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Acute toxicity evaluation of mixture of neem *(Azadirachta indica)* and moringa (*Moringa oleifera)* seed oils in rats

Ilesanmi J. O. Y.[1*], Gungula D. T. [2] and Nadro M. S.[3]

[1]Department of Food Science and Technology, Modibbo Adama University of Technology, Yola, Nigeria.
[2]Department of Crop Production and Horticulture, Modibbo Adama University of