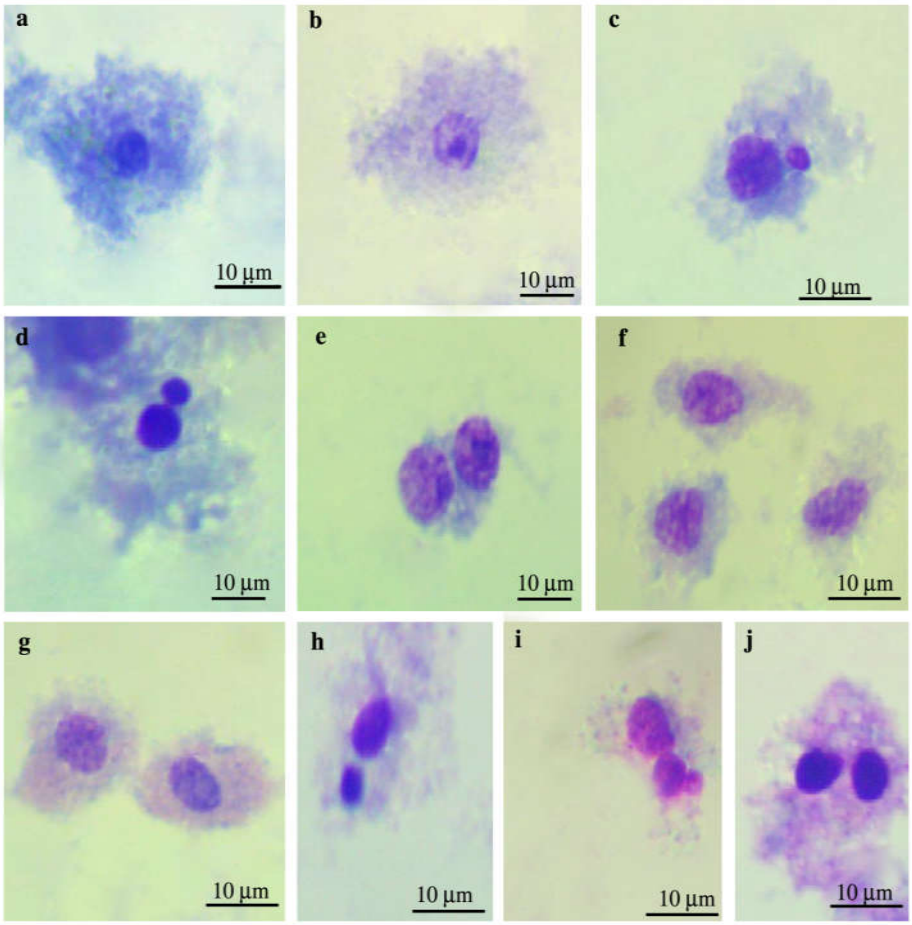

Fig. 3 Effect of fullerene C_{60} showing nuclear abnormalities in liver and gill cells of *Anabas testudineus*. Liver cells (a-e): (a) Control cell, (b) DMSO-exposed cell, (c) Micronucleus, (d) Nuclear bud, and (e) Binucleated cell; Gill cells (f-j): (f) Control cell, (g) DMSO-exposed cell, (h) Micronucleus, (i) Nuclear bud, (j) Binucleated cell

