CHAPTER 1

Acute and sub-chronic toxicological evaluation of *Anethum graveolens* L. seed extract

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INTRODUCTION

Medicinal plants are widely used in traditional medicine and play an important role in the development of novel pharmacological agents (Munari *et al*., 2010; de Azevedo Neta Mahon *et al*., 2014). In recent times, herbal medicine has been gaining wide acceptance. One reason for this trend is the cost of orthodox medicines which put them beyond the reach of many people particularly in resource poor countries (Osujih, 1993; Cohen-Kohler, 2007). World Health Organisation (WHO) stressed its commitment in encouraging the development of public policies with the objective of inculcating herbal medicine in national health system of its member states in its global strategy on traditional, complementary and alter-native medicine, for the period 2014–2023 (WHO, 2013).

The one of the Indian traditional medicinal system is Ayurveda and it has been mentioned even in the ancient Vedas and other scriptures. It has been evolved between 2500 and 500 BC in India and it is based on the use of plants for the remediation (Pandey *et al*., 2013). Approximately 70 percent of rural population has been using traditional Ayurvedic system of medicine. The Ayurvedic practitioners of the traditional systems of medicine prepare formulations by their own recipes and used for healing ailments. Approximately 40 per cent of people are using the herbal medicine for the treatment of various diseases in western countries (Pandey *et al*., 2013).

The herbal drugs, tinctures, extracts, resin, and latex are derived from the plants; they are derived from natural sources and have been used considered as non-

toxic. However, evidences on the toxicity risks associated with a wide variety of such remedies have emerged in the last few years (Jesus *et al*., 2012 and de Azevedo Neta Mahon *et al*., 2014). Hence, it is important that safety assessments should be conducted on natural products for which certain medicinal uses have been scientifically validated (Ernst, 2005 and Saad *et al*., 2006).

Despite the interesting results obtained from the pharmacological studies and their potential therapeutic usefulness, there are no reports on acute and sub-chronic toxicological studies done on AG seed extract. In this study, we have evaluated acute and sub-chronic toxicity of aqueous extract of AG seeds.

MATERIALS AND METHODS

Collection of Plant

The seeds of AG were purchased locally from the market, sown and allowed to grow. Fully grown plants were harvested, identified and authenticated at the Department of Botany, Faculty of Science, The M. S. University of Baroda, Vadodara.

Preparation of Plant Extract

Hundred grams of dried seeds were soaked in 100 ml ethyl alcohol: water (1:1) for 3 days. Resulting filtrate was concentrated by heating to get a semi solid paste that was later freeze dried to obtain a net yield of 6% W/W. Known amount of the extract was weighed and reconstituted in appropriate volume of 0.5% Carboxy Methyl Cellulose (CMC).

Experimental Animals

Adult Swiss albino mice weighing between 20-25 gm were obtained from Zydus Cadilla Research Centre, Ahmedabad, Gujarat, India. They were housed and maintained in clean poly-propylene cages placed in animal house conditions (temperature: 23±2°C; photoperiod: 12 h light and 12 h dark; humidity: 45-50%). They were fed with standard laboratory pellets (M/S Pranav agro, Ltd., Baroda, India) and water *ad libitum*. The experimental protocol was carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) India and approved by the animal ethical committee of Department of Zoology, The M.S. University of Baroda, Vadodara (Approval No.827/ac/04/CPCSEA).

Experimental Design

Acute Oral Toxicity

The acute oral toxicity study was conducted according to the guidelines of Organization for Economic Cooperation and Development (OECD) test 420. Twenty four mice were randomly divided into four groups of six animals in each group. Various doses (1000 mg/kg, 2000 mg/kg, and 3000 mg/kg and 5000 mg/kg body weight, respectively) of seed extract of AG were administered orally to respective groups of mice. Mice were observed closely for toxic symptoms and behavioural changes for two hours after administration and possible mortality was recorded within twenty four hours.

Sub-chronic Oral Toxicity

The sub chronic oral toxicity study was conducted according to the guidelines of OECD Test 407. The mice were divided into four groups of six mice per group. Group I served as normal control (NC) and animals were administered vehicle (0.5% CMC) via gastric incubation. Groups II, III and IV were administered 1000 mg/kg, 2000 mg/kg and 3000 mg/kg doses of aqueous extract of AG respectively via gastric incubation for 28 days consequently. Food and water intake of each group were monitored regularly.

Body Weight

The body weight of each mouse was assessed before commencement of dosing, every 7 days during the dosing period and on the day of sacrifice. The relative body weight (RBW) of each animal was then calculated using the formula RBW= [absolute body

weight of one time interval(g)/body weight of mouse on commencement of dosing day (g)]×100.

Collection of Blood and Autopsy of Tissues

At the end of 28 days, overnight fasted animals were given mild ether anaesthesia and blood was collected into ethylene diamine tetra acetate (EDTA) coated vials by retro orbital sinus puncture. The vials were centrifuged at 3000 rpm for 10 minutes at 4°C and plasma was collected and stored at -80 ºC for biochemical analysis. Animals were sacrificed by cervical dislocation under mild ether anaesthesia and dissected. Liver, kidney, heart, lungs, adrenal and spleen were excised and rinsed with 0.9% saline. All tissues were blotted dried and weighed.

Biochemical Parameters

Plasma levels of Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Acid phosphatase (ACP), Alkaline phosphatase (ALP), Blood glucose, Urea, Creatinine, Total protein, Bilirubin, Lipid profile [Triglyceride (TG), Total cholesterol (TC) and HDL-C] were analyzed using commercially available kits (Recon diagnostic, Vadodara). Very low density lipoprotein (VLDL) and Low density lipoprotein (LDL) were calculated by Friedewald's formula (Friedewald *et al*., 1972).

Hematology

At the end of 28 days, overnight fasted animals were given mild ether anaesthesia and blood was collected into EDTA coated vials by retro orbital sinus puncture for hematological analysis. White blood cell (WBC), Red blood cell (RBC), Haemoglobin (Hb), Mean corpuscular volume (MCV), Mean corpuscular haemoglobin (MCH), Mean corpuscular haemoglobin concentration (MCHC) and

Red cell distribution width (RCDW) estimations were carried out using an automated globular counter (BC 2300 Hematology Analyzer, Mindray)

Statistical Analysis

Statistical analysis of the data was done by one way ANOVA followed by Bonferroni's multiple comparison tests. The results were expressed as mean ± S.E.M using Graph Pad Prism version 3.0 for Windows, Graph Pad Software, San Diego California USA.

RESULTS

Acute Oral Toxicity

The mice treated with the dose 1000, 2000, 3000 and 5000mg/kg body weight did not show any behavioural changes and mortality for 24hrs after administration.

Sub-chronic Oral Toxicity

No remarkable changes were observed in general behaviour or other activities during 28 days study.

Relative Body Weight and Food Intake

No significant alterations were recorded in body weight and food intake (Table 1.1 and Figure 1.1).

Relative Organ Weight

There were no significant changes in the relative weight of Heart, Lungs, Liver, Spleen, Kidney and adrenal gland (Table 1.2 and Figure 1.2).

Hematology

The hematological parameters of experimental and control groups are presented in Table 1.3 and Figure 1.3. The results suggest that all parameters (RBC, WBC, HB, MCV, MCH, MCHC, RCDW, monocytes, lymphocytes, eosinophil and platelets) remained within normal physiological range throughout 28 days.

