Article

Toxicological Screening of 4-Phenyl-3,4-dihydrobenzo[h]quinolin-2(1H)-one: A New Potential Candidate for Alzheimer's Treatment

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ABSTRACT: Toxicity studies are necessary for the development of a new drug. Naphthalene is a bicyclic molecule and is easy to derivatize. In our previous study, a derivative of naphthalene (4-phenyl,3,4-dihydrobenzoquinoline-2(H)one) was synthesized and reported its in vitro activity on different enzymes. This study was a probe to investigate the toxicity potential of that compound (SF3). Acute oral (425), subacute (407), and teratogenicity (414) studies were planned according to their respective guidelines given by organization of economic cooperation and development (OECD). Acute oral, subacute, and teratogenicity studies were carried out on 2000, 5−40, and 40 mg/kg doses. Blood samples were collected for hematological and biochemical analyses. Vital organs were excised for oxidative stress (superoxide dismutase, catalase, glutathione, and malondialdehyde) and histopathological analysis. LD_{50} of SF3 was higher than 2000 mg/kg. In acute and subacute studies, levels of alkaline phosphates and aspartate transaminase were increased. Teratogenicity showed no resorptions, no skeletal or soft tissue abnormalities, and no cleft pallet. Oxidative stress biomarkers were close to the normal, and no increase in the malondialdehyde level was seen. Histopathological studies revealed normal tissue architecture of the selected organs, except kidney, in acute oral and subacute toxicity studies at 40 mg/kg. The study concluded that SF3 is safer if used as a drug.
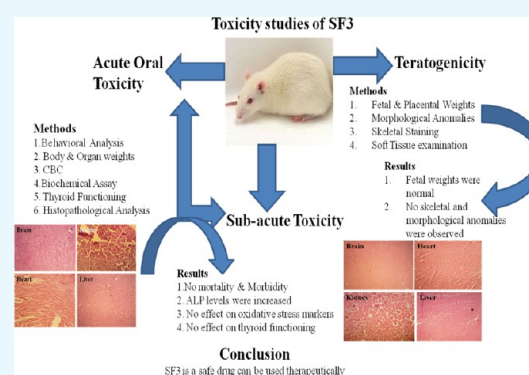
INTRODUCTION

The naphthalene nucleus provides a flexible and diverse scaffold in the field of chemistry, particularly medicinal chemistry.[1] In the
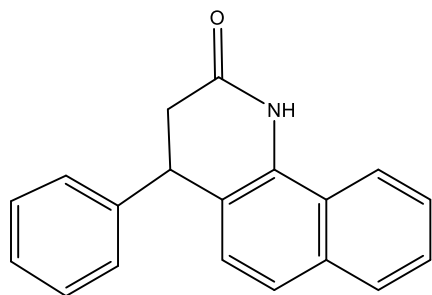
4-Phenyl-3,4-dihydrobenzo[h]quinolin-2(1H)-one

Figure 1. Chemical structure of the test compound SF3.

last few decades, organic compounds containing the naphthalene nucleus have gained particular interest in drug discovery.[1,2] Simple structural modification and diverse biological activities highlight it in drug designing.[3] Moreover, it is also a part of nature in the form of phytochemicals having

attractive biological potentials, for example, rifampicin (antitubercular),[4] patentiflorin A (anti-HIV),[5] justicidin A (anticancer),[6] and bis-ANS 82 (tubulin inhibitor).[7] Different drugs in the market with a naphthalene moiety are bedaquiline,[8] nafcillin,[9] naproxen,[10] nafimidone,[11] and so forth.

Naphthalene is used in various therapeutic applications for its cytotoxicity. It is intracellularly converted to the reactive metabolites, that is, naphthalene epoxides and naphthoquinones. These metabolites covalently interact with the cysteine residue of the various cellular proteins and produce toxicity. Naphthalene oxides react with the cysteine's sulfhydryl group by SN_1 and SN_2 reactions producing naphthoquinones.[12] These naphthoquinones follow the Lipinski rule of 5 as a drug moiety with two hydrogen-bond acceptors and a log P of 1.71. It has been reported that 1-naphthol is converted into naphthoquinone by a tyrosinase enzyme.[13] These naphthoquinones
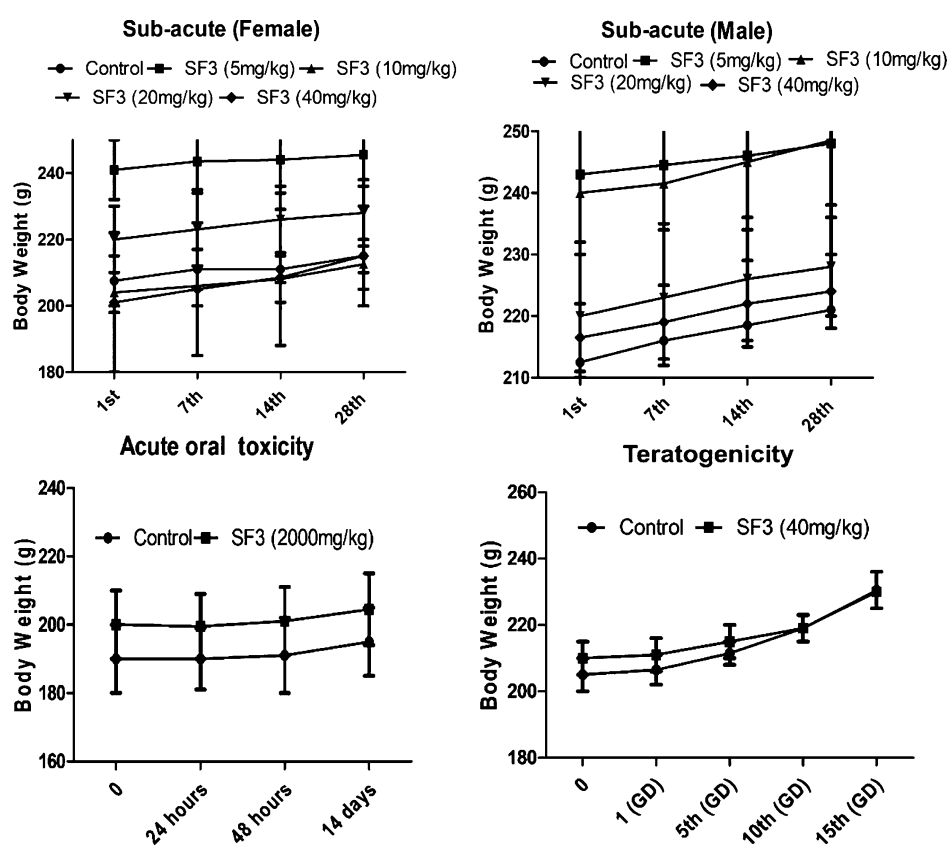
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Figure 2. Effects of treatments on body weights (g) in acute oral, subacute, and teratogenicity studies.

generate reactive oxygen species (ROS) that are toxic to cancer cells. Similarly, hepatic metabolism of different naphthalene-containing compounds also generated ROS. Naphthoquinone has also been observed to stimulate microsomal oxygen consumptions in the presence of NADPH. The generation of ROS by the metabolism of naphthalene-containing drugs makes it suitable as cancer therapeutics.[3] The naphthalene scaffold possesses various antagonistic activities and used as anticancer agents,[14] antimicrobial,[15] neurodegenerative diseases,[16] anti-inflammatory,[17] antidiabetic,[18] anticonvulsant,[19] antihypertensive,[20] antidepressant,[21] antipsychotic,[22] antitubercular, and antiviral.[23] The introduction of different functional groups on the naphthalene nucleus could provide a promising strategy to achieve various biological activities. These compounds could also lower the toxicity, improve the pharmacokinetics, and avoid the interactions and adverse drug reactions. These characteristics are necessary to design drugs to treat multifactorial diseases, for example, Parkinson and neurodegenerative diseases.[24]

Toxicity studies indicate the probability of the adverse effect caused by interacting the drugs with the cells. Toxicity studies of the drugs/substances/any molecule that tends to be a drug are critical because it protects or predicts the hazardous effects of the substances on the living cells.[25] Toxicity studies are encircled in acute oral (up to 14 days), subacute (28 days), subchronic (90 days), chronic (6–12 months), and teratogenicity according to the exposure period.[26,27] In drug discovery, toxicity studies play an essential role in the development of the drug. In our previous studies, a naphthalene derivative 4-phenyl-3,4-dihydrobeno-quinolin-2-one (SF3) was synthesized, and its *in silico* studies showed promising binding with the acetylcholinesterase (AChE) enzyme.[28,29] The previous study also showed its *in*

vitro enzyme kinetic analysis against AChE. Before targeting their specific role in Alzheimer disease, toxicity studies are necessary to predict this compound's hazardous effect. The current study was aimed to evaluate the toxicity potential of SF3 (Figure 1) on acute, subacute exposure, and pregnancy.