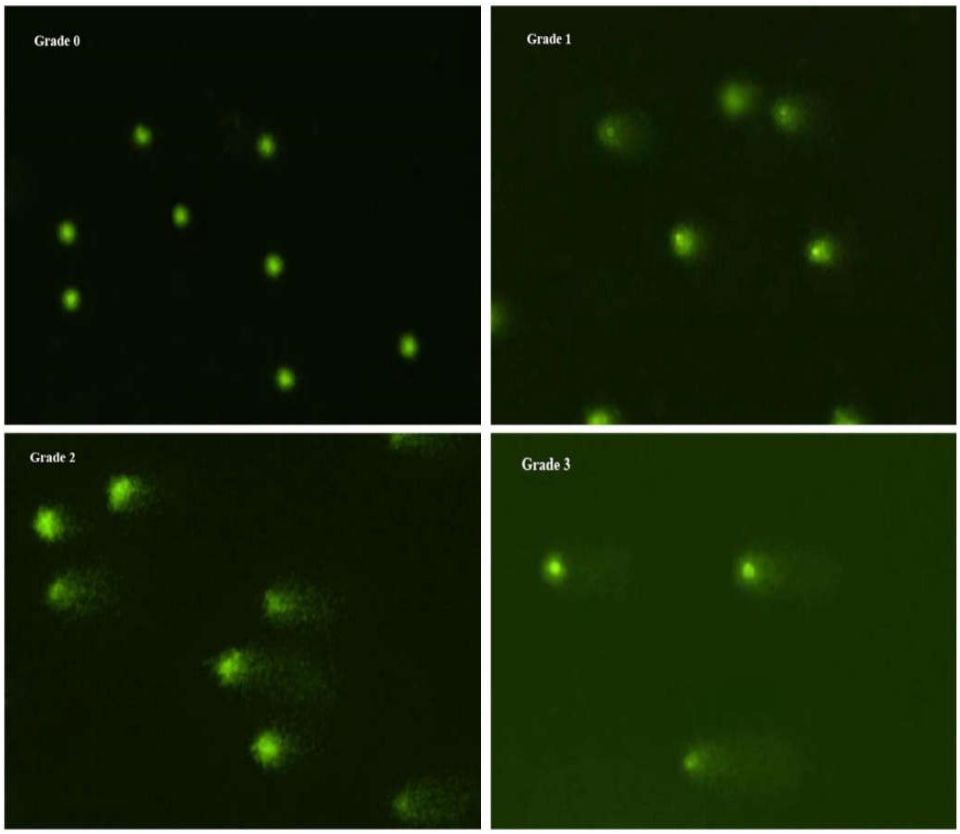

Fig. 4 Representative images of grades of DNA damage in *Anabas testudineus*: Grade 0-control and vehicle-control cells; Grades 1 to 3-Fullerene C_{60}-exposed cells

Treatment groups		Micronucleus	Nuclear Bud	Irregular nucleus	Notched Nucleus	Binucleated cell
Control		0	0.30±0.48	0.1±0.21	0	0
Vehicle		0	0.40±0.52	0.15±0.24	0	0
C_{60}-5 mg/ L	**4 d**	6.50±1.78*	2.00±0.82*	5.2±1.25*	2.00±0.82*	1.50±0.53*
	7 d	11.5±1.35*	17.2±1.75*	15.3±1.43*	4.90±0.88*	2.40±0.52*
	15 d	14.6±2.63*	24.6±1.71*	23.55±0.85*	8.10±0.88*	6.80±1.32*
	30 d	2.90±0.88*	12.5±1.78*	14.9±2.74*	2.00±0.82*	0
	60 d	2.00±0.82*	5.20±1.32*	6.25±1.5*	0.60±0.70	0
Control		0	0.30±0.48	0.1±0.21	0	0
Vehicle		0	0.40±0.52	0.15±0.24	0	0
C_{60}-10 mg/ L	**4 d**	9.10±0.88*	6.90±2.18*	10.35±2.04*	2.70±0.48*	2.20±0.63*
	7 d	14.1±0.88*	21.9±2.18*	18.85±1.52*	8.10±0.88*	4.50±1.35*
	15 d	18.3±1.25*	28.8±1.32*	27.8±1.82*	11.1±0.88*	8.71±1.25*
	30 d	3.30±1.25*	13.9±3.57*	17.95±2.92*	3.60±1.51*	0.10±0.32
	60 d	2.20±0.63*	6.50±1.35*	9.35±1.45*	1.00±0.94*	0

Table 1 Effect of C_{60} fullerene on nuclear abnormalities in the peripheral erythrocytes of the fish, *Anabas testudineus* (Mean ± SD; asterisks (*) indicated significance at $P<0.05$ against the control groups; n = 10/ group)