Biochemical Parameters

The values of different biochemical parameters are showed in Table 1.4 and Figure 1.4. The activities of marker enzymes AST, ALT, ACP and ALP also did not show any significant change. The chronic oral administration of AG extract did not alter the

levels of creatinine, blood glucose, total protein and bilirubin. There were no noticeable changes in lipid profile (TC, TG, HDL, LDL and VLDL).

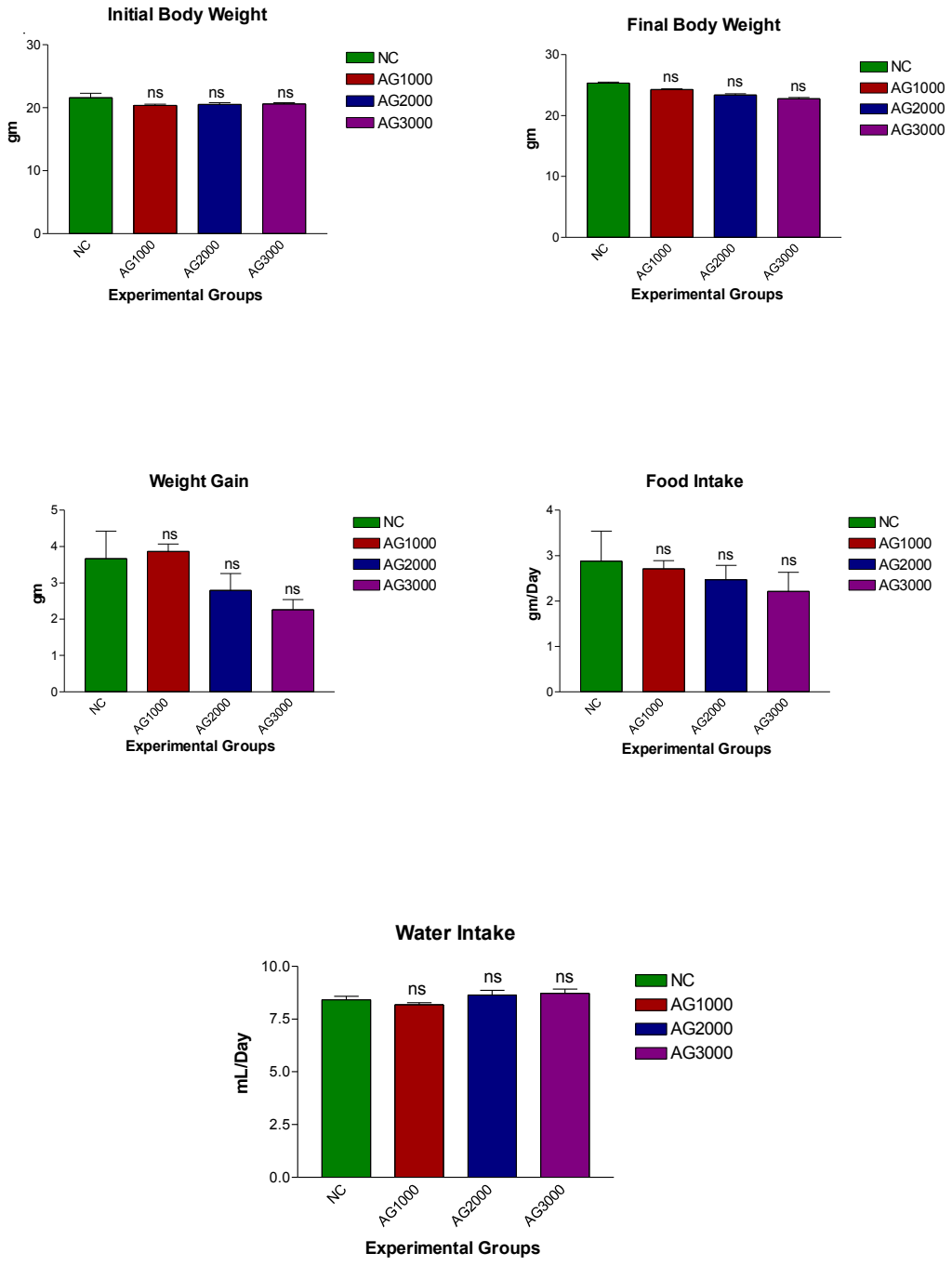
TABLES AND FIGURES

Table 1.1: Effect of *Anethum graveolens* L. Seed Extract on Body Weight, Food Intake and Water Intake

Groups	Body weight (gm)		Weight gain gm	Food intake gm/day	Water intake ml/day
	Initial	Final			
NC	21.60 ± 0.72	25.32 ± 0.17	3.669 ± 0.76	2.88 ± 0.66	8.42 ± 0.17
AG1000	20.36 ± 0.19^{ns}	24.27 ± 0.13^{ns}	3.863 ± 0.21^{ns}	2.71 ± 0.18^{ns}	8.18 ± 0.10^{ns}
AG2000	20.53 ± 0.28^{ns}	23.38 ± 0.18^{ns}	2.798 ± 0.46^{ns}	2.47 ± 0.32^{ns}	8.65 ± 0.21^{ns}
AG3000	20.62 ± 0.20^{ns}	22.75 ± 0.20^{ns}	2.260 ± 0.28^{ns}	2.21 ± 0.42^{ns}	8.72 ± 0.20^{ns}

Where, n=6. Data are expressed as mean ± S.E.M, A ($p<0.05$), B ($p<0.01$) and C ($p<0.001$) and ns: non significant when NC v/s *Anethum graveolens* (AG1000, 2000 and 3000 mg/kg body weight)

Figure 1.1: Effect of *Anethum graveolens* L. Seed Extract on Body Weight, Food Intake and Water Intake



Where, n=6. Data are expressed as mean ± S.E.M, A ($p<0.05$), B ($p<0.01$) and C ($p<0.001$) and ns: non significant when NC v/s *Anethum graveolens* (AG1000, 2000 and 3000 mg/kg body weight)

Table 1.2: Effect of *Anethum graveolens* L. Seed Extract on Relative Organ Weights

Organs (gm)	NC	AG1000	AG2000	AG3000
Liver	4.59 ± 0.01	4.12 ± 0.20^{ns}	4.10 ± 0.10^{ns}	4.05 ± 0.08^{ns}
Kidney	1.47 ± 0.01	1.38 ± 0.05^{ns}	1.29 ± 0.03^{ns}	1.08 ± 0.07^{ns}
Heart	0.63 ± 0.01	0.60 ± 0.02^{ns}	0.54 ± 0.01^{ns}	0.49 ± 0.02^{ns}
Adrenal	0.05 ± 0.003	0.05 ± 0.004^{ns}	0.04 ± 0.007^{ns}	0.04 ± 0.005^{ns}
Lungs	0.63 ± 0.07	0.72 ± 0.06^{ns}	0.70 ± 0.07^{ns}	0.65 ± 0.10^{ns}
Spleen	0.45 ± 0.03	0.73 ± 0.10^{ns}	0.58 ± 0.04^{ns}	0.54 ± 0.03^{ns}

Where, n=6. Data are expressed as mean ± S.E.M, A ($p<0.05$), B ($p<0.01$) and C ($p<0.001$) and ns: non significant when NC v/s *Anethum graveolens* (AG1000, 2000 and 3000 mg/kg body weight)

Figure 1.2: Effect of *Anethum graveolens* L. Seed Extract on Relative Organ Weights