■ RESULTS

Effect of SF3 Treatment on Behavioral and Physical Changes in the Acute Oral Toxicity Study. No mortality and morbidity were observed throughout 14 days after administering the 2000 mg/kg dose through the oral route. Thus, the LD_{50} of the SF3 was higher than 2000 mg/kg. The rats did not show any behavioral change and physical change (Figure 2).

Effect of SF3 Treatments on Organ and Body Weights in Acute, Subacute, and Teratogenicity. Animals treated with SF3 gained the body weight in parallel to the control group in each study. The selected organs did not show any abnormality. No change in the color and weight of the organs was observed (Figure 2, Table 1).

The results are presented as mean ± SEM; $n = 5$ for acute oral and $n = 10$ for teratogenicity and subacute toxicity studies.

Estimation of Teratogenic Parameters after the Administration of SF3. A detailed examination (physical, soft tissue, and skeletal examinations) of toxicity related to the fetus was analyzed in the teratogenicity study. Resorptions, early resorptions, the number of dead and alive fetuses, the fetus's weight, and skeletal and soft anomalies were analyzed critically after the administration of SF3. No skeletal and soft tissue anomalies were observed (Figures 3 and 4). Weights of the fetuses and no of alive fetuses were parallel to the control (Table 2). No physical sign of toxicity was observed. Placental and fetal

Table 1. Variations in the Different Organ Weights (g) in Different Treatments[a]

| | acute toxicity | | subacute toxicity | | | | | | | | | | teratogenicity | |
| | | | male | | | | | female | | | | | | |
organs	control	2000 mg/kg	control	5 mg/kg	10 mg/kg	20 mg/kg	40 mg/kg	control	5 mg/kg	10 mg/kg	20 mg/kg	40 mg/kg	control	40 mg/kg
brain	1.5 ± 0.2	1.56 ± 0.2	1.3 ± 0.3	1.3 ± 0.1	1.4 ± 0.2	1.3 ± 0.6	1.2 ± 0.6	1.5 ± 0.4	1.6 ± 0.6	1.5 ± 0.2	1.5 ± 0.3	1.4 ± 0.1	1.4 ± 0.2	1.5 ± 0.12
heart	0.3 ± 0.1	0.44 ± 0.1	0.5 ± 0.2	0.6 ± 0.2	0.5 ± 0.3	0.5 ± 0.8	0.6 ± 0.5	0.6 ± 0.3	0.5 ± 0.3	0.6 ± 0.1	0.6 ± 0.2	0.65 ± 0.3	0.62 ± 0.3	0.6 ± 0.32
spleen	0.2 ± 0.1	0.3 ± 0.8	0.3 ± 0.4	0.5 ± 0.3	0.4 ± 0.4	0.3 ± 03	0.4 ± 0.3	0.4 ± 0.1	0.5 ± 0.3	0.4 ± 0.2	0.4 ± 0.3	0.3 ± 0.5	0.54 ± 0.1	0.53 ± 0.40
kidney	1.2 ± 0.1	1.5 ± 0.1	1.2 ± 0.3	1.2 ± 0.2	1.5 ± 0.8	1.4 ± 0.5	1.4 ± 0.1	1.1 ± 0.5	1.4 ± 0.2	1.2 ± 0.1	1.1 ± 0.5	1.3 ± 0.3	1.2 ± 0.1	1.23 ± 0.13
lungs	0.7 ± 0.3	0.92 ± 0.3	0.8 ± 0.5	0.8 ± 1.3	0.8 ± 0.6	0.7 ± 0.7	0.8 ± 0.6	0.9 ± 0.2	0.7 ± 0.1	0.7 ± 0.2	0.8 ± 0.3	0.8 ± 0.8	0.75 ± 0.2	0.76 ± 0.52
liver	6.9 ± 0.4	6.4 ± 0.5	5.8 ± 0.5	6.9 ± 0.1	6.3 ± 1.2	6.4 ± 0.3	6.6 ± 0.2	6.1 ± 0.4	6.6 ± 0.3	6.2 ± 0.3	6.9 ± 0.6	6.7 ± 0.5	6.24 ± 1.2	6.7 ± 1.3
stomach	0.9 ± 0.8	1.1 ± 1.2	1.1 ± 0.5	1.2 ± 0.0	1.6 ± 0.9	1.6 ± 0.8	1.9 ± 0.3	1.2 ± 0.3	1.2 ± 0.5	1.1 ± 0.5	1.5 ± 0.7	1.6 ± 0.8	1.2 ± 0.2	0.90 ± 0.62
ovaries	0.2 ± 1.5	0.1 ± 0.9						0.1 ± 0.1	0.1 ± 1.2	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.9	0.32 ± 0.4	0.23 ± 0.3
testis	2.2 ± 1.2	2.6 ± 1.4	2.6 ± 0.4	2.2 ± 0.3	2.2 ± 0.5	2.1 ± 0.7	2.1 ± 0.1							

[a]Data are presented as mean ± SEM; n = 5 for acute oral toxicity and n = 10 for teratogenicity and subacute toxicity studies.

weights are intact with the control. No significant toxicity sign was observed after the SF3 treatment. SF3 was quite a safe drug in pregnancy as it had no teratogenic effect.

Effect on Sperm Count and Morphological Studies in Subacute Toxicity Studies. Sperm count and its morphology were examined after 28 days in the subacute study. Table 3 reveals that treatment of SF3 at different doses significantly increased the sperm count compared to the control. Any abnormality in morphology was also decreased when animals were treated with SF3 compared to the control (Figure 5).

Effect of Acute, Subacute, and Teratogenic Studies on Hematological Parameters. Complete blood count (CBC) was performed at the end of acute, subacute, and teratogenic studies. Blood was collected by cardiac puncture. Table 4 shows the complete results of CBC.

WBC's count was increased significantly in acute oral and teratogenic studies ($P < 0.05$). While in subacute studies, WBC's count was normal in both male and female animals. No effect was observed in the levels of HB, MCH, MCHC, MCV, and HCT.

Effect of Acute and Subacute Studies on Biochemical Parameters. Biochemical marker estimation is necessary to determine the toxicity levels in the major organs (liver and kidney). For the liver function, AST, ALP, and ALT levels were used as toxicity markers. In acute toxicity (2000 mg/kg) studies, levels of ALP and ALT were significantly increased ($P < 0.05$) in comparison with the control. However, the lipid profile and renal functioning seem to be normal (Table 5).

In subacute toxicity, ALP levels were significantly increased at 10 mg/kg dose in females while in males, 5 and 10 mg/kg doses increased the ALP levels considerably compared to the control. Levels of proteins were increased at a high dose (40 mg/kg) in male and female animals. No change was observed in the lipid profile in both male and female animals (Figure 6).