Treatment groups	Gill		Liver	
	C_{60}-5 mg/ L	C_{60}-10 mg/ L	C_{60}-5 mg/ L	C_{60}-10 mg/ L
Control	0.60±0.52	0.60±0.52	0.20±0.42	0.20±0.42
Vehicle	0.50±0.53	0.50±0.53	0.30±0.48	0.30±0.48
4 d	14.0±1.63*	17.2±1.75*	4.90±1.66*	9.40±1.71*
7 d	19.7±1.70*	21.8±1.75*	9.90±2.73*	18.4±1.71*
15 d	26.3±1.25*	28.7±1.25*	18.8±1.32*	25.2±1.32*
30 d	29.8±1.32*	32.3±1.91*	24.2±1.32*	28.8±0.79*
60 d	33.8±1.32*	36.0±0.82*	28.1±0.88*	31.5±1.78*

Table 2 Effect of C_{60} fullerene on micronucleus formation in gill and liver cells of the fish, *Anabas testudineus* (Mean±SD; asterisks (*) indicated significance at $P<0.05$ against the control groups; n = 10/ group)

Treatment groups		Grades of DNA damage					Total Score (%)
		Grade 0	Grade 1	Grade 2	Grade 3	Grade 4	
Control		89.00±1.15	10.70±1.25	0	0	0	**1.1**
Vehicle		88.60±2.22	10.40±2.22	0	0	0	**1.3**
C_{60}-5 mg/ L	**4 d**	78.80±1.32*	16.00±1.63*	5.10±1.45*	0	0	**2.5**
	7 d	71.40±1.26*	18.50±1.35*	11.20±1.32*	0	0	**4.0**
	15 d	60.30±1.25*	24.80±1.32*	14.80±1.75*	0	0	**5.5**
	30 d	53.80±1.32*	22.60±2.22*	16.10±0.88*	12.00±0.82*	0	**8.5**
	60 d	40.30±1.25*	24.70±2.06*	18.00±0.82*	17.20±1.32*	0	**11.2**
C_{60}-10 mg/L	**4 d**	67.00±1.63*	28.40±1.26*	6.90±1.85*	0	0	**3.9**
	7 d	60.00±1.63*	28.20±1.32*	11.80±1.32*	0	0	**5.2**
	15 d	44.70±1.25*	42.10±0.88*	15.80±1.32*	0	0	**6.9**
	30 d	40.00±1.63*	25.50±1.35*	19.50±1.35*	17.30±1.25*	0	**11.2**
	60 d	32.40±1.71*	30.20±1.75*	20.50±1.78*	21.10±1.20*	0	**12.5**

Table 3 Grades of DNA damages after C_{60} fullerene exposure on erythrocytes of the fish, *Anabas testudineus* (Mean ± SD; asterisks (*) indicated significance at $P<0.05$ against the control groups; n = 10/ group)