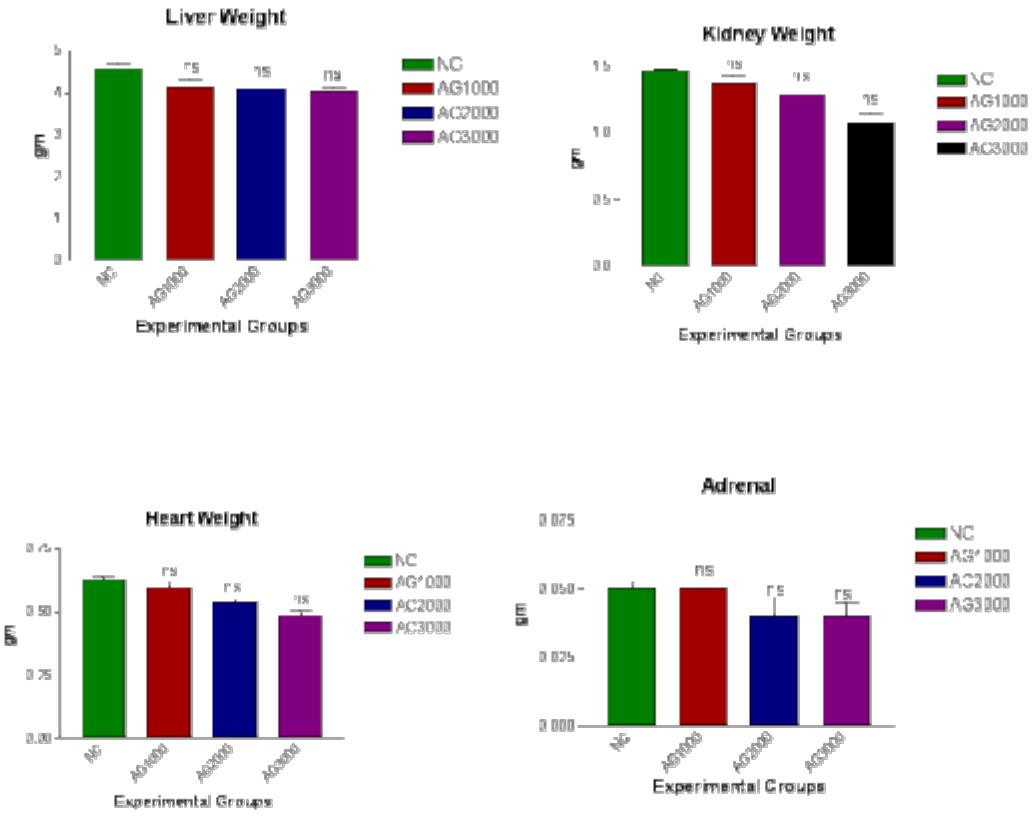


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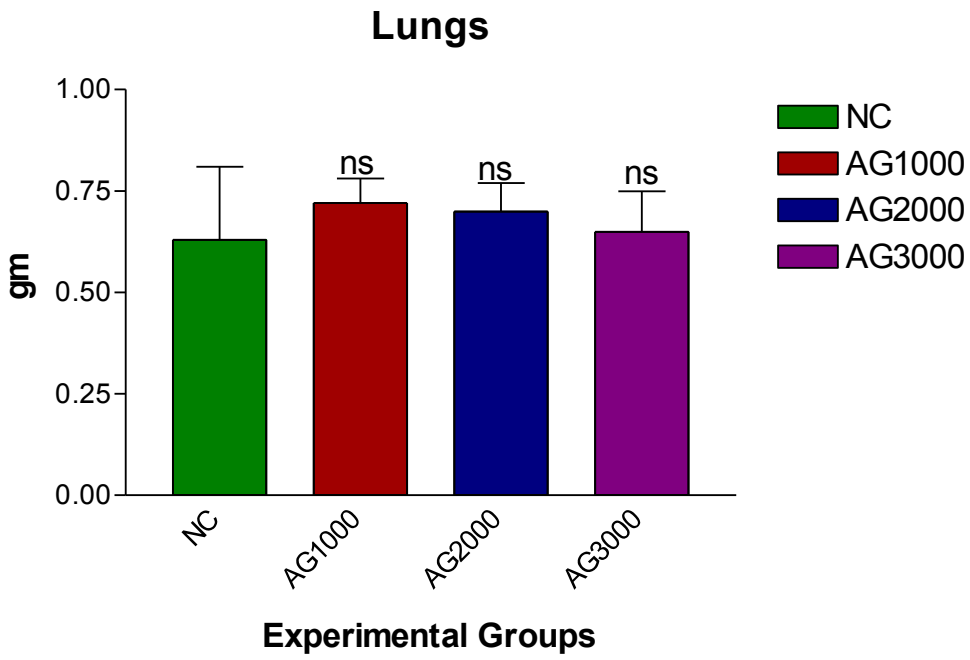
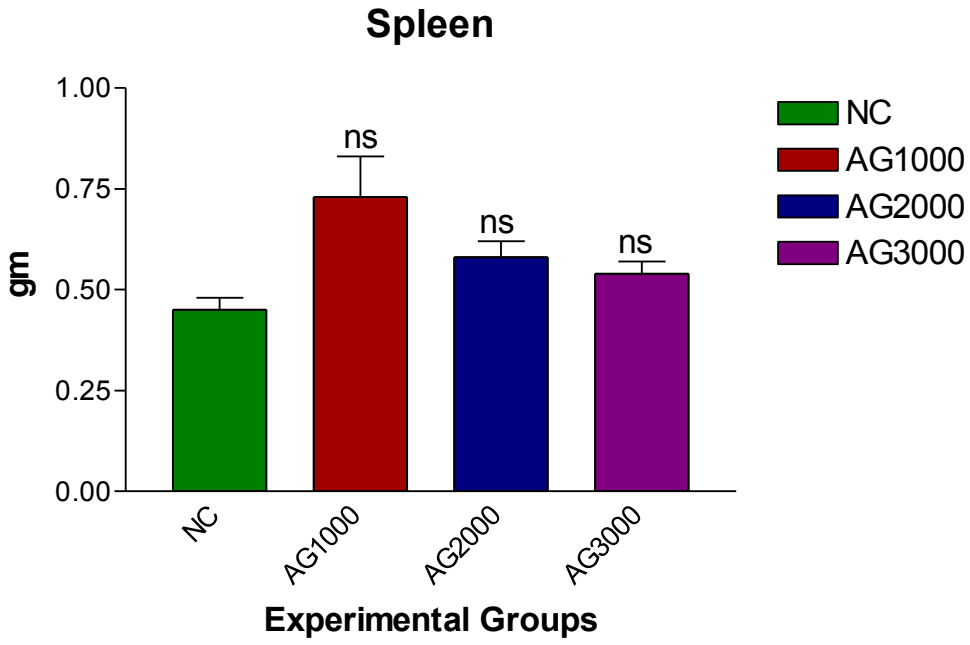
Figure 1.2 (Continued)





Where, n=6. Data are expressed as mean ± S.E.M, A ($p<0.05$), B ($p<0.01$) and C

(p<0.001) and ns: non significant when NC v/s *Anethum graveolens* (AG1000, 2000 and 3000 mg/kg body weight)

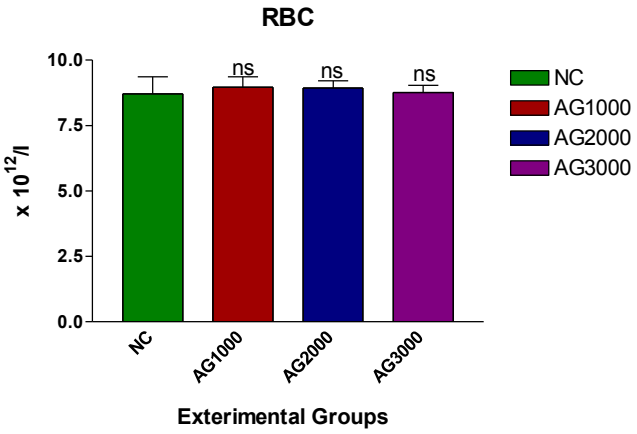
Table 1.3: Effect of *Anethum graveolens* L. Seed Extract on Haematological Parameters