Estimation of Thyroid Function in Acute and Subacute Toxicity Studies. Table 6 reveals that SF3 showed non-significant changes in the levels of T_3, T_4, and TSH in both acute and subacute toxicity studies when compared to the control group.

Effect of Acute, Subacute, and Teratogenic Studies on Oxidative Stress Biomarkers. Different oxidative stress markers (SOD, CAT, GSH, and MDA) were estimated at the end of the subacute study for analyzing any toxicity at the tissue level. In subacute toxicity studies, antioxidants such as SOD, CAT, and GSH were decreased in the kidney and spleen tissues of male rats. The heart was also affected as GSH levels were reduced at lower doses but increased when treated with high doses. In male and female rats, MDA levels seem to be normal in almost all tissues compared with the control (Figure 7). CAT levels were normal in nearly all tissues except the spleen. In the spleen, lower test doses reduced it, while higher doses brought it close to the normal value. Results showed that in female rats, levels of GSH were declined in liver and kidney tissues at higher doses (20 and 40 mg/kg) in comparison with the control group. CAT and SOD levels were also affected in the spleen and liver tissues of female rats.

ELISA Analysis. Levels of testosterone and histamine were analyzed in the subacute study. Results showed that SF3 significantly elevated testosterone levels in females compared with the control, while in males, the levels were in parallel to the control. No effect was seen in histamine levels in both male and female animals (Figure 8).
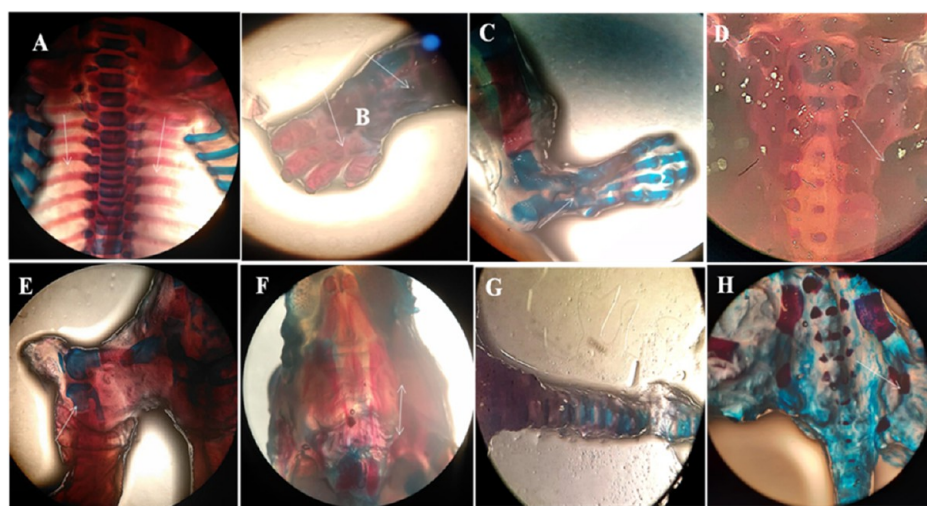
Figure 3. Effects of SF3 (40 mg/kg) treatment on skeletal anomalies during teratogenicity studies; (A) No ribs fusion, (B) no shortening of normal ossified bones of forelimbs, (C) no shortening of completely ossified hind limb bones, (D) normal vertebral column, (E) normal bone sizes, (F) no cleft palate, (G) normal tail, and (H) normal lower vertebral column bones.
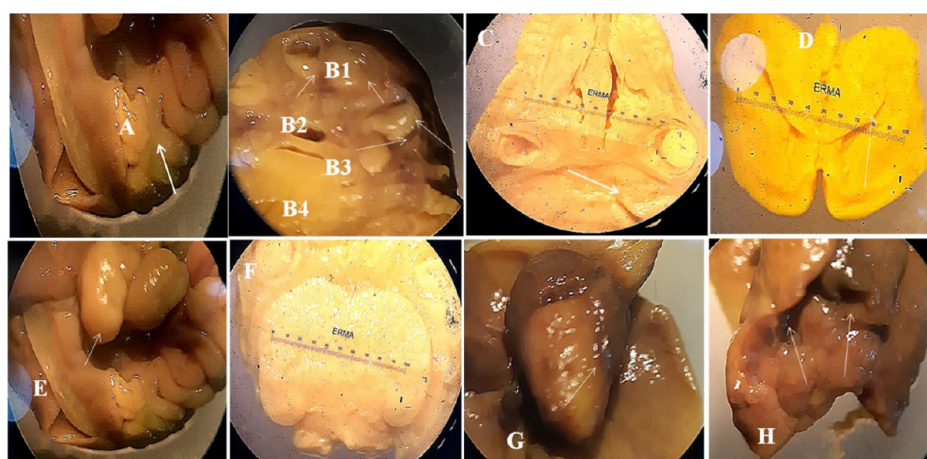

Figure 4. Soft tissue examination of animals pubs treated with SF3 (40 mg/kg). (A) Normal intestine, (B1) normal head section, (B2) olfactory bulb, (B3) retina, (B4,C) two representation of the normal palate, (D) no hydronephrosis, (E) normal kidney size with no hydronephrosis, (F) normal frontal lobe section, (G) normal heart size, and (H) normal liver size.

Table 2. Fetal and Placental Weights and Morphological Anomalies during the Teratogenic Study[a]

	groups	
anomalies	normal control	treated (40 mg/kg)
cleft palate	0.0 ± 0.0	0.0 ± 0.0
spina bifida (microns)	40 ± 0.3	42 ± 0.5
rib malformation	0.0 ± 0.0	0.0 ± 0.0
delayed cervical ossification	0.0 ± 0.0	0.0 ± 0.0
early resorption	0.0 ± 0.0	0.0 ± 0.0
late resorptions	0.0 ± 0.0	0.0 ± 0.0
abortions	0.0 ± 0.0	0.0 ± 0.0
no. of litters	09 ± 0.3	07 ± 0.4
no. of live fetuses	08 ± 0.3	06 ± 0.4
maternal death rate	01 ± 0.1	02 ± 0.2
fetal weight (gm)	5.3 ± 0.1	5.2 ± 1.3
placental weight (gm)	0.7 ± 0.2	0.65 ± 0.2

[a]Data are represented as mean ± SEM (n = 10).

Histopathological Analysis. Histopathology revealed that all treated groups on 14th (acute oral toxicity), 19th (teratogenicity), and 28th day (subacute toxicity) showed no cellular changes in the major organs, except kidney, in comparison with the control group (Figures 9, 10 and 11). In the kidney, cellular changes were observed at 40 mg/kg dose level. Low doses did not show toxicity. All other organs showed normal architecture.