Treatment groups		Head DNA	Percentage Head DNA	Tail length	Tail DNA	Percentage Tail DNA	Tail Moment	Olive Moment
Control		51626.02± 5123.33	92.40±4.37	2.54±1.57	4589.58±2651.75	7.60±4.37	1.22±1.18	0.96±0.66
Vehicle		54162.30± 6349.10	92.75±4.34	2.31±1.42	3882.24±2210.49	7.25±4.34	1.16±1.27	0.89±0.68
C_{60}-5 mg/ L	**4 d**	52104.25±7480.19	85.58±2.09*	4.57±1.49*	8260.29±2070.28*	14.42±2.09*	3.12±1.32*	2.10±0.57*
	7 d	49298.70±5634.32	80.89±1.88*	6.79±1.27*	12199.97±1848.57*	19.11±1.88*	5.48±1.12*	3.12±0.51*
	15 d	39037.65±4752.49*	74.80±2.95*	8.50±1.95*	14482.79±3819.83*	25.20±2.95*	6.93±1.53*	3.96±0.83*
	30 d	35964.96±4939.53*	64.30±3.13*	11.30±2.09*	20395.55±2881.29*	35.70±3.13*	9.67±1.46*	5.46±1.05*
	60 d	31081.40±4377.79*	45.21±5.39*	18.51±3.67*	33494.48±4052.63*	50.60±4.26*	17.26±3.61*	8.83±1.07*
Control		51626.02± 5123.33	92.40±4.37	2.54±1.57	4589.58±2651.75	7.60±4.37	1.22±1.18	0.96±0.66
Vehicle		54162.30± 6349.10	92.75±4.34	2.31±1.42	3882.24±2210.49	7.25±4.34	1.16±1.27	0.89±0.68
C_{60}-10 mg/ L	**4 d**	47689.77±6807.63	83.78±3.00*	5.04±0.95*	8711.49±1793.99*	16.22±3.00*	3.65±1.20*	2.14±0.46*
	7 d	42231.00±7953.42*	78.41±4.92*	6.69±1.00*	11680.66±2878.64*	21.59±4.92*	5.49±1.71*	3.08±0.73*
	15d	36880.43±4955.07*	69.64±3.91*	10.91±3.00*	16079.04±3319.67*	30.36±3.91*	9.17±3.10*	5.11±1.53*
	30d	31339.97±7741.76*	56.80±6.60*	13.78±2.96*	26267.27±4589.48*	44.73±5.28*	12.54±2.86*	6.46±1.19*
	60d	22831.15±6586.93*	42.10±4.50*	20.53±4.60*	37498.22±5666.90*	58.91±4.07*	18.50±3.72*	10.73±1.74*

Table 4 Effect of C_{60} fullerene on comet parameters in peripheral erythrocytes of the fish, *Anabas testudineus* (Mean ± SD; asterisks (*) denote significance at $P<0.05$ against the control groups; n = 10/ group)

Treatment groups		Grades of DNA damage					Total Score (%)
		Grade 0	Grade 1	Grade 2	Grade 3	Grade 4	
Control		81.90±2.18	17.10±1.85	0	0	0	**1.9**
Vehicle		80.70±2.06	18.50±1.78	0	0	0	**2.2**
C_{60}-5 mg/ L	**4 d**	65.10±0.88*	33.30±3.09*	0	0	0	**3.4**
	7 d	44.90±1.85*	54.80±1.75*	1.00±0.82*	0	0	**5.7**
	15 d	29.90±2.18*	57.80±1.03*	11.70±1.25*	0	0	**8.2**
	30 d	17.80±2.25*	61.80±1.32*	18.50±1.35*	7.50±1.35*	0	**11.4**
	60 d	12.60±2.22*	55.50±1.08*	26.10±0.88*	8.60±2.22*	0	**12.9**
C_{60}-10 mg/ L	**4 d**	47.50±1.35*	44.30±1.25*	6.50±1.78*	0	0	**5.8**
	7 d	31.60±2.63*	57.70±2.36*	8.80±1.75*	0	0	**7.7**
	15 d	18.00±2.71*	62.20±1.32*	17.80±0.79*	5.20±1.75*	0	**10.7**
	30 d	13.20±2.25*	59.80±1.75*	22.90±1.85*	6.50±1.35*	0	**12.8**
	60 d	7.00±1.63*	52.50±1.35*	26.40±2.22*	12.60±2.22*	0	**14.4**

Table 5 Grades of DNA damages after C_{60} fullerene exposure in gill of the fish, *Anabas testudineus* (Mean ± SD; asterisks (*) indicated significance at $P<0.05$ against the control groups; n = 10/ group)

