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Safety evaluation of *Syzygium aromaticum* oil in male and female wistar rats

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Abstract
The present study was planned to evaluate safety of *Syzygium aromaticum* oil (clove oil) following oral administration at dose of 50, 100 and 200 mg/kg body weight once daily for 28 days in male and female wistar rats. Body weight and feed consumption of animals were monitored at weekly interval. At the end of experiment, blood sample were collected for the hematological and serum biochemical investigations as well as organs are collected for histopathological examination. No significant difference was observed in body weight and feed consumption of clove oil treated male and female rats as compared to control rats. No significant changes have been observed in Hb, RBCs, PCV, TLCs, MCV, MCH and MCHC in clove oil treated male and female rats at the end of experiment as compared to control rats. No significant change have been observed in serum creatinine, BUN, total bilirubin, AST, ALT, total cholesterol, total protein and albumin in clove oil treated male and female rats as compared to control rats at the end of experiment. Results of the present study suggest that the clove oil was found safe following repeated oral administration @ 50, 100 and 200 mg/kg for 28 days in male and female wistar rats.

Keywords: *Syzygium aromaticum*, clove oil, safety evaluation, wistar rats

1. Introduction
Essential oils (EOs) also called volatile or ethereal oils are aromatic oily liquid obtained from diffrent plant material (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots). Essential oils are complex mixtures of low molecular weight (usually less than 500 daltons) compounds extracted by steam distillation, hydrodistillation or solvent extraction [1]. Use of EOs in traditional systems of medicine is being practiced since ancient times in human history. Researchers from all over the world are trying to characterize a range of biological properties of EOs which includes antimicrobial, antiviral, antimutagenic, anticancer, antioxidant, anti-inflammatory, immunomodulatory and antiprotozoal activities [2].
Plants of the genus Eugenia (Syzygium), comprising of about 100 species, grow in tropical climate in which *Syzygium aromaticum* or *Eugenia caryophyllata* plant is a high (up to 15 m), evergreen tree of the family Myrtacae [3]. It is commonly called Clove in English, Laung in Hindi and Laving in Gujarati. The clove is aromatic, dry, fully grown, but unopened flower bud of clove tree. Clove is a tree which is growing in islands of Indonesia, Tanzania, Sri Lanka, Madagascar, India and Malaysia [4]. In India clove is mostly grown in the hilly tracts of Tamilnadu, Karnataka and Kerala. Clove relieves stomach pain, nausea and vomiting [5]. Clove has a deodorizing property and so used in perfumes and cosmetics [6].
Clove oil is obtained by distillation of the leaves, flowers, buds and stem of the clove tree [7]. Clove bud oil is a colourless or yellow liquid. Clove buds contain 15 to 20 % of oil by weight. The main oil constituents are eugenol (70–95 %), eugenol acetate (up to 20 %) and β-caryophyllene (12–17%). The essential oil of clove has biocidal activity against *Aedes albopictus* (tiger mosquitos), thereby helping in the control of malaria [8]. Stress which is very common in every individual can also be relieved with the help of hydro-alcoholic extracts of clove oil [9]. Clove and its essential oil has been found effective in poultry to improve growth performance, control some intestinal pathogens and stimulate digestion and also showed strong antimicrobial, antifungal, anti-inflammatory, anesthetic, anti-carcinogenic, anti-parasitic and antioxidant activities [10]. The alcoholic extract of clove buds showed significant antibacterial activity against *Propionibacterium acne* and *Staphylococcus epidermis* [11]. Various activities of clove oil were reported like an anti-inflammatory, analgesic, antiseptic, deworming, disinfectants and antibacterials because it inhibits the growth or kills most pathogens [12]. *S. aromaticum* ethanolic extract showed the potential anti-inflammatory effect of the extract against rat paw edema at dose rate of 250 and 500 mg/kg b.wt. [13]. Clove oil is also used for the treatment of sore throat, colds, catarrh and inflammation of the mucous

membranes of the mouth. It is also helps to deal with any breathing problems, general weakness and neuralgia [14]. Hence in the present study research was done to evaluate safety of clove oil following its repeated oral administration in wistar male and female rats.