■ DISCUSSION

Toxicological studies are essential to study the molecules that were assumed to have therapeutic potential. The OECD provides guidelines for the toxicity studies of chemicals/ molecules/compounds. According to the OECD guidelines, rodents are the preferred species for toxicity studies.[30] A lot of literature studies suggested that females are more sensitive than males, that is, OECD studies preferred female rats. Therefore, the acute oral toxicity study was performed on female rats. However, in subacute studies, both sexes were recommended (male and female).[31]

Metabolism of naphthalene is carried out by the microsomal enzyme system, and converted into the 1-naphthol and 1,2-epoxide. 1-Naphthol is directly toxic, depleting the glutathione

Table 3. Sperm Count and Morphology of SF3-Treated Animals (Male) at Different Dose Levels in the Subacute Toxicity Study[a]

treatment group	sperm count (×10⁶ sperm/mL)	normal sperm	hock less	bent	coiled/folded	detached head
control	157 ± 3.4	74 ± 1.2	9 ± 2.1	55 ± 3.2	8.6 ± 2.3	10 ± 0.6
SF3 5 mg/kg	338 ± 2.1***	274 ± 2.2***	8 ± 0.9	25 ± 2.6	22 ± 2.1	9 ± 0.9
SF3 10 mg/kg	340 ± 5.1***	222 ± 3.4***	5 ± 0.12	21 ± 2.8	73 ± 1.5	19 ± 1.3
SF3 20 mg/kg	208 ± 4.6***	118 ± 4.6*	14 ± 2.1	12 ± 3.6	26 ± 1.9	28 ± 1.5
SF3 40 mg/kg	215 ± 6.2***	149 ± 3.2**	4 ± 1.3	18 ± 4.1	39 ± 2.3	5 ± 2.6

[a]The data are represented as mean ± SEM with $n = 5$, *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ were given in comparison with the control.
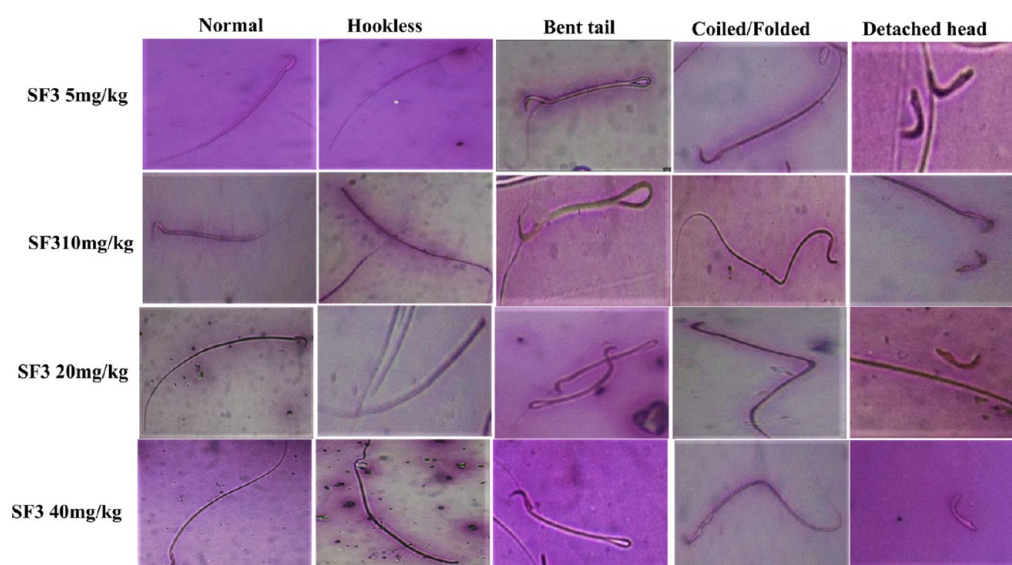

Figure 5. Sperm morphology after the treatment with selected doses of SF3 in subacute toxicity studies.

level, while 1,2-epoxide is nontoxic.[32] A previous study reported that naphthalene toxicity was enhanced using the P450 cytochrome inducers and preventing pretreatment with the prostaglandin synthetase inhibitors.[33] Our compound is the derivative of the naphthalene; that is why a detailed toxicity study was planned to assess the potential of toxicity generated by SF3.

The present study revealed the complete analysis of the toxicity potential of the SF3, a naphthalene derivative. Single-dose or short-term exposure hazardous effects of the chemicals/molecules/compounds are observed in the acute toxicity study. This study will majorly evaluate the physical change in behavior, body weight, morbidity, and mortality caused by toxic agents.[34] In the present study, acute toxicity results showed that the LD_{50} of SF3 is more significant than the 2000 mg/kg dose as no toxic symptoms were observed throughout the study. Estimation of liver enzymes is an indicator of liver functioning. ALP and ALT rose significantly in the animals treated with 2000 mg/kg dose while other biochemical parameters remained normal, indicating the normal functioning of the kidney and lipid profile.

Toxic effects based on repeated-dose administration were analyzed in the subacute toxicity study. It provided in-depth information on the toxic effects of the test drug on body weight, organ weight, biochemical, physiological, hematological, oxidative stress, and histopathological changes. Estimations of the parameters mentioned above are required according to the guidelines of the regulatory bodies.[35,36] In recent years, sex-related toxicity studies have gained considerable attention. Gender-dependent metabolism causes changes in the pharmacological and toxicological effects. In the toxicological studies, female animals were found to be more sensitive than males.[37] In

this study, a subacute toxicity study of SF3 was performed on both male and female animals for 28 days. No death and clinical sign and symptoms of toxicity were observed throughout the 28 days of treatment. The behavior of all animals was the same and comparable to the normal group. The weights of the organs were parallel to the control as no hypertrophy or megaly was seen. No significant alteration was observed in the hematological parameters. However, WBC levels were increased significantly, indicating the immunostimulant potential of the SF3 as increased levels of WBC's boosted the immune system. Liver and renal function tests are crucial due to the liver and kidney's role in the metabolism and excretion of the waste products. To evaluate the toxicity of any compound, estimation of the functioning state of these organs is vital. Biochemical marker analysis verified the functional state of vital organs.[38] In the subacute study, 5 mg/kg dose in male and female and 10 mg/kg in male animals increased the ALP levels while all other parameters remained normal in both genders. In both male and female rats, SF3 significantly decreased the urea level on test doses compared to the control.

The thyroid function test is an important marker of metabolism. No effect was observed in the thyroid functioning test in acute and high doses of subacute toxicity studies. Surprisingly, SF3 at all dose levels significantly elevated the sperm count, and the percentage of normal sperm count was also increased. The testosterone level was normal at all dose level. Sperm morphology is the primary biomarker of fertility in male. SF3 elevated the sperm count and increased the number of normal sperm meaning that SF3 increases the rats' fertility profile.[39] As reported previously, naphthalene increased the levels of reactive oxygen species (ROS). Hence, it is essential to

Table 4. Effect of Acute, Subacute, and Teratogenicity on Hematological Parameters[a]

hematology parameters	control	acute oral toxicity	terato-genicity	subacute toxicity							
				5 mg/kg		10 mg/kg		20 mg/kg		40 mg/kg	
				M	F	M	F	M	F	M	F
WBC's ($\times 10^3/\mu L$)	3.7 ± 0.12	13.8 ± 0.1*	12.8 ± 0.2*	3.6 ± 0.42	6.8 ± 0.2*	5.2 ± 0.2*	5.2 ± 0.1*	4.0 ± 0.3	8.0 ± 0.3*	6.0 ± 0.1*	8.8 ± 0.2*
RBC's	5.95 ± 0.32	6.67 ± 0.6	5.65 ± 1.1	6.12 ± 0.3	5.92 ± 1.1	6.13 ± 0.1	5.11 ± 1.2	6.94 ± 1.2	6.53 ± 2.1	5.89 ± 1.1	6.55 ± 1.3
platelets	817 ± 0.14	603 ± 0.52	800 ± 0.2	659 ± 0.4	750 ± 0.2	615 ± 0.21	750 ± 0.3	817 ± 0.12	888 ± 0.1	650 ± 0.32	806 ± 0.3
Hb	12.0 ± 1.2	12.8 ± 0.3	12.0 ± 0.14	12.9 ± 0.1	12.7 ± 0.1	13.0 ± 0.1	11.2 ± 0.1	12.9 ± 0.1	12.5 ± 0.1	12.6 ± 1.1	12.9 ± 0.1
LYM ($\times 10^3/\mu L$)	3.1 ± 1.4	7.0 ± 2.1*	10.9 ± 1.1*	2.9 ± 1.0	4.6 ± 1.0	3.0 ± 1.1	3.0 ± 1.1	5.8 ± 0.9	4.3 ± 1.0	4.8 ± 1.0	5.1 ± 1.1
MID ($\times 10^3/\mu L$)	0.4 ± 1.3	5.9 ± 1.3	1.5 ± 0.5	0.7 ± 0.62	0.8 ± 0.3	0.8 ± 0.7	0.8 ± 0.5	6.3 ± 0.5	1.1 ± 0.6	5.4 ± 0.6	1.1 ± 0.6
GRA ($\times 10^3/\mu L$)	0.1 ± 0.02	0.8 ± 0.1*	0.4 ± 0.1	1.0 ± 0.1*	0.4 ± 0.0	0.4 ± 0.12	0.4 ± 0.2	0.5 ± 0.13	0.6 ± 0.6	0.3 ± 0.1	0.6 ± 0.1
MCH (pg)	20.30 ± 0.1	19.1 ± 0.8	19.6 ± 0.2	21.0 ± 0.3	21.6 ± 0.1	21.2 ± 0.2	22 ± 0.2	20.1 ± 0.2	20.6 ± 0.2	18 ± 0.12	19.9 ± 0.2
MCHC (g/dL)	36.5 ± 0.96	35.8 ± 0.2	33.5 ± 0.8	35.5 ± 0.7	34.1 ± 0.8	36.2 ± 0.8	36.1 ± 0.8	34.9 ± 0.8	35.9 ± 0.9	34.4 ± 0.7	35.1 ± 0.7
MCV (fl)	55.4 ± 1.2	53.6 ± 1.0	58.6 ± 0.2	59.1 ± 0.2	53.3 ± 0.1	58.6 ± 0.1	51.0 ± 0.2	57.7 ± 0.1	57.5 ± 0.1	52 ± 0.2	56.7 ± 0.2
HCT (%)	33.0 ± 3.2	36.2 ± 2.1	33.1 ± 1.2	36.2 ± 1.3	37.5 ± 1.2	35.6 ± 1.5	31.2 ± 1.5	22.7 ± 1.1	37.5 ± 1.1	30.9 ± 2.4	37.1 ± 1.2