Treatment groups		Head DNA	Percentage Head DNA	Tail length	Tail DNA	Percentage Tail DNA	Tail Moment	Olive Moment
Control		54705.70±6115.12	91.61±2.77	4.80±6.80	5019.90±1821.28	4.06±2.51	8.39±2.77	0.30±0.33
Vehicle		52800.88±7184.28	91.95±4.23	4.20±3.05	5100.34±1696.12	6.31±1.85	8.05±4.23	0.34±0.33
C_{60}-5 mg/ L	4 d	47473.61±8280.56*	75.32±3.27	8.37±2.03	14070.92±2182.08	4.48±2.08	24.58±3.23	3.21±1.24
	7 d	44134.51±8093.64*	73.33±4.43*	11.53±1.25	16303.87±3028.28*	15.04±4.20*	27.22±3.99*	3.54±0.91
	15 d	38845.62±7979.30*	53.59±6.16*	18.44±3.57*	35355.36±6261.95*	20.77±5.43*	46.24±5.09*	11.85±4.96*
	30 d	32448.97±6188.74*	39.25±5.60*	21.96±5.59*	38703.76±4015.01*	36.73±6.47*	61.78±4.31*	20.17±2.15*
	60 d	16235.87±4560.91*	23.05±6.50*	24.86±2.79*	44862.68±9871.36*	57.67±5.89*	75.90±4.23*	22.81±7.25*
Control		54705.70±6115.12	91.61±2.77	4.80±6.80	5019.90±1821.28	4.06±2.51	8.39±2.77	0.30±0.33
Vehicle		52800.88±7184.28	91.95±4.23	4.20±3.05	5100.34±1696.12	6.31±1.85	8.05±4.23	0.34±0.33
C_{60}-10 mg/ L	4 d	48050.94±5727.73*	76.47±2.08*	9.72±4.22*	14341.74±9031.87*	18.68±4.37*	22.33±4.65*	2.89±2.15*
	7 d	41237.67±8323.00*	68.12±3.60*	9.88±2.01*	16177.80±5011.65*	35.23±4.81*	32.52±2.83*	7.33±1.49*
	15 d	38638.12±7454.25*	49.51±7.09*	19.04±3.88*	30409.31±3554.17*	46.29±4.89*	50.12±5.30*	13.15±3.21*
	30 d	26365.31±4947.48*	36.68±2.35*	28.45±5.42*	46040.67±7164.58*	59.30±2.33*	63.32±2.35*	21.39±3.54*
	60 d	11632.06±4948.04*	20.22±5.54*	31.22±6.28*	57507.04±8980.28*	64.83±5.14*	78.96±4.65*	24.33±3.75*

Table 6 Effect of C_{60} fullerene on comet parameters in gill of the fish, *Anabas testudineus* (Mean ± SD; asterisks (*) indicated significance at $P<0.05$ against the control groups; n = 10/ group)

Treatment groups		Grades of DNA damage					Total Score (%)
		Grade 0	Grade 1	Grade 2	Grade 3	Grade 4	
Control		76.60±2.22	23.90±0.88	0	0	0	**2.4**
Vehicle		74.90±2.18	24.80±1.75	0	0	0	**2.8**
C_{60}-5 mg/ L	**4 d**	67.70±2.06*	33.30±1.25*	0	0	0	**3.2**
	7 d	34.60±2.63*	56.10±2.18*	7.00±1.63*	0	0	**7.4**
	15 d	31.90±1.85*	46.30±2.06*	23.30±2.87*	0	0	**8.6**
	30 d	27.20±1.75*	45.40±2.63*	17.30±2.63*	6.00±2.71*	0	**9.8**
	60 d	29.00±2.71*	40.70±1.70*	22.10±2.18*	11.20±1.75*	0	**11.4**
C_{60}-10 mg/ L	**4 d**	56.60±2.22*	27.60±2.22*	15.60±3.20*	0	0	**6.1**
	7 d	47.90±2.18*	32.30±2.06*	22.30±2.63*	0	0	**7.4**
	15 d	36.60±2.07*	35.90±1.85*	27.40±2.22*	2.20±1.32*	0	**9.6**
	30 d	21.20±1.75*	38.50±1.35*	33.40±2.63*	6.80±1.75*	0	**12.4**
	60 d	17.50±1.35*	46.20±1.75*	28.00±3.06*	13.80±2.25*	0	**13.6**

Table 7 Grades of DNA damages after C_{60} fullerene exposure in liver of the fish, *Anabas testudineus* (Mean ± SD; asterisks (*) indicated significance at $P<0.05$ against the control groups; n = 10/ group)