2. Materials and Methods

2.1 Experimental animals

The study was conducted on adult healthy male and female wistar rats. Twenty male rats (335 to 355 g) and 20 female rats (220 to 240 g) of 8-10 weeks of age were procured from Cadila Healthcare Ltd. (R & D Centre), Ahmedabad, Gujarat. All the protocols as per the CPCSEA guidelines on the care and use of laboratory animals were followed and approved by the Institutional Animal Ethics Committee (Project No. IAEC/279/VPT/2018) of Veterinary College, AAU, Anand. Rats were kept under constant observation during entire period of study. The animals were housed in standard polypropylene cages and maintained under controlled room temperature (22 ± 2^0C) and humidity (55 ± 5%) with 12 h light and12 h dark cycle. All the rats were fed normal pellet diet and deionized water was provided *ad libitum* throughout the course of the experiment. All the rats were kept under acclimatization for 5 days prior to grouping and initiation of experiment. Rats were kept under constant observation during entire period of study. All necessary managemental procedures were adopted to keep the rats free from stress.

2.2 Drugs and chemicals

Clove essential oil (Natural, Functional grade) was purchased from Sigma-Aldrich, India. All other reagents used of analytical grade like biochemical kits for estimation of serum biochemical parameters were purchased from Coral Clinical System (Goa, India).

2.3 Experimental design

Forty rats were divided into eight groups, each group contains 5 males and 5 females. Group I were served as the male control and group V served as female control group. Clove oil was administered orally at dose of 50, 100 and 200 mg/kg body weight once daily for 28 days to male rats of group II, III and IV and female rats of group VI, VII and VIII, respectively. The solutions were administered orally to rats directly into stomach by using rat oral feeding gavage for 28 days.

Group I- Male control (untreated)
Group II- Clove oil – male (50 mg/kg)
Group III- Clove oil – male (100 mg/kg)
Group IV- Clove oil – male (200 mg/kg)
Group V- Female control (untreated)
Group VI- Clove oil – female (50 mg/kg)
Group VII- Clove oil – female (100 mg/kg)
Group VIII- Clove oil – female (200 mg/kg)

2.4 Body weight and feed consumption

Body weight and feed consumption of all rats were measured weekly interval for 28 days.

2.5 Hematological estimation

On termination of experiment, blood samples were collected from retro-orbital plexuses under light anesthesia with the help of capillary tube. Blood samples collected in test tubes with K_3EDTA were utilized for estimation of various hematological parameters (Hb, RBCs, PCV, TLCs, MCV, MCH and MCHC) by hematology auto analyzer (Mindray, BC-2800 Vet, Garnerville, New York).

2.6 Serum biochemical estimation

Serum biochemical parameters like serum creatinine, blood urea nitrogen (BUN), serum aspartate aminotransferase (AST), serum alanine aminotransferase (ALT), total cholesterol (TC), total bilirubin, total protein and total albumin were estimated by using auto serum chemistry analyser (Mindray BS-120, Mumbai, India).

2.7 Histopathology

After opening the carcass, gross lesions were recorded and collected tissues like kidney, liver, spleen and heart were fixed in 10 % formalin. The formalin fixed tissues were processed by paraffin wax embedding method of tissue sectioning. Sections from the tissues were cut at 5-6 microns thickness with automatic section cutting machine (Leica, Germany) and were stained with Haematoxylin and Eosin (H & E) stains.

2.8 Statistical analysis

All the data have been presented as mean ± SE. Statistical comparisons of the means were made by one way analysis of variance (ANOVA) using software SPSS (Version 25). Significant differences ($p < 0.05$) between different experimental groups were analyzed by Duncan's test.

3. Results

3.1 Effect on body weight and feed consumption

There was no significant difference observed in body weight of male rats group (II, III and IV) and female rats group (VI, VII and VIII) of different treatment on day 7, 14, 21 and 28 at dose of 50, 100 and 200 mg/kg as compared to rats of male and female control group (I & V). Similarly, no significant difference observed in feed consumption of male rats group (II, III and IV) and female rats group (VI, VII and VIII) of different treatment on week 1, 2, 3 and 4 at dose of 50, 100 and 200 mg/kg as compared to rats of male and female control group (I & V). Body weight and feed consumption are presented in Table 1 & 2, respectively.