[a]The data are represented as mean ± SEM with n = 5 for acute toxicity, n = 10 for teratogenicity, and n = 10 for subacute toxicity studies. *P < 0.05 was the increased level of significance compared to the control. M = male and F = female.

Table 5. Estimation of Biochemical Markers in the Acute Oral Toxicity Study[a]

biochemical markers	units	control	SF3 (2000 mg/kg)
uric acid	(mg/dL)	5.0 ± 1.2	4.22 ± 0.54
protein	(g/dL)	7.65 ± 1.2	13.63 ± 0.78*
creatinine	(mg/dL)	1.12 ± 2.1	0.60 ± 0.69
bilirubin	(mg/dL)	1.2 ± 1.42	1.66 ± 1.35
ALP	U/L	185 ± 1.97	289 ± 1.33***
ALT	U/L	42 ± 0.34	106 ± 0.24***
AST	U/L	65 ± 1.45	12.2 ± 0.32
urea	mg/dL	30.2 ± 0.12	2.1 ± 3.2
cholesterol	mg/dL	65.12 ± 2.1	48.1 ± 2.1
HDL	mg/dL	20.3 ± 1.23	10.2 ± 0.89
triglyceride	mg/dL	56.84 ± 1.2	64.1 ± 3.42
LDL	mg/dL	33.32 ± 0.32	25.3 ± 2.1

[a]The data are represented as mean ± SEM, n = 5. *P < 0.05 and ***P < 0.001 were given in comparison with the control.

measure the oxidative stress in vital organs to measure any change at the biochemical level generated by the SF3. Endogenous antioxidants (SOD, CAT, and GSH) and oxidant (MDA) levels were measured in the different organs for analyzing any toxicity generated by the SF3. The heart, liver, kidney, spleen, stomach, brain ovaries, and testis were selected to estimate oxidative stress levels. No significant change in the level of MDA was observed in all tissues. Levels of CAT were decreased in the spleen of male and female animals treated with a low dose of SF3 (5 mg/kg). GSH was decreased in the spleen and kidney tissues of male animals at 10, 20, and 40 mg/kg dose levels. Oxidative stress plays a significant role in the progression of inflammation; that is why histamine levels were measured.[40] SF3 did not show any effect on the levels of serum histamine levels. SF3 did not produce a significant level of toxicity. The toxicities produced are of a negligible amount. Teratogenesis is the major problem when the new drug development and investigation are concerned. Teratogenicity is a fundamental part of toxicity studies. No teratogenic signs were observed in soft tissue and skeletal level studies during teratogenicity. Histopathology revealed normal tissue architecture in selected organs, except kidney in acute oral and subacute toxicity studies.

CONCLUSIONS

This study provides valuable data on the toxic profile of SF3. It was concluded that SF3 LD_{50} was higher than 2000 mg/kg, and repeated dose administration did not significantly alter the test parameters evaluated in the study. Moreover, it also determined that SF3 did not have teratogenic potential. Results indicated that SF3 would be used for further pharmacological studies in animal models.

MATERIALS AND METHODS

Drugs and Chemicals. Pyrogallol solution and Alcian blue were purchased from Oxford Labs (India). Elman's reagent and Alizarin red S were purchased from Omicron Sciences Limited (UK). Follin's reagent, carboxymethyl cellulose, picric acid, EDTA, N-1-naphthyl ethylene amine dihydrochloride, sulpha-nilamide, phosphoric acid, thiobarbituric acid, sodium phosphate dibasic heptahydrate and sodium phosphate monobasic monohydrate, sodium carbonate, sodium hydroxide, copper sulphate and sodium-potassium tartrate, DTNB, and Griess reagent were purchased from Sigma-Aldrich, USA. IL-6 (Cat#EH21L6), histamine (KA2589), testosterone (MA5-
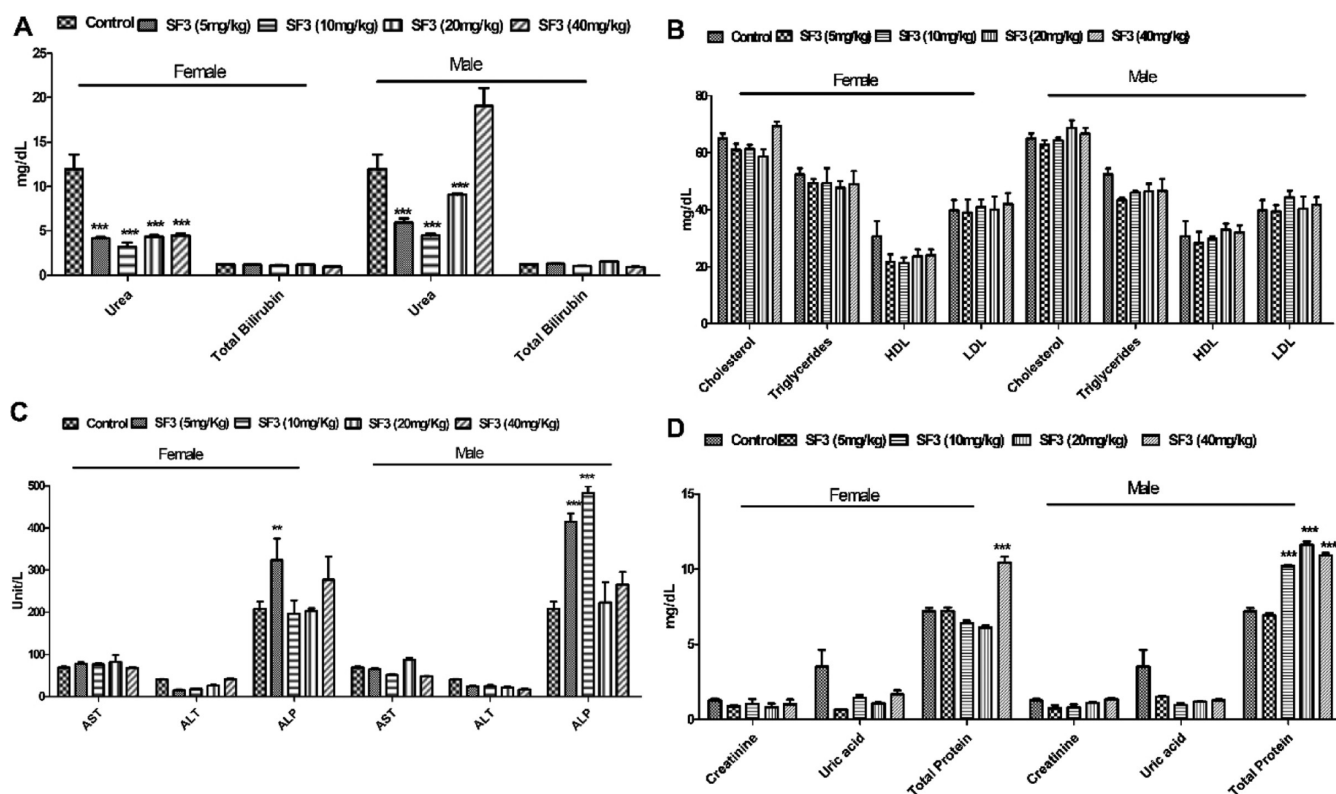
Figure 6. Biochemical parameter estimation after 28 days of treatment with different dose levels of SF3 in the subacute study. Data are represented as mean ± SEM, $n = 10$. **$P < 0.01$ and ***$P < 0.001$ were given in comparison with the control.