Treatment groups		Head DNA	Percentage Head DNA	Tail length	Tail DNA	Percentage Tail DNA	Tail Moment	Olive Moment
Control		45036.89±7446.32	93.28±3.74	2.60±1.27	3144.21±1836.34	5.54±1.98	5.54±1.98	0.46±0.31
Vehicle		49763.53±4863.58	93.10±6.19	3.62±1.34	4180.47±3365.79	4.92±1.74	4.92±1.74	0.44±0.47
C_{60}-5 mg/ L	**4 d**	47513.66±4849.40	86.86±6.76*	5.46±3.20*	8639.45±5229.75*	13.14±4.79*	13.14±4.79*	3.65±3.45*
	7 d	42291.27±2968.19	76.36±2.40*	8.83±1.96*	13866.26±3723.62*	23.64±2.40*	23.64±2.40*	6.09±2.12*
	15 d	38470.69±7035.65*	66.94±3.78*	11.51±3.67*	20255.70±6655.79*	33.06±3.78*	33.06±3.78*	9.13±3.43*
	30 d	32818.62±5968.72*	48.76±4.54*	18.63±3.75*	29805.34±5137.99*	51.24±4.54*	51.24±4.54*	18.43±2.96*
	60 d	23788.67±2948.97*	36.74±2.65*	21.38±2.47*	36230.01±4256.48*	63.26±2.65*	63.26±2.65*	20.11±3.85*
Control		45036.89±7446.32	93.28±3.74	2.60±1.27	3144.21±1836.34	5.54±1.98	5.54±1.98	0.46±0.31
Vehicle		49763.53±4863.58	93.10±6.19	3.62±1.34	4180.47±3365.79	4.92±1.74	4.92±1.74	0.44±0.47
C_{60}-10 mg/ L	**4 d**	47792.72±4010.41	85.59±3.85*	6.74±3.32*	11141.86±5878.62*	14.41±3.85*	14.41±3.85*	4.05±1.96*
	7 d	48685.47±5408.68	75.63±5.91*	7.90±2.19*	13250.41±4005.25*	21.67±3.18*	21.67±3.18*	5.59±2.76*
	15 d	36914.59±6538.26*	60.35±4.67*	14.26±2.34*	27765.57±6859.02*	40.35±3.86*	40.35±3.86*	12.07±2.38*
	30 d	32065.28±4950.52*	46.53±4.61*	20.73±4.31*	30230.61±4073.68*	55.08±2.93*	55.08±2.93*	20.32±3.39*
	60 d	21274.54±4900.39*	34.29±3.64*	24.22±1.82*	36940.68±2049.14*	65.71±3.64*	65.71±3.64*	22.71±2.22*

Table 8 Effect of C_{60} fullerene on comet parameters in liver of the fish, *Anabas testudineus* (Mean ± SD; asterisks (*) indicated significance at $P<0.05$ against the control groups; n = 10/ group)

5.1 Effect of fullerene C_{60} on nuclear and cytoplasmic abnormalities in peripheral erythrocytes

Carbon-based nanomaterials have been shown to induce genotoxic stress either by the direct interaction with DNA and mitotic apparatus or indirect interaction by the induction of oxidative stress or inflammatory response (Schins and Knaapen, 2007; Attia *et al*., 2018). Fullerenes has the ability to bind directly with the double-strand DNA either at the nucleotide minor groove or at the hydrophobic ends, and also with the single-strand DNA leading to distorted nucleotides (Zhao *et al*., 2005; Prylutska *et al*., 2017). Some studies have reported the indirect toxicity of fullerenes by the generation of reactive oxygen species leading to decline in antioxidant enzymes, mitochondrial dysfunction and oxidative stress-mediated DNA damage (Sumi and Chitra, 2017; Chen *et al*., 2018). Besides, several literatures have demonstrated both genotoxic and non-genotoxic effects of carbon nanomaterials, and this controversy could be due to various factors such as concentration of nanomaterial exposed, duration and route of exposure, solubilzing effect of dispersing agent and so on. The present study aimed to evaluate if sublethal exposure of fullerene C_{60} nanomaterial could induce genotoxicity in the fish, *Anabas testudineus* by the assessment of cytonuclear abnormalities in the peripheral erythrocytes.