3.2 Effect on hematological parameters

No significant changes have been observed in Hb, RBCs, PCV, TLCs, MCV, MCH and MCHC levels in clove oil treated male rats (II, III and IV) and female rats (VI, VII and VIII) as compared to control rats (I & V, respectively) at the end of experiment on 28th day as depicted in Table 3.

Table 1: Effect of repeated oral administration of clove oil (*Syzygium aromaticum*) for 28 days on body weight (g) of male (I-IV) and female (V-VIII) rats (Mean ± S.E.; n=5)

Group	Treatment	0 day	7 day	14 day	21 day	28 day
I	Control-M	336 ± 8.12	350 ± 10.95	354 ± 8.72	369 ± 7.65	375 ± 7.58
II	SA-50-M	344 ± 15.68	352 ± 19.85	364 ± 18.87	369 ± 20.27	380 ± 17.03
III	SA-100-M	348 ± 5.39	374 ± 5.10	381 ± 6.96	387 ± 1020	401 ± 3.32
IV	SA-200-M	353 ± 4.90	367 ± 6.63	375 ± 15.49	389 ± 7.14	398 ± 9.17
V	Control-F	222 ± 3.74	228 ± 3.74	226 ± 4.00	234 ± 8.12	249 ± 5.57

VI	SA-50-F	232 ± 7.35	246 ± 2.45	248 ± 4.90	244 ± 5.10	250 ± 7.07
VII	SA-100-F	228.4 ± 2.66	236 ± 2.45	236 ± 8.12	238 ± 2.00	246 ± 2.92
VIII	SA-200-F	238 ± 4.90	244 ± 13.27	238 ± 11.58	244 ± 7.48	244 ± 5.10

SA-50-M= *Syzygium aromaticum* oil @ 50 mg/kg b.wt in male rats
SA-100-M= *Syzygium aromaticum* oil @ 100 mg/kg b.wt in male rats
SA-200-M= *Syzygium aromaticum* oil @ 200 mg/kg b.wt in male rats
SA-50-F= *Syzygium aromaticum* oil @ 50 mg/kg b.wt in female rats
SA-100-F= *Syzygium aromaticum* oil @ 100 mg/kg b.wt in female rats
SA-200-F= *Syzygium aromaticum* oil @ 200 mg/kg b.wt in female rats

Table 2: Effect of repeated oral administration of clove oil (*Syzygium aromaticum*) for 28 days on feed consumption (g) of male (I-IV) and female (V-VIII) rats (Mean ± S.E.; n=5)

Group	Treatment	Week 1	Week 2	Week 3	Week 4
I	Control-M	19.76 ± 0.24	20.69 ± 1.69	21.07 ± 0.36	21.86 ± 0.43
II	SA-50-M	19.19 ± 2.05	17.98 ± 0.12	19.29 ± 0.71	20.95 ± 0.48
III	SA-100-M	20.60 ± 1.55	20.12 ± 2.02	20.95 ± 1.90	21.90 ± 0.95
IV	SA-200-M	18.02 ± 0.83	18.31 ± 1.12	18.67 ± 0.67	20.36 ± 1.21
V	Control-F	21.05 ± 1.49	21.93 ± 0.79	19.62 ± 0.40	21.64 ± 0.21
VI	SA-50-F	19.05 ± 0.57	20.57 ± 0.86	19.79 ± 1.07	19.00 ± 0.43
VII	SA-100-F	18.83 ± 0.98	19.29 ± 0.43	19.59 ± 0.84	18.93 ± 0.79
VIII	SA-200-F	18.57 ± 0.71	20.71 ± 0.57	18.44 ± 0.94	19.02 ± 1.69

Table 3: Effect of repeated oral administration of clove oil (*Syzygium aromaticum*) for 28 days on Hb, RBCs, PCV, TLCs, MCV, MCH and MCHC of male (I-IV) and female (V-VIII) rats (Mean ± S.E.; n=5).