Table 6. Thyroid Functioning Marker Estimation in Treated Groups during Acute and Subacute Toxicity Studies[a]

parameters	units	control	acute toxicity (2000 mg/kg)	subacute toxicity (40 mg/kg)			
				control	male	control	female
T_3	ng/dL	46.2 ± 1.6	42.23 ± 1.3	42.3 ± 1.6	40.58 ± 1.4	47.3 ± 1.7	42.58 ± 0.9
T_4	μg/dL	4.9 ± 1.2	3.63 ± 0.2	5.2 ± 1.6	5.1 ± 0.5	4.6 ± 0.5	4.07 ± 0.7
TSH	μIU/mL	0.037 ± 0.032	0.01 ± 0.01	0.05 ± 0.01	0.06 ± 0.032	0.06 ± 0.01	0.083 ± 0.02

[a]Data are represented as mean ± SEM, $n = 5$ for acute and $n = 10$ for subacute toxicity study.

14715), and NF-κB (Cat#85-86081-11) were purchased from Thermo-Fischer Scientific, USA.

Experimental Animals. Adult Wistar rats, 2−3 months old, weighing 150−250 g were housed in the animal house of Riphah Institute of Pharmaceutical Sciences, Riphah International University Lahore campus. Experimental animals were provided with the standard environmental conditions 22 ± 2 °C temperature, 40−50% humidity, 12/12 h light/dark cycle, and free access to food and water. Experimental protocols acute oral, subacute oral, and teratogenicity were approved from Ethical committee of Riphah Institute of Pharmaceutical Sciences, Lahore Campus, with the voucher number of REC/RIPS-LHR/035 for further considerations under the rules and regulations of National Institute of Health (NIH) guide for the care and use of laboratory animals.

Acute Oral Toxicity. This study was performed according to the 425 guidelines of the Organization of Economic Cooperation and Development (OECD) for acute toxicity studies of chemicals. Twelve female rats (180−215 g weight) were randomly divided into two groups ($n = 6$). Group 1 served as the control group and received 0.5% carboxymethylcellulose (CMC) 1 mL/kg, while group II was treated with a 2000 mg/kg dose of SF3. Initially, only one animal from each group was treated with a single oral dose of SF3 and CMC *via* an oral gavage feeding needle. After administering the dose, clinical signs of toxicity such as morbidity and mortality were observed at different intervals, that is, 30 min and 1, 2, 3, 4, and 24 h. If no mortality was observed after 24 h, other animals of both groups also received their respective doses. Toxicity was observed 14 days in total. Overall observations that were made to detect the toxicity were general behavior, skin, fur, eye changes, secretions from the mucous membrane, respiratory and autonomic or CNS disturbances, morbidity, and mortality. After 14 days, female rats were anesthetized using isoflurane (2−3%) diluted with oxygen and blood was collected by cardiac puncture.[27]

Subacute Toxicity. Healthy male and female rats with weights ranging between 210−250 and 200−240 g, respectively, were used in this study. Fifty rats were randomly divided into five groups (5 males and 5 females in each group). Group 1 received CMC 1 mL/kg and designated as the control; group II-V received SF3 at 5, 10, 20, and 40 mg/kg dose level, respectively. All the treatments were given for consecutive 28 days once daily through the oral route. Any change in body weight and physical appearance was recorded throughout the study period.[41]

Teratogenicity. The highest dose of the SF3 in the subacute toxicity study was selected for the teratogenic studies. An OECD
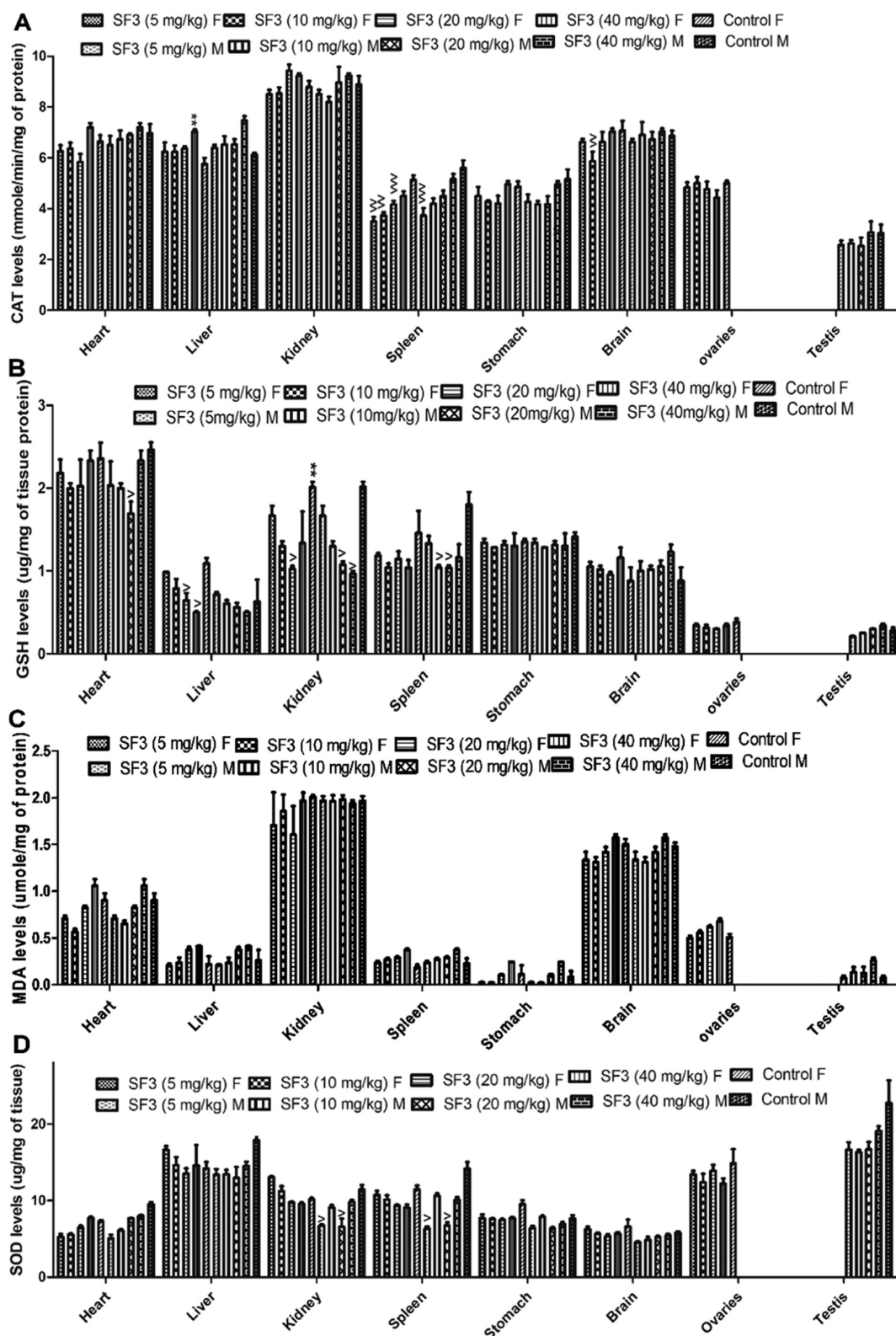
Figure 7. Estimation of oxidative stress markers in the selected organs of male and female rats during the subacute toxicity study. The data are presented as mean ± SEM of $n = 3$ where $*P < 0.05$ is the level of significance during increase and $\hat{P} < 0.05$ is the level of significance during decrease in comparison with the control.

414 guideline for teratogenicity was used to design the protocol. Twenty female rats (200−240 g) were divided into two groups one group is a control group, and the second group ($n = 10$) received SF3 at the 40 mg/kg dose level. Three female rats were housed with one male, and the day at which a vaginal plug was

observed, labeled as 0 days of gestation. Treatments were continued from gestational day 5−15 *via* the oral route. On the 19th day of gestation, C-section was performed, and fetuses were removed carefully.[42] Fetuses and placenta were weighed, and any deformity in each fetus was recorded.[42,43]
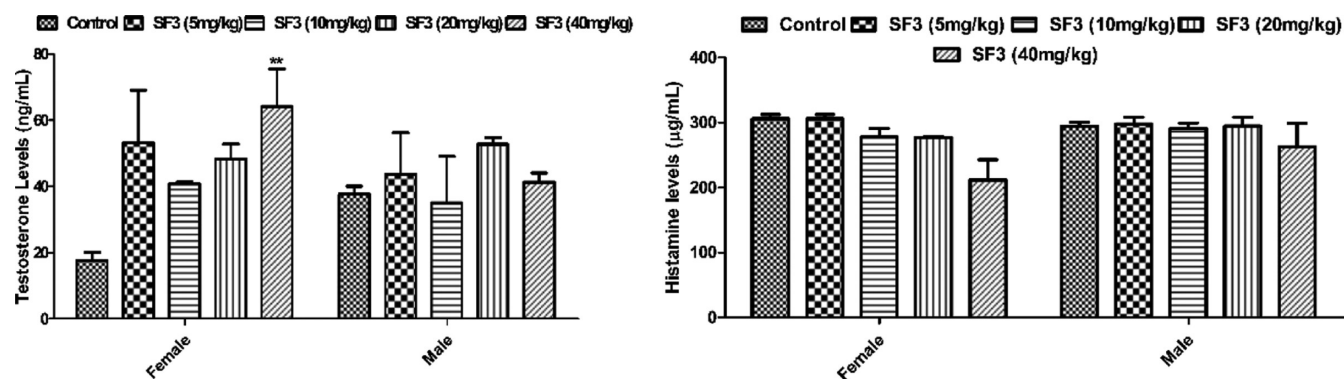
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Figure 8. Serum histamine and testosterone levels in the subacute toxicity study. The data are presented as mean $\pm$ SEM of $n = 3$, where **$P < 0.01$ is the significance level compared to the control.
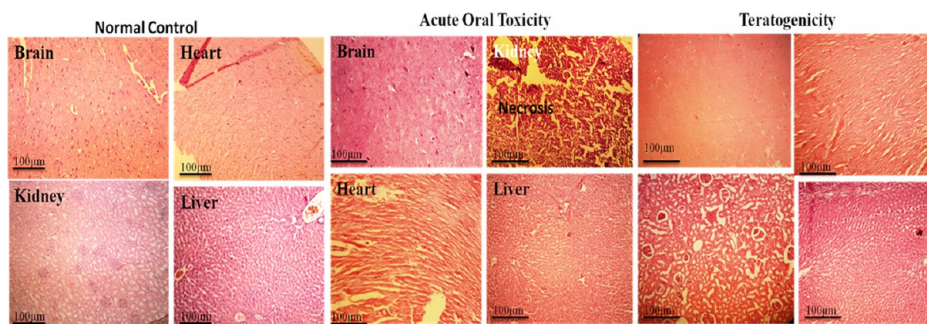

Figure 9. Histopathological studies on selected organs in control, acute oral toxicity, and teratogenicity studies.
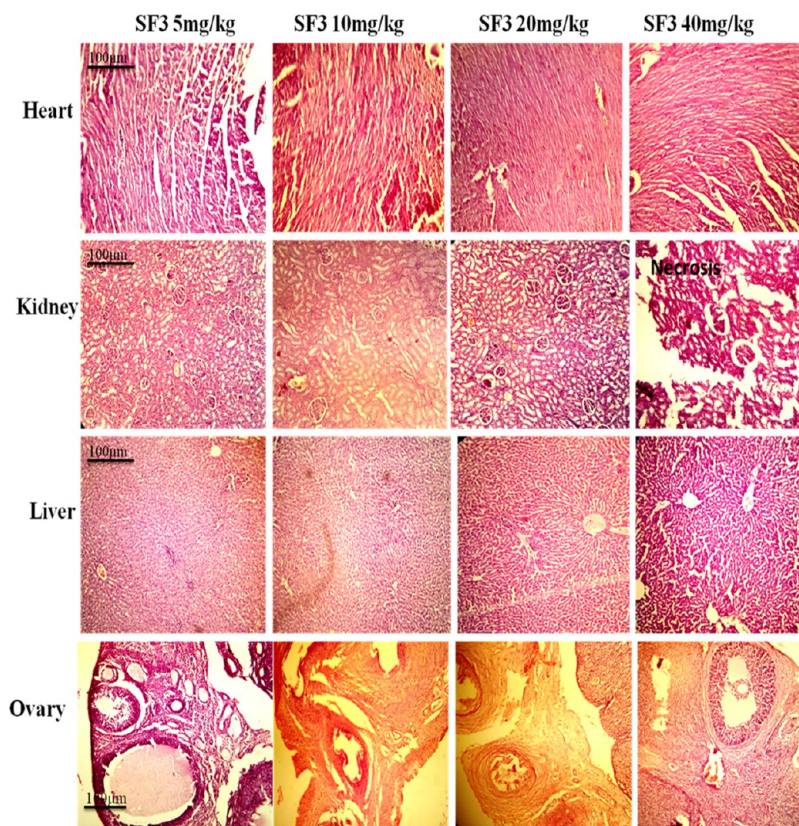

Figure 10. Histopathological studies on selected organs of female animals in subacute toxicity studies.

Staining of the Fetal Skeleton. In teratogenic studies, fetuses were removed and soaked into the 4% NaCl solution overnight to remove the muscular mass. Muscles and organs were removed carefully from the skeleton,[44] and the bone
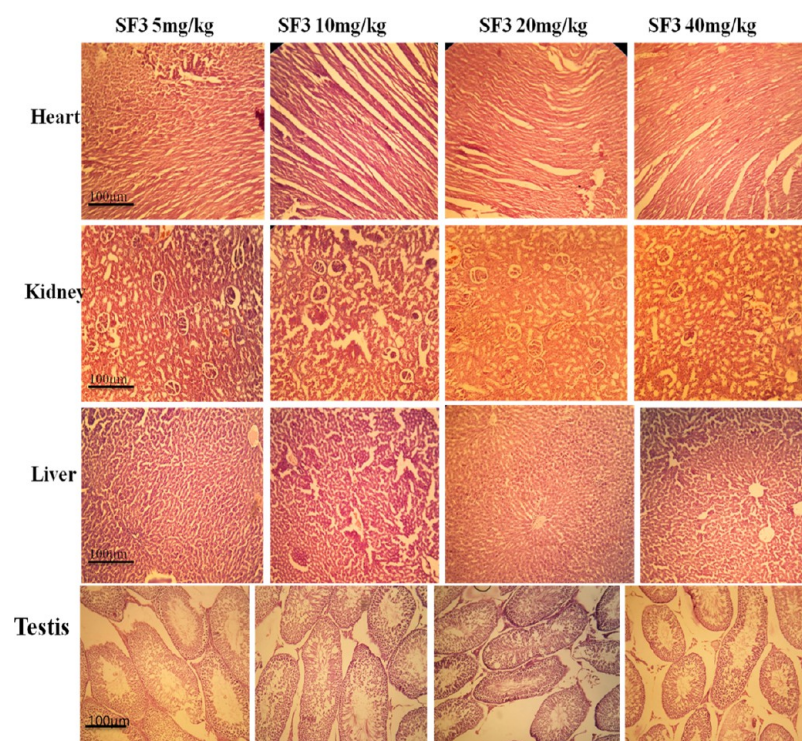
Figure 11. Histopathological studies on selected organs of male animals in subacute toxicity studies.

skeleton was stained with acidic staining (Alzarian red, pH 2.8, 24 h). After staining, moisture was reduced by soaking the skeleton in absolute alcohol. After dehydration, the specimen was soaked in the basic stain (Alcian blue, 24−30 h). The stained specimens were placed in the clearing solution (1:1, 70% ethanol/glycerin) for 6−8 h. The specimens were analyzed under a dissecting microscope for bone ossification, spina bifida, rib and limb deformities, and cleft palate.[44,45]

Soft Tissue Examination. For the rapid and gross examination of soft tissues, Wilson's technique was used. Fetuses were soaked for 8−10 days in the Bouin solution (Saturated solution of picric acid). After 8−10 days of soaking in Bouin solution, fetuses were soaked in distilled water to remove any irritant of picric acid. The skin was removed, and tissues were observed separately. Different transverse and longitudinal cuts were made for the head analysis. Organs were examined for any megaly and visual abnormality.[46]

Sperm Analysis. In the subacute toxicity study, the diffusion method was used for the collection of sperm. Orchidectomy was performed by the castration method. The incision was made on the prescrotal region of the testis, and testicles were oozed out.[47] Cauda epididymis was poured into the Petri dish containing phosphate buffer pH 7.4. Sperm suspension was made by swirling the Petri plate, and sperm suspension was analyzed for the count and morphological features.[39]

Hematological Analysis. At the end of each study, animals were given anesthesia using the 2−3% isoflurane dilute with oxygen. Blood was collected through cardiac puncture. One milliliter of blood was collected in the EDTA-coated tube, rolled on a rolling mixture, and kept at room temperature for hematological analysis. Hematological parameters RBC's, WBC's, platelets, hemoglobin, MCH, MCV, and % leukocyte count were determined as toxicity indices.[48−50]

Biochemical Analysis. After completing each study, 2−3 mL of blood was kept in the EDTA tube and centrifuged at 4000

rpm for 15 min. The separate plasma was stored for biochemical analysis. Biochemical parameters AST, ALT, ALP,[51] bilirubin, urea, creatinine, HDL, LDL, VLDL, and total cholesterol were measured using specific kits of the chemistry analyzer (Merck, USA).[52] Serum was collected by putting the blood into the EDTA free tube; it was centrifuged at 4000 rpm for 15 min. Serum was separated, and TSH, T3, and T4 levels were measured using ELISA kits.

Oxidative Stress Biomarker Analysis. After blood collection, animals were sacrificed by the cervical dislocation method. The animals were dissected, and different organs such as the brain, kidney, heart, liver, ovary, testis, and spleen were removed and weighed. The homogenates of these organs were prepared in the phosphate buffer (0.1 M, pH 7.4). The homogenates were centrifuged at 6000 rpm, 4 °C for 10 min. The supernatant was collected to estimate SOD, CAT, GSH, and MDA levels.[53]

Superoxide Dismutase Analysis. In 0.1 mL of tissue homogenate, 0.1 mL of pyrogallol solution and 2.8 mL of potassium phosphate buffer (pH 7.4, 0.1 M) were added and mixed thoroughly. Absorbance was measured at 325 nm.[54] Levels of SOD were calculated using the following regression line of the standard

$$Y = 0.0095X + 0.1939$$

Catalase Analysis. A total of 1.95 mL of phosphate buffer (pH 7, 50 mM) and 50 μL of tissue homogenate were mixed in 1 mL of H_2O_2 (30 mM) solution. The mixture was vortexed thoroughly, and wavelength was noted at 240 nm.[54] The following formula was used for CAT level measurements

$$CAT\ levels = \frac{OD}{E} \times vol.\ of\ sample \times mg\ of\ protein$$

OD is the change in absorbance and E is the extinction coefficient of H_2O_2 (0.071 mmol/cm). Total protein was estimated using the Lowery method.

Total Glutathione Analysis. Tissue homogenate (1 mL), trichloroacetic acid (10%), 4 mL of phosphate buffer (pH 8), and 0.5 mL of DTNB solution were mixed. After the precipitation, the supernatant was removed, and absorbance was measured at 412 nm.[55] GSH levels were measured using the following formula

$$GSH = Y - \frac{0.00314}{0.034} \times \frac{DF}{BT} \times VU$$

DF = dilution factor, BT = tissue homogenate, VU = volume used, and Y = absorbance at 412 nm.

Malondialdehyde Analysis. Thiobarbituric acid (3 mL) reagent was added in tissue homogenate (1 mL). The mixture was mixed well and incubated at room temperature for 15 min. The mixture was heated at 80 °C for 15 min and cooled on an ice bath. The supernatant was removed and measured at 532 nm. Levels of MDA were analyzed using the following formula.[42]

$$\text{Conc. of MDA} = \text{Abs}_{532} \times 100 \times V_T/1.56 \times 105 \times W_T \times V_U$$

where V_T = total volume of the mixture, which is 4 mL, W_T = weight of the dissected brain, and V_U = aliquot volume.

Histamine and Testosterone Analysis. In the subacute toxicity study, blood serum of both male and female rats was used to estimate histamine and testosterone levels. Each protein was combined with HRP-labeled antibody and make an antigen−antibody complex. Then, this complex was mixed with TBM solution, and the reaction was stopped by adding the stop solution. Absorbance was measured at 450 nm using an ELISA reader. Levels of histamine and testosterone were calculated using their specific standard regression lines.[56]

Histopathological Studies. At the end of acute oral, subacute, and teratogenicity studies, selected organs were fixed in 4% formaldehyde solution, embedded in paraffin wax, and sliced. Sliced sections were fixed on slides and stained with H and E staining. Sections were observed under a microscope (100×) for analyzing any change in the cells.[57]

Statistical Analysis. All the data were expressed as mean ± SEM. Graphpad Prism 5.0 software was used for the interpretation of the experimental data. One-way ANOVA or two-way ANOVA was used to analyze the data, followed by Tukey comparison and Bonferroni post hoc test. *$P < 0.05$ was considered as the level of significance. **$P < 0.01$ and ***$P < 0.001$ labeled as moderate and highly significant levels.

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Experimental work and data collection: F.A., T.I., A.U.R., I.A., and U.S.; designing the study: B.A. and S.A.; statistical analysis: M.U.M.; and interpretation of the data and critical revision of the manuscript: S.A. and I.A.

Notes

The authors declare no competing financial interest.

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