The genotoxic data analyzed after sublethal fullerene C_{60} exposure at 5 and 10 mg/ L concentrations showed nuclear

abnormalities like micronucleus, binucleated, nuclear bud, and cells with irregular, vacuolated, and serrated nucleus. The cytoplasmic abnormalities observed were in the form of echinocyte, acanthocyte, anisochromatic cells, sticky cells, vacuolated cytoplasm, cytoplasmic degeneration, and erythrocytes with irregular membrane. Micronucleus or Howell-Jolly bodies are remnants of erythrocyte nuclei that indicate chromosomal damage correlated with DNA double-strand breaks (Lal and Ames, 2011). The cytonuclear abnormalities observed after fullerene C_{60} exposure indicated that the nanomaterial functioned as clastogen and aneugen agents, and the data showed similarity on the genotoxicity induced by carbon nanotubes in human lymphocytes (Cveticanin *et al*., 2010). Thus induction of micronucleus demonstrated aneuploidogenic effects and chromosome rupture of C_{60} fullerene caused by the physical disturbances in the mitotic apparatus or spindle formation mediated through the induction of oxidative stress. The other nuclear abnormalities formed designated the gene amplification in order to remove the toxicant from the nucleus (Carrasco *et al*., 1990). Besides, the micronucleus formation in erythrocytes has been found related to cell kinetics and replacement, which also reflected the genomic instability (Cavas and Ergene-Gozukara, 2003b).

The present results showed a duration- and concentration-dependent increase in the nuclear and cytoplasmic abnormalities up to 15 d of fullerene exposure, followed by decrease in the lesions. Therefore, the current study illustrated the indirect genotoxic effects

of fullerene C_{60} associated with oxidative stress-mediated DNA damage. Such indirect mechanism of genotoxicity induced by ROS generation has been reported after exposure to other nanoparticles such as silicon dioxide, aluminium oxide, titanium dioxide, and iron oxide nanoparticles in *Oreochromis mossambicus* (Vidya and Chitra, 2018), silver nanoparticles in *Labeo rohita* (Khan *et al*., 2017), copper oxide nanoparticles in *Cyprinus carpio* (Nikdehghan *et al*., 2018), and titanium dioxide nanoparticles in *Trachinotus carolinus* (Vignardi *et al*., 2014). In addition to nuclear abnormalities, the cytoplasmic anomalies, particularly formation of vacuolization after fullerene C_{60} exposure could be due to changes in altered haemoglobin distribution (Ateeq *et al*., 2002). The observations can be correlated to the reduction in the concentration of haemoglobin and haematocrit value after sublethal exposure of fullerene C_{60} which has been discussed in chapter 1.

It has been reported that the genomic lesions appeared in the form of micronuclei formation in the erythrocytes of fish, which can be repaired by DNA repair mechanisms, and are easily eliminated from the blood (Russo *et al*., 2004; Bucker *et al*., 2006). The DNA damage no longer existed in long-term exposure groups of fullerene C_{60}, but expected to be formed in various organs, thus nuclear abnormalities in gill and liver cells have been examined in the fish, *Anabas testudineus*.

5.2 Effect of fullerene C_{60} on nuclear abnormalities in gill and liver cells

Evaluation of micronucleus frequencies in fish gill and liver cells are considered as the most sensitive index than diagnosed in erythrocytes (Cavas and Ergene-Gozukara, 2003b). Gill cells are continuously dividing, and constantly exposed to toxicants thus highly susceptible to micronuclei induction than erythrocytes (Al-Sabti and Metcalfe, 1995). Liver cells are the centre for xenobiotic metabolism, and regarded as the most suitable tissue for the micronucleus test (Williams and Metcalfe, 1992). In the present study, a dose- and duration dependent increase in the micronucleus formation and nuclear abnormalities like nuclear bud and binucleated cells were observed in both gill and liver cells. The present findings clearly illustrated that gill and liver cells showed more sensitive response to genotoxic effects of fullerene C_{60} than the erythrocytes with about 10 to 16-fold increase in micronucleus formation.