Group	Treatment	Hb (g/dl)	RBCs (10^6/µl)	PCV (%)	TLCs (10^3/µl)	MCV (fl)	MCH (pg)	MCHC (g/dl)
I	Control-M	15.46 ± 0.58	9.06 ± 0.49	44.76 ± 2.03	6.63 ± 0.31	50.09 ± 3.67	17.28 ± 1.11	34.74 ± 1.72
II	SA-50-M	16.76 ± 0.87	10.03 ± 0.66	50.36 ± 2.22	6.42 ± 0.16	51.31 ± 4.93	16.80 ± 0.54	33.70 ± 2.62
III	SA-100-M	15.80 ± 0.37	9.80 ± 0.35	50.34 ± 1.45	6.60 ± 0.18	51.71 ± 2.70	16.17 ± 0.34	31.54 ± 1.50
IV	SA-200-M	16.46 ± 0.45	9.93 ± 0.40	49.45 ± 2.89	6.40 ± 0.39	49.80 ± 2.05	16.62 ± 0.27	33.62 ± 1.63
V	Control-F	15.18 ± 0.58	8.98 ± 0.76	46.95 ± 1.45	6.73 ± 0.42	53.33 ± 3.48	17.32 ± 1.39	32.55 ± 2.08
VI	SA-50-F	15.88 ± 0.15	8.87 ± 0.12	44.62 ± 0.72	5.98 ± 0.41	50.28 ± 0.52	17.91 ± 0.24	35.61 ± 0.45
VII	SA-100-F	15.30 ± 0.16	8.78 ± 0.03	44.62 ± 2.07	6.52 ± 0.18	51.87 ± 2.28	17.79 ± 0.13	34.55 ± 1.44
VIII	SA-200-F	16.76 ± 0.35	10.63 ± 1.03	50.56 ± 3.03	6.36 ± 0.13	48.64 ± 3.60	16.19 ± 1.12	33.47 ± 1.35

3.4 Effect on serum biochemical parameters

No significant changes have been observed in serum creatinine level, blood urea nitrogen (BUN), serum aspartate aminotransferase (AST), serum alanine aminotransferase (ALT), total cholesterol (TC), total bilirubin, total protein and total albumin in clove oil treated male rats (II, III and IV) and female rats (VI, VII and VIII) as compared to control male and female rats (I & V, respectively) at the end of experiment on 28th day as depicted in Table 4.

Table 4: Effect of repeated oral administration of clove oil (*Syzygium aromaticum*) for 28 days on creatinine, BUN, AST, ALT, Bilirubin, total cholesterol, total protein and albumin of male (I-IV) and female (V-VIII) rats (Mean ± S.E.; n=5)

Group	Treatment	Creatinine (mg/dl)	BUN (mg/dl)	AST (U/l)	ALT (U/l)	Bilirubin (mg/dl)	TC (mg/dl)	Total Protein (g/dl)	Albumin (g/dl)
I	Control-M	0.38 ± 0.02	17.07 ± 0.83	66.28 ± 2.99	137.95 ± 7.24	0.67 ± 0.05	102.45 ± 4.09	6.00 ± 0.35	4.38 ± 0.26
II	SA-50-M	0.50 ± 0.07	16.61 ± 1.75	60.63 ± 5.49	136.45 ± 5.57	0.55 ± 0.16	95.35 ± 6.06	5.87 ± 0.41	4.13 ± 0.19
III	SA-100-M	0.55 ± 0.07	18.92 ± 0.81	80.55 ± 9.98	147.75 ± 6.97	0.57 ± 0.10	89.44 ± 4.17	6.07 ±0.16	4.08 ± 0.06
IV	SA-200-M	0.58 ± 0.06	19.12 ± 0.49	69.08 ± 3.32	144.63 ± 4.94	0.75 ± 0.04	103.80 ± 6.10	5.73 ± 0.26	4.12 ± 0.16
V	Control-F	0.50 ± 0.03	18.42 ± 0.65	61.69 ± 6.67	137.87 ± 9.09	0.77 ± 0.09	83.32 ± 5.92	5.91 ± 0.16	4.44 ± 0.12
VI	SA-50-F	0.43 ± 0.09	19.83 ± 0.81	68.24 ± 9.36	141.01 ± 5.62	0.51 ± 0.02	88.17 ± 9.54	6.35 ± 0.14	4.45 ± 0.10
VII	SA-100-F	0.54 ± 0.05	16.91 ± 0.62	65.23 ± 3.83	137.25 ± 2.31	0.57 ± 0.09	90.85 ± 10.41	6.48 ± 0.35	4.28 ± 0.20
VIII	SA-200-F	0.47 ± 0.07	17.81 ± 1.69	63.10 ± 3.48	134.24 ± 4.56	0.70 ± 0.10	87.46 ± 3.60	6.38 ± 0.32	4.48 ± 0.06

3.5 Histopathology

In the present study, histopathology of organs like kidney, liver, spleen and heart from control rats (group I) did not revealed any gross or microscopic changes. Whereas histopathology of kidney, liver, spleen and heart from clove oil treated male rats (II, III and IV) and female rats (VI, VII and VIII) did not show any marked gross or histopathological changes and are depicted in Fig. 1 to 8.

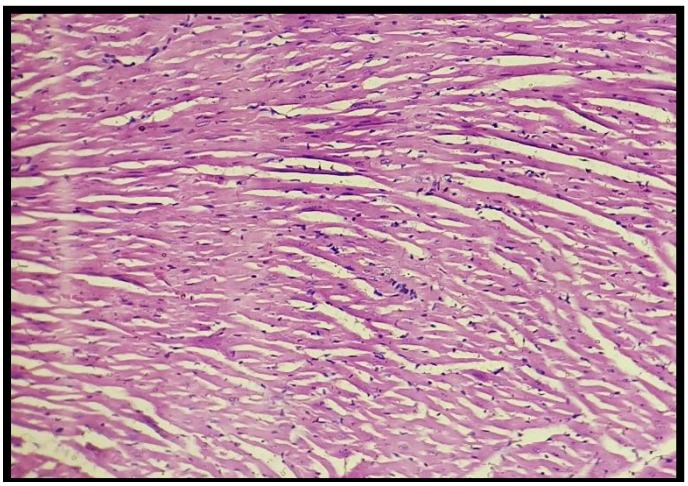

Fig 1: Section of heart from male control rats (group I) showing normal architecture (H & E stain X 120).

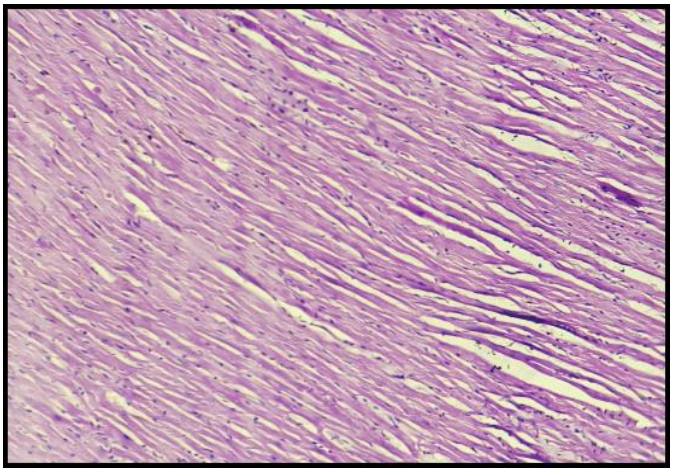

Fig 2: Section of heart from clove oil treated male rats of group III (200mg/kg) showing no pathological alterations in architecture (H & E stain X 120).

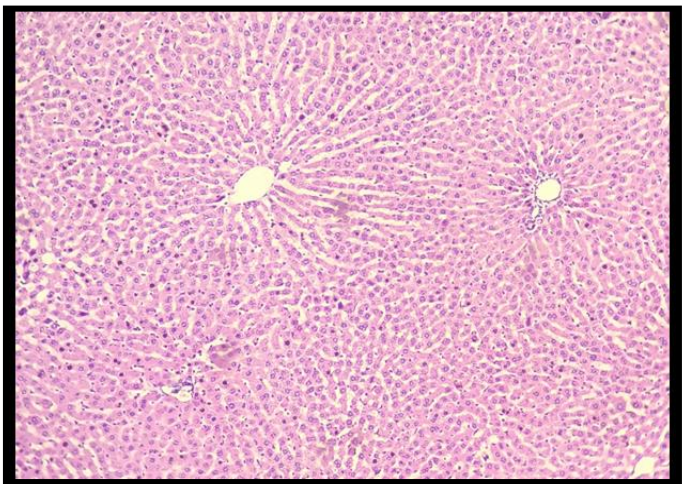

Fig 3: Section of liver from female control rats (group V) showing normal architecture (H & E stain X 120).

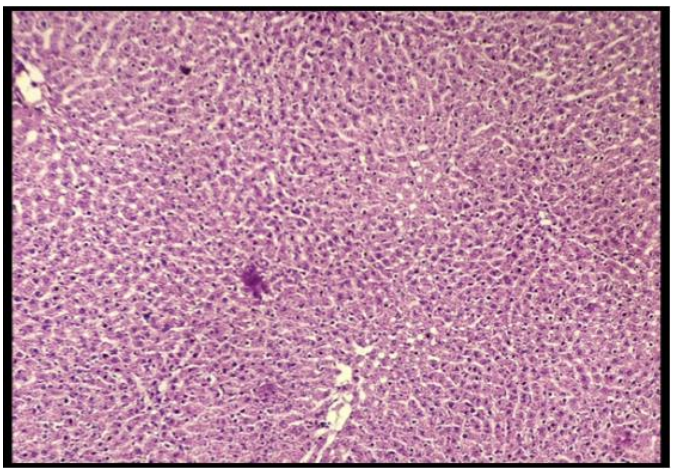

Fig 4: Section of liver from clove oil treated female rats of group VIII (200mg/kg) showing no pathological alterations in architecture (H & E stain X 120).

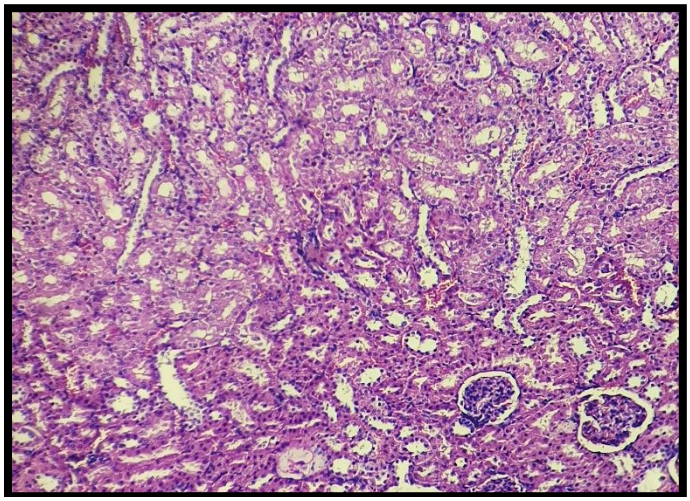

Fig 5: Section of kidney from male control rats (group I) showing normal architecture (H & E stain X 120).

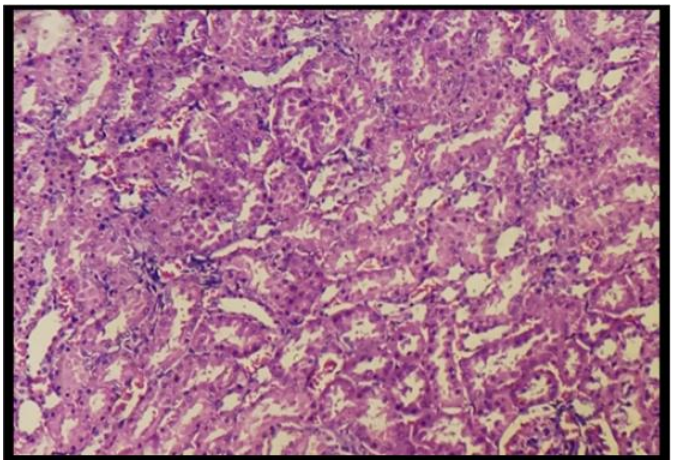

Fig 6: Section of kidney from clove oil treated male rats of group III (200mg/kg) showing no pathological alterations in architecture (H & E stain X 120).

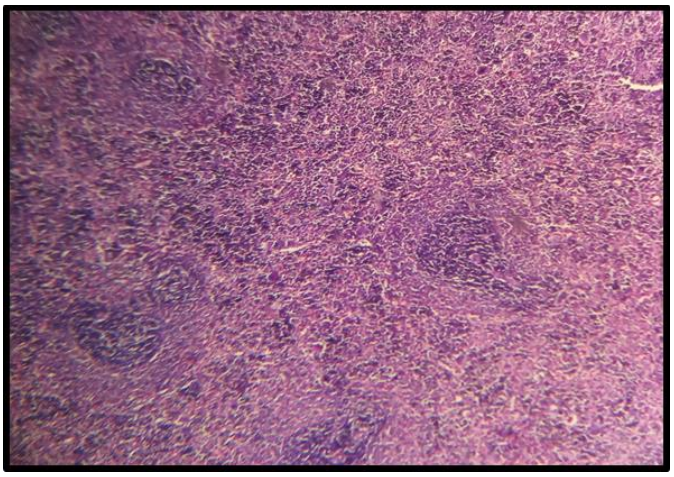

Fig 7: Section of spleen from female control rats (group V) showing normal architecture (H & E stain X 120).

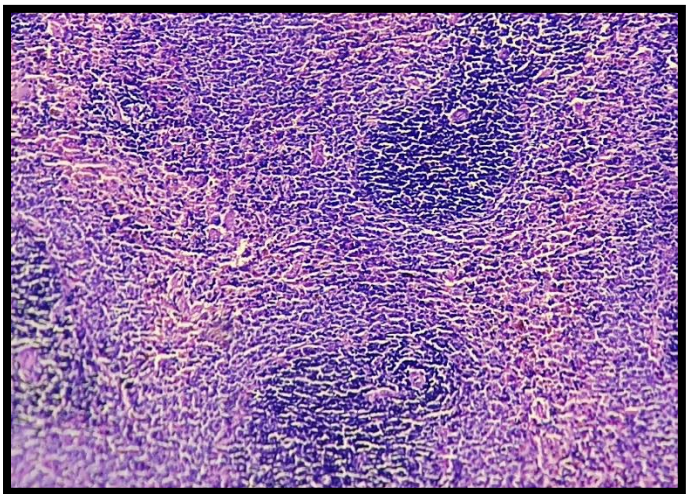

Fig 8: Section of spleen from clove oil treated female rats of group VIII (200mg/kg) showing no pathological alterations in architecture (H & E stain X 120).

4. Discussion

In the present study, no clinical signs or mortality observed in all clove oil treated male and female rats as well as control group rats also did not show any noticeable clinical signs or mortality. Similarly, Sub-acute toxicity studies of clovinol (polyphenol-rich extract of clove buds) at doses of 0.5, 1.0 and 2.5 g/kg b.wt. confirmed that it did not induce any mortality, abnormal clinical or behavior signs [15]. Likewise another safety assessment of a standardized polyphenolic extract of clove buds reported that oral administration of clovinol at 0.25, 0.5 and 1.0 g/kg body weight did not produce any mortality, adverse effects or clinical sign during the 14 days acute toxicity study [16]. Similarly, report safety assessment of ethanolic extract of *Syzygium aromaticum* in albino rats at 250, 500, and 1000 mg/kg showed no mortality or clinical signs in rats [13].

In present study, no significant change in body weight and feed consumption were observed in all clove oil treated male and female rats as compared to control group of male and female rats. Similarly, sub-acute toxicity studies of clovinol at doses of 0.5, 1.0 and 2.5 g/kg b.wt., showed that clove oil treated rats did not induce any significant changes in body weight and feed consumption as compared to that of control group [15]. Another report of safety assessment of *Syzygium aromaticum* flower bud (clove) extract with respect to testicular function in mice @ 15, 30 and 60 mg/kg showed that treatment had no significant effect on the body weight of animals [17]. Additionally similar report of safety assessment of a standardized polyphenolic extract of clove buds daily administered at 0.25, 0.5 and 1.0 g/kg body weight for 90 days showed no significant difference in the weight gain, growth rate and feed consumption among the male and female animals when compared to the untreated control group of animals [16].

The results of present study showed no significant changes in Hb, RBCs, PCV, TLCs, MCV, MCH and MCHC levels in clove oil treated male and female rats as compared to control group of rats. Similar results were reported in sub-acute toxicity testing of clovinol administered at doses of 0.5, 1.0 and 2.5 g/kg b.wt. for 28 days in wistar rats that clovinol did not produce any significant ($p > 0.05$) changes in haemoglobin, WBCs and RBCs counts of treated animals [15]. Another similar report of safety assessment of a standardized polyphenolic extract of clove buds daily administered at 0.25, 0.5 and 1.0 g/kg body weight for 90 days in wistar rats showed that clovinol did not produce any significant ($p > 0.05$) changes in the hematological parameters like hemoglobin, WBCs, RBCs and platelets count[16].Similarly, repeated oral administration of *Syzygium aromaticum* in albino rats at 250, 500 and 1000 mg/kg showed no significant ($P > 0.05$) changes in Hb, PCV, WBCs and RBCs as compared to control rats were also reported [13].

In present study, no significant changes have been observed in serum creatinine level, BUN, AST, ALT, total cholesterol, total bilirubin, total protein and total albumin in clove oil treated male and female rats as compared to control group of rats. Similarly, repeated oral administration of *Syzygium aromaticum* flower bud (clove) hexane extract at the dose of 15, 30 and 60 mg/kg for 35 days in mice showed no significant difference in creatinine, AST and ALT levels as compared to control mice [17]. Similar study of sub-acute toxicity of clovinol at doses of 0.5, 1.0 and 2.5 g/kg b.wt administered daily once for 28 days in both male and female rats showed no significant ($p > 0.05$) change in biochemical parameters like creatinine, urea, hepatic function and cholesterol levels as compare to control group [15]. Another similar safety assessment of a standardized polyphenolic extract of clove buds administered daily once at 0.25, 0.5 and 1.0 g/kg body weight for 90 days in wistar rats showed that treatment group did not produce any significant changes on biochemical parameters related to renal function, hepatic function and cholesterol level in compared to untreated control male and female rats [16]. Additionally another safety assessment of ethanolic extract of *Syzygium aromaticum* in albino rats at 250, 500 and 1000 mg/kg for 21 days in rats showed no significant ($P > 0.05$) changes in the levels of bilirubin, urea, creatinine, AST, ALT, total protein and albumin as compared to control rats [13].

The results of present study showed no significant changes in histopathology of organs like kidney, liver, spleen and heart in clove oil treated male and female rats as well as control group of rats. Similar observations were reported for toxicological effects of essential oil from clove (500, 1000, 1500, 2000, and 2500 mg/b.wt.) on albino rats showed that the structure of the hepatocytes appeared more or less like to be normal but few specimens also revealed slight congestion of the blood vessels in the portal area and associated with inflammatory infiltration [18]. Similar results were also reported for sub-acute toxicity studies of clovinol at doses of 0.5, 1.0 and 2.5 g/kg b.wt. reported that the histopathological examination of various organs of animals treated with 2.5

g/kg b.wt. clovinol showed normal cellular architecture similar to control rats. The liver section of clovinol treated rats showed normal portal triads and central venous system. The tissue sections of spleen from clovinol treated rats showed normal lymphoid follicles with area of prominent in germinal center. The kidney tissues of clovinol treated rats showed normal glomeruli with bowman's capsule and renal tubules [15]. Oral administration of clove oil @ 300 and 600 mg/kg showed no alterations in morphology [19]. Safety assessment of a standardized polyphenolic extract of clove buds at 0.25, 0.5 and 1.0 g/kg body showed normal cellular architecture similar to the control group. The liver section of polyphenolic extract of clove treated rats showed normal portal triads and central venous system, the tissue sections of spleen also showed normal lymphoid follicles and the kidney tissues showed normal glomeruli with Bowman's capsule and renal tubules as compare to control rats [16].

5. Conclusions

The present study revealed that clove oil was found safe following repeated oral administration @ 50, 100 and 200 mg/kg b.wt. for 28 days in male & female wistar rats.

6. Acknowledgements

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7. Conflict of interest

The authors declare that they have no conflict of interest.

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