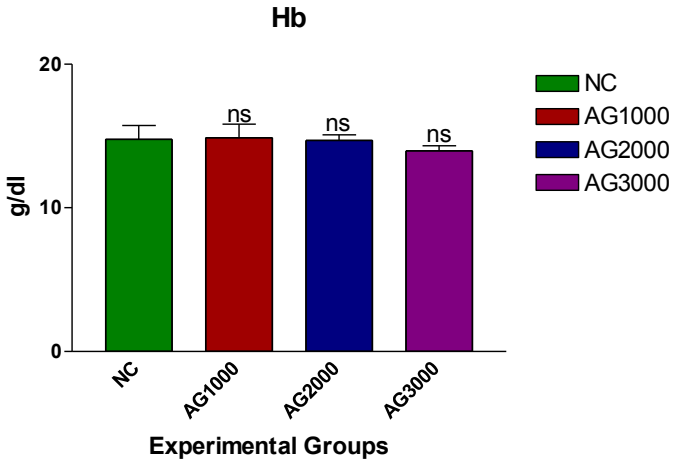
Organs (gm)	NC	AG1000	AG2000	AG3000
RBC[@]	8.71 ± 0.65	8.97 ± 0.39[ns]	8.94 ± 0.27[ns]	8.77 ± 0.27[ns]
Hb[§]	14.78 ± 0.96	14.87 ± 0.95[ns]	14.70 ± 0.40[ns]	13.98 ± 0.34[ns]
MCV[£]	42.83 ± 1.34	41.43 ± 0.53[ns]	41.27 ± 0.32[ns]	41.47 ± 0.13[ns]
MCH[¥]	16.87 ± 0.45	16.90 ± 0.46[ns]	16.65 ± 0.52[ns]	16.56 ± 0.44[ns]
MCHC[§]	39.76 ± 0.49	39.53 ± 0.82[ns]	38.50 ± 0.47[ns]	38.30 ± 0.40[ns]
RCDW[ψ]	17.83 ± 0.83	17.60 ± 0.42[ns]	16.70 ± 0.40[ns]	16.30 ± 0.40[ns]
WBC[θ]	6.03 ± 1.21	6.12 ± 0.64[ns]	4.76 ± 0.57[ns]	4.82 ± 1.07[ns]
Monocytes[ψ]	1.76 ± 0.34	2.10 ± 1.00[ns]	1.56 ± 0.33[ns]	1.42 ± 0.37[ns]
Lymphocytes[ψ]	22.77 ± 4.81	27.67 ± 1.55[ns]	32.00 ± 1.42[ns]	37.33 ± 3.80[ns]
Eosinophils[ψ]	2.30 ± 0.34	2.36 ± 0.33[ns]	3.60 ± 0.57[ns]	3.30 ± 0.57[ns]
Platelet[θ]	580.6 ± 26.49	612.6 ± 51.77[ns]	631.6 ± 27.76[ns]	645.4 ± 28.62[ns]

Where, @ = x 10^{12}/l, § = g/dl, £ = fl, ¥ = pg, ψ = %, θ = x 10^{3}/µl

RBC, red blood cell; Hb, Haemoglobin; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; RCDW, red cell distribution width; WBC, white blood cell. Where, n=6. Data are expressed as mean ± S.E.M, A (p<0.05), B (p<0.01) and C (p<0.001) and ns: non significant when NC v/s *Anethum graveolens* (AG1000, 2000 and 3000 mg/kg body weight)

Figure 1.3: Effect of *Anethum graveolens* L. Seed Extract on haematological Parameters





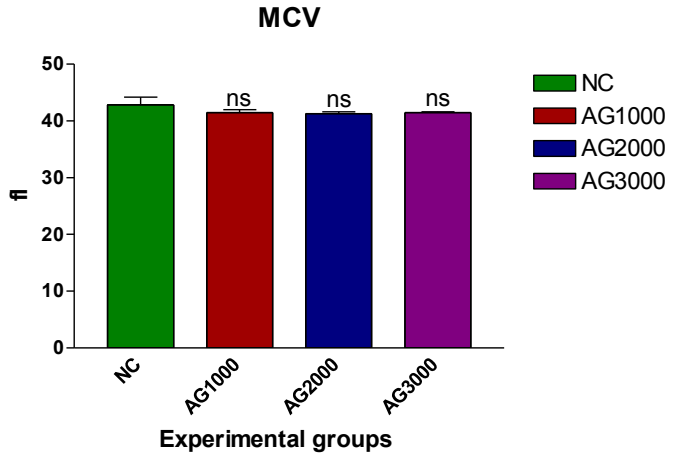


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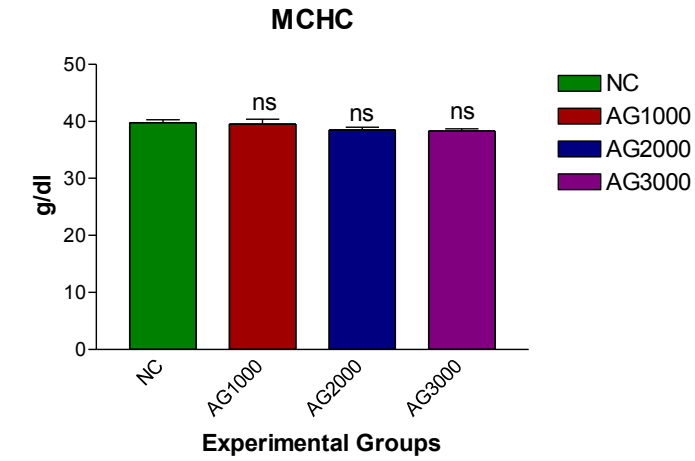
Figure 1.3 (Continued)



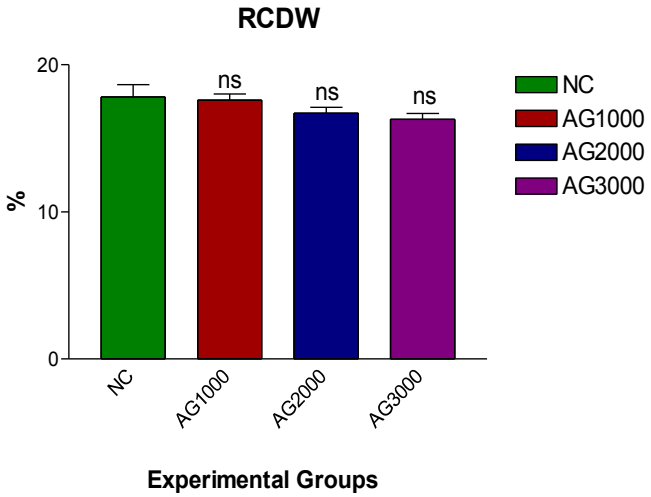


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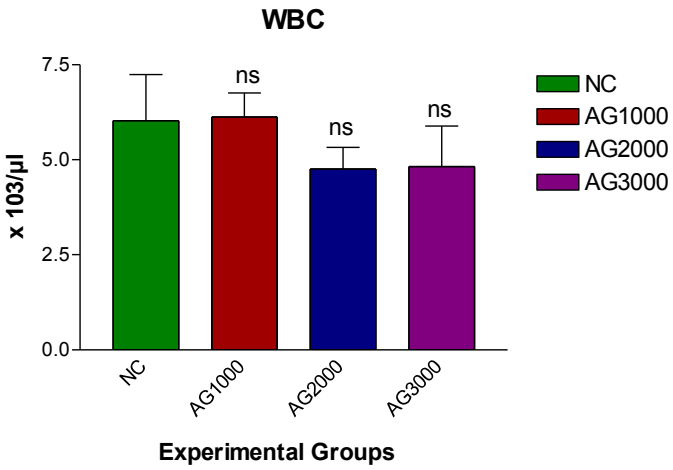
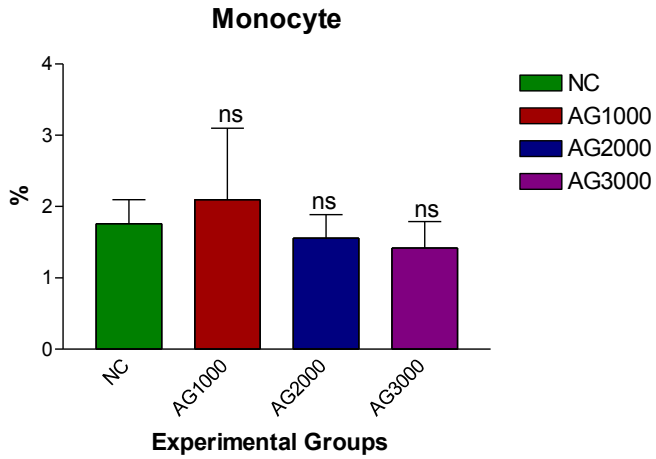
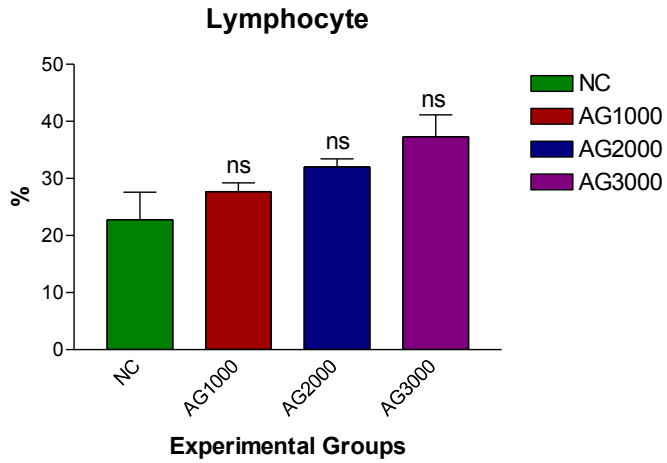
Figure 1.3 (Continued)







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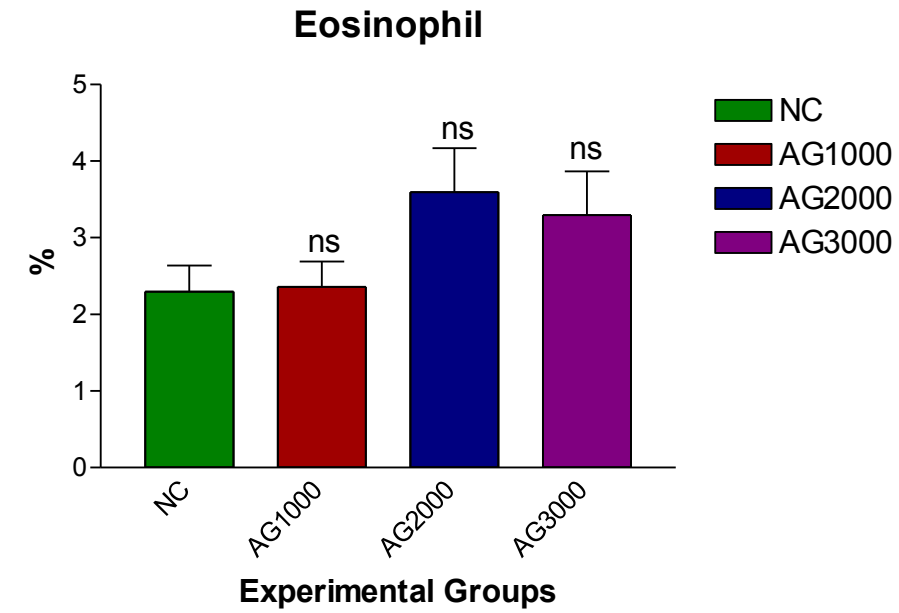

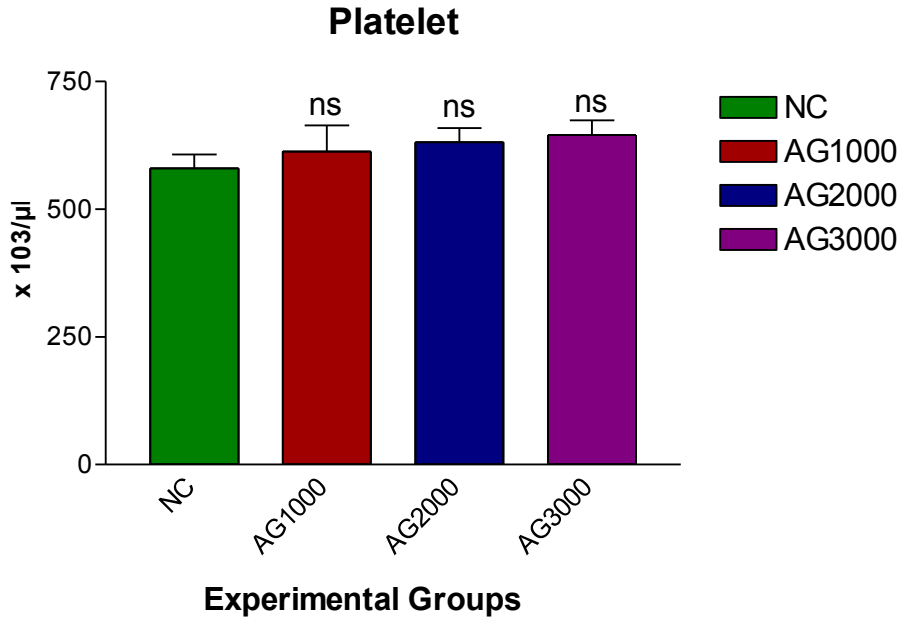

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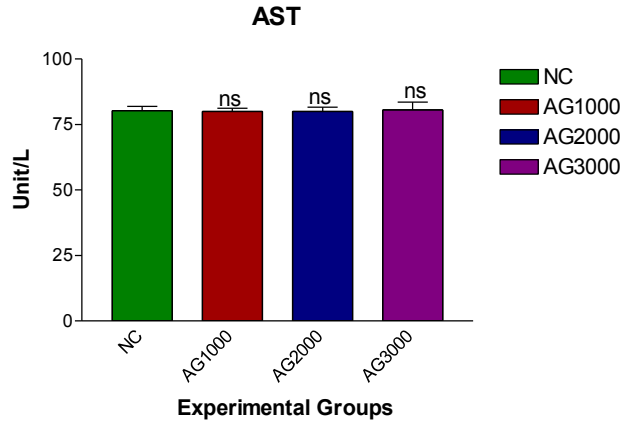
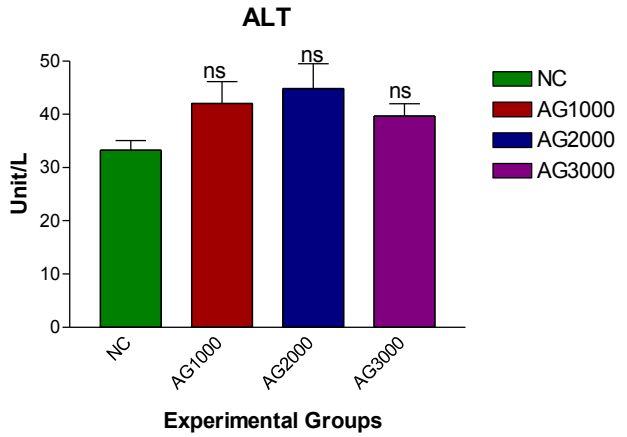
Table 1.4: Effect of *Anethum graveolens* L. Seed Extract on Biochemical Parameters

Organs (gm)	NC	AG1000	AG2000	AG3000
AST†	80.27 ± 1.72	80.00 ± 1.24^{ns}	80.07 ± 1.59^{ns}	80.60 ± 2.91^{ns}
ALT†	33.27 ± 1.78	42.00 ± 4.15^{ns}	44.87 ± 4.65^{ns}	39.66 ± 2.31^{ns}
ACP†	0.30 ± 0.02	0.33 ± 0.03^{ns}	0.37 ± 0.04^{ns}	0.33 ± 0.09^{ns}
ALP†	18.06 ± 0.84	18.2 ± 0.92^{ns}	19.3 ± 1.19^{ns}	19.8 ± 0.76^{ns}
Urea€	71.48 ± 5.56	80.90 ± 2.82^{ns}	84.41 ± 2.43^{ns}	86.35 ± 5.94^{ns}
Creatinine€	0.48 ± 0.02	0.50 ± 0.02^{ns}	0.51 ± 0.02^{ns}	0.63 ± 0.03^{B}
Blood glucose€	169.3 ± 6.54	157.7 ± 7.65^{ns}	165.2 ± 5.72^{ns}	178.50 ± 4.10^{ns}
Total protein§	4.65 ± 0.20	4.62 ± 0.22^{ns}	4.85 ± 0.26^{ns}	4.71 ± 0.26^{ns}
Bilirubin€	0.36 ± 0.05	0.30 ± 0.03^{ns}	0.37 ± 0.05^{ns}	0.32 ± 0.03^{ns}
TC€	66.83 ± 2.93	59.00 ± 5.23^{ns}	60.170 ± 3.99^{ns}	63.00 ± 2.38^{ns}
TG€	103.7 ± 3.70	97.00 ± 5.7^{ns}	109.8 ± 3.48^{ns}	95.50 ± 4.86^{ns}
HDL€	20.75 ± 1.04	24.77 ± 1.48^{ns}	21.65 ± 0.97^{ns}	20.19 ± 1.30^{ns}
VLDL€	20.75 ± 1.04	24.77 ± 1.48^{ns}	21.65 ± 0.97^{ns}	20.19 ± 1.30^{ns}
LDL€	20.73 ± 0.74	19.40 ± 1.14^{ns}	21.97 ± 0.69^{ns}	19.10 ± 0.97^{ns}

Where, † = Unit/L, €= mg/dl, § = g/dl
Where, n=6. Data are expressed as mean ± S.E.M, A ($p<0.05$), B ($p<0.01$) and C ($p<0.001$) and ns: non significant when NC v/s *Anethum graveolens* (AG1000, 2000 and 3000 mg/kg body weight)

Figure 1.4: Effect of *Anethum graveolens* L. Seed Extract on Biochemical Parameters





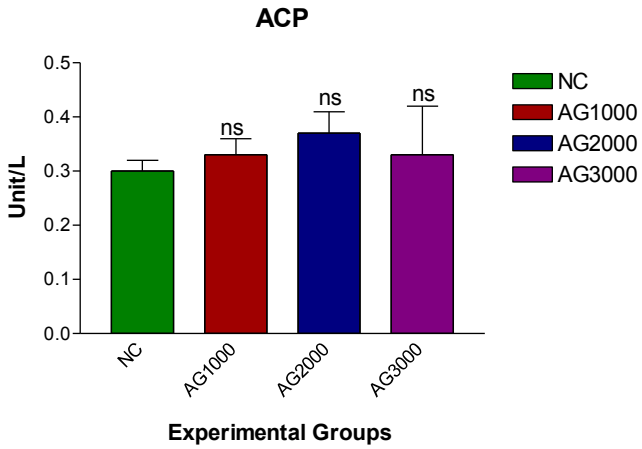


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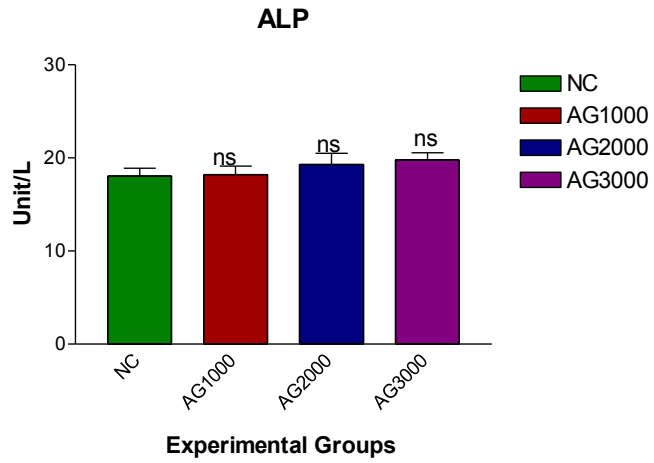
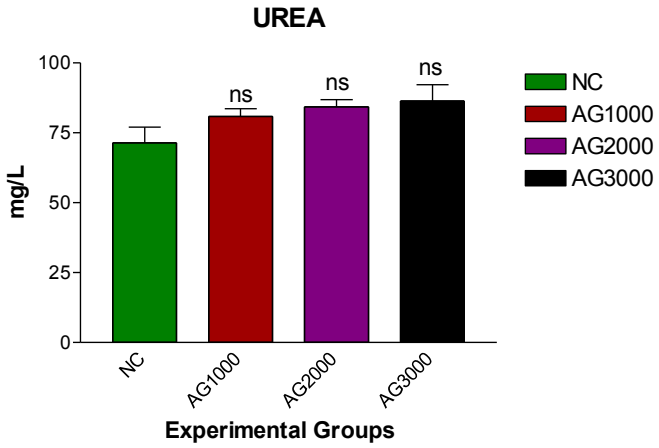
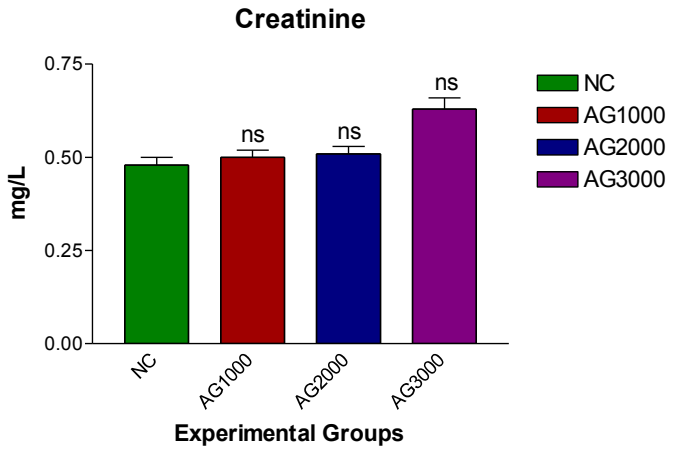
Figure 1.4 (Continued)







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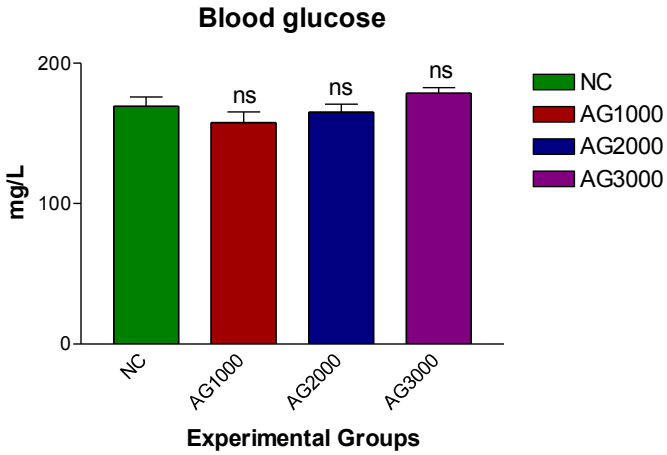
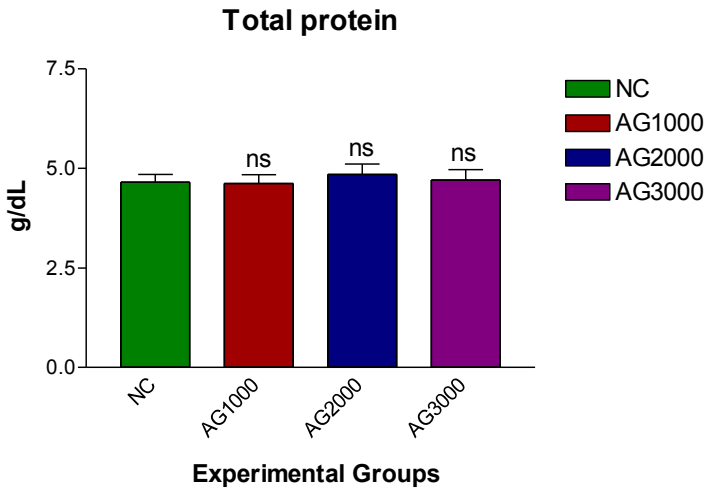
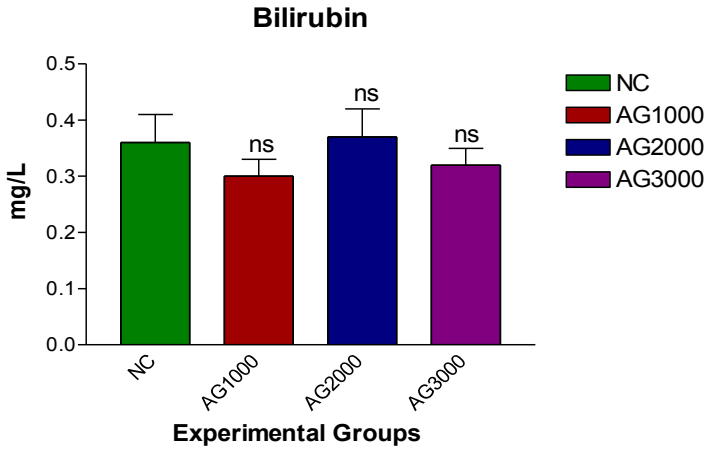
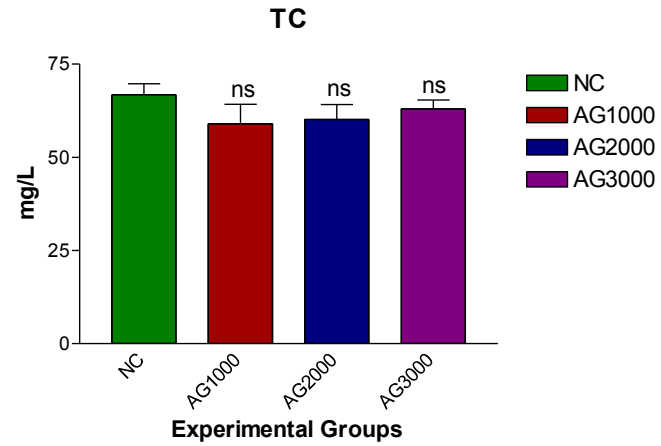


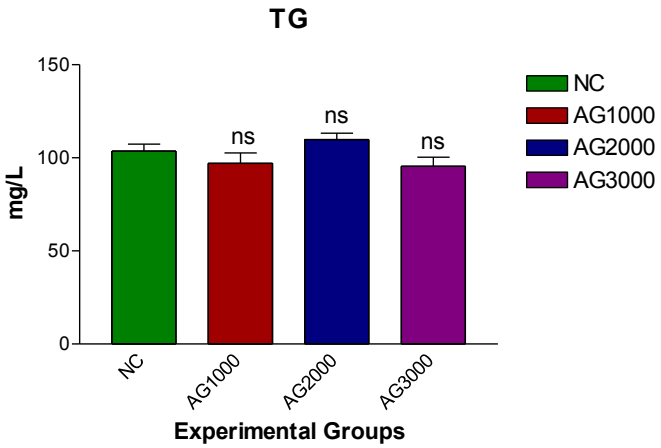


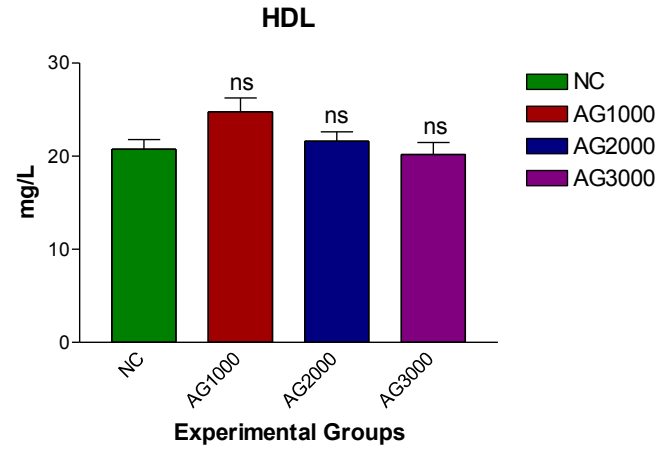


Where, n=6. Data are expressed as mean ± S.E.M, A ($p<0.05$), B ($p<0.01$) and C ($p<0.001$) and ns: non significant when NC v/s *Anethum graveolens* (AG1000, 2000 and 3000 mg/kg body weight)

Figure 1.4 (Continued)

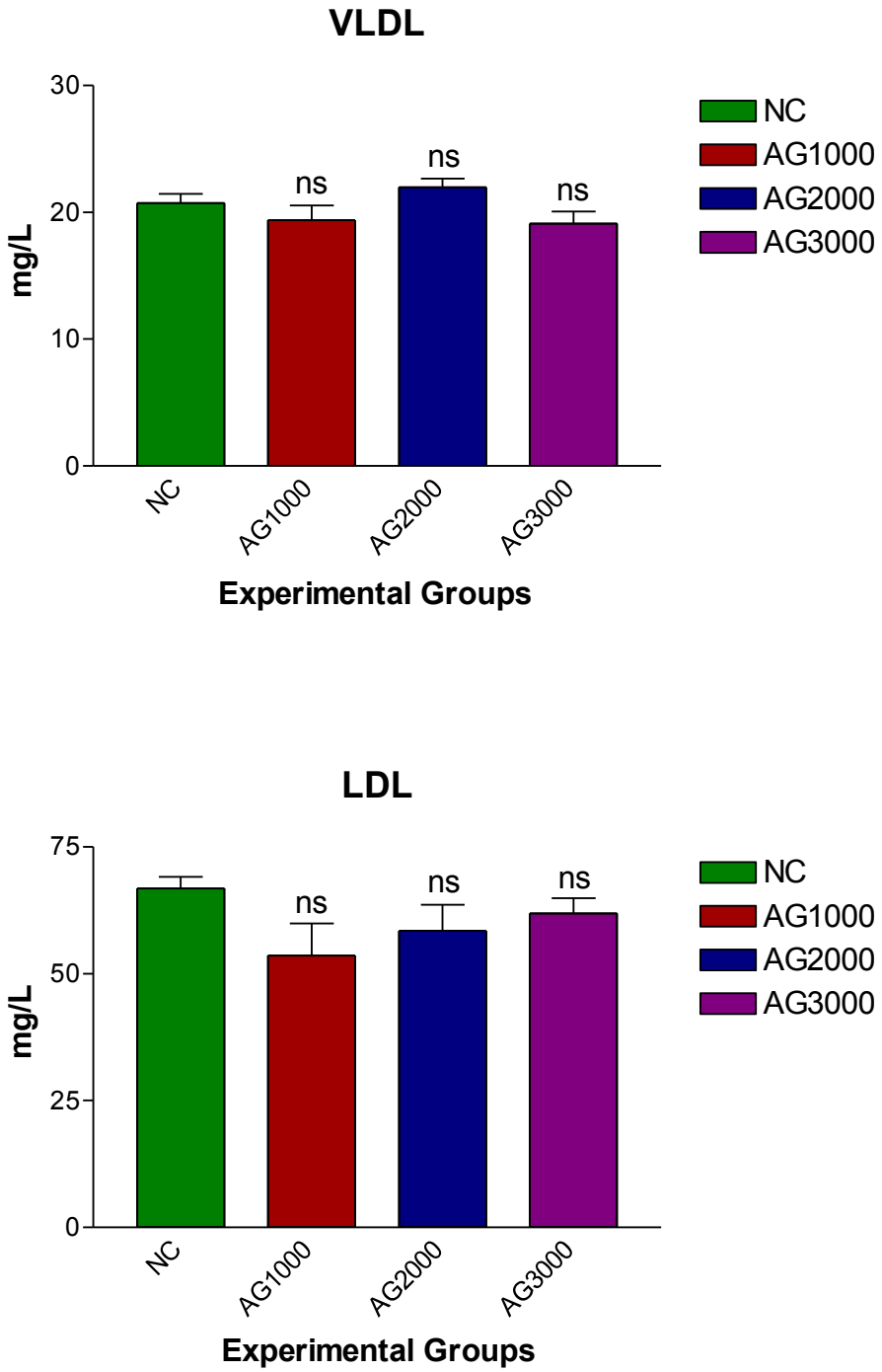






Where, n=6. Data are expressed as mean ± S.E.M, A ($p<0.05$), B ($p<0.01$) and C ($p<0.001$) and ns: non significant when NC v/s *Anethum graveolens* (AG1000, 2000 and 3000 mg/kg body weight)

Figure 1.4 (Continued)



Where, n=6. Data are expressed as mean ± S.E.M, A ($p<0.05$), B ($p<0.01$) and C ($p<0.001$) and ns: non significant when NC v/s *Anethum graveolens* (AG1000, 2000 and 3000 mg/kg body weight)

DISCUSSION

Since ancient times plants have been exemplary sources of medicine. 25 percent of modern medicines are derived from plants and the global market for herbal medicines currently stands over US$ 60 billion annually and growing steadily (WHO, 2009). Increased popularity of herbal medicines might be due to easy access, relative low cost and lesser side effects than synthetic drugs. In present study, we evaluated the possible toxicity of aqueous extract of *Anethum graveolens* L. seeds. This type of study is needed before a phytotherapeutic agent can be generally recommended for use (Abdel- Daim *et al*., 2013).

Acute toxicity evaluation reveals neither behavioural alterations nor mortality. Since AG extract was non-toxic even at 5000 mg/kg body weight, its LD_{50} value can be predicted to be more than 5000 mg/kg body weight. Hence, AG extract can be considered non-toxic and fit for human consumption (OECD guidelines, 1995; Diezi, 1989). Decrement in body and organ weights is co-related with adverse effect of drugs (El-Hilaly *et al*., 2004; Bakoma *et al*., 2011; Tahraoui *et al*., 2010). In the present study, AG extract fed mice did not record any significant alterations in body or organ weights, food and water intake. Hematology is one of the important indices of physiological and pathological status in man and animal (Mukinda and Syce, 2007). In our findings, there were no significant changes in the hematological parameters like White blood cell (WBC), Red blood cell (RBC), Haemoglobin (Hb), Mean corpuscular volume (MCV), Mean corpuscular haemoglobin (MCH), Mean corpuscular haemoglobin concentration (MCHC), Red cell distribution width (RCDW) and platelet counts as compared to control group suggesting non-toxic effects of this plant extract.

AST and ALT are well known enzymes for indication of liver function (Hilaly *et al*., 2004; Ferreira *et al*., 2014) and biomarkers for predicting possible toxicity (Rahman *et al*., 2001). Generally, the elevation of AST and ALT in serum indicates liver damage (Mdhluli, 2003). In the present study, the AST and ALT levels were normal as compared to control group indicating that given doses were non-toxic.

Creatinine is a good indicator of renal function (El-Hilaly *et al*., 2004). The rise in Creatinine level indicates kidney damage (Lameire *et al*., 2005; Palm and Lundblad, 2005). There were no significant changes in Urea and Creatinine levels in this study. A rise in total Bilirubin level in the blood can be caused by liver cell damage (Cheesbrough, 1991). In our finding, there was no significant change in bilirubin level. Decrease in total protein is related to high tissue demands due to liver disease associated with a reduction of protein synthesis (Cheesbrough, 1991). Increase in plasma total protein indicates tissue damage (Emerson *et al*., 1993). Here, with respect to control group non-significant changes were recorded in total protein levels. In conclusion, this study does not show any significant alteration in haematological and biochemical parameters. The acute and subchronic toxicity profiles are considered as non-toxic. This finding forms a base for further *in vitro* and *in vivo* clinical study.

SUMMARY

Anethum graveolens L. (AG) belonging to *Apiaceae* (*Umbelliferae*) family, is an annual aromatic herb known for culinary and medicinal use since ancient times. This study was aimed to assess the toxicity profile of seed extract of AG by determining its effects after administration of acute and sub-chronic doses in swiss albino mice. Swiss albino mice were divided into four groups of six animals in each group. In acute toxicity, study the mice were administered single oral dose of 1000, 2000, 3000 and 5000mg/kg body weight and general behaviour, adverse effects and possible mortality were observed for 24 hours after administration. In the chronic dose study, the extract was administered orally at the doses of 1000, 2000 and 3000mg/kg body weight as treatment groups and Carboxy Methyl Cellulose (0.5%) as control group for 28 days. There was neither adverse effect nor death in treated groups. There were no alterations in organ weight, hematological profile and biochemical parameters. The overall finding of this study indicates that AG extract is absolutely non-toxic and can be considered safe for human consumption as a therapeutic herb.