The increase in micronucleus, nuclear bud and binucleated cells after fullerene exposure indicated the possible accumulation of nanomaterial in gill and liver cells. The present study was in agreement with the genotoxicity assessment of micronuclei and other nuclear abnormalities in gill and liver cells exposed to cadmium chloride and copper sulfate in *Cyprinus carpio*, *Corydoras paleatus* and *Carassius gibelio* (Cavas *et al*., 2005), and methyl methanesulfonate in zebrafish, *Danio rerio* (FaBbender and Braunbeck, 2013).

5.3 Effect of fullerene C_{60} on DNA damage in peripheral erythrocytes, gill and liver cells using comet score

Comet assay is widely used to assess the genetic damage in various tissues including erythrocytes, liver, gill and kidney in the form of DNA strand breaks (Dhawan *et al*., 2009). It is used in genotoxicity testing of chemicals in wide variety of eukaryotic organisms and in various *in vitro* systems. Nanogenotoxicology prefer comet assay as the robust, versatile and reliable standard genotoxicity test to detect the accumulation of DNA breaks. Over several years, comet assay has been successfully performed in erythrocytes of fish because the blood can be drawn without killing the animal (Sunjog *et al*., 2014). However, in order to evaluate the tissue-specific DNA breaks several other cells derived from liver, gill, sperm and epithelial cells were used. The present study measured the DNA damage from the peripheral erythrocytes, gill and liver cells of the fish, *Anabas testudineus*. The best descriptor used in comet assay to determine the DNA damage includes head DNA content, percent head DNA, tail DNA content, percent tail DNA, tail length, tail and olive moment (De Boeck *et al*., 2000).

In the comet image, the undamaged DNA nucleoid is referred as 'head' and the damaged part is known as 'tail'. Thus both head and tail DNA content and percent DNA were scored after fullerene exposure in order to plot the difference between the undamaged and damaged DNA. Percent tail DNA denotes the actual percentage of DNA damage that occurred in a particular cell. Among the tail parameters, tail moment has been considered as the

appropriate measure to detect the induced DNA damage, where it extend between the centre of the head to the centre of the tail (De Boeck *et al*., 2000). However, olive tail moment describe heterogeneity within a cell population, which can differentiate variation in the DNA distribution within the tail (Olive *et al*., 1990).

In the present study, exposure of fullerene C_{60} at both sublethal concentrations for 30 and 60 d showed grade 3 DNA damage in erythrocytes. However, grade 3 DNA damage was observed after 15 d of fullerene exposure at high sublethal concentrations in gill and liver cells, and after 30 d in the low sublethal exposure groups. On comparison of the total score percentage, the high level of DNA damage was observed in gill cells followed by liver cells and erythrocytes. The present findings showed dose- and time-dependent increase in the total score of DNA damage in erythrocytes, gill and liver cells, which indicate a positive correlation between fullerene and genetic damage. Such grade 3 DNA damage has been observed after metal oxide nanoparticles exposure in the erythrocytes of the freshwater fish, *Oreochromis mossambicus* (Vidya and Chitra, 2018). Similarly, genotoxicity of carbon black, a carbonaceous nanoparticle, investigated using comet assay showed a remarkable DNA strand break in the mouse hepatocytes (Zhang *et al*., 2019).

1. Sublethal exposure of fullerene C_{60} induced micronucleus formation and other nuclear abnormalities in peripheral erythrocytes as well as in gill and liver cells of the fish, *Anabas testudineus*, which indicated the DNA double-strand break.

2. Cytoplasmic abnormalities observed in the erythrocytes suggested the possible penetration, but failure in the elimination of nanomaterial through DNA repair mechanism.

3. Genomic instability and DNA damaging effects of fullerene C_{60} was evidenced by various grades of DNA damage and altered comet parameters in erythrocytes, gill and liver cells.

4. The present findings illustrated that the high degree of DNA damage, based on the micronuclei formation and grades of comet score, was noticed in gill cells, followed by liver cells and erythrocytes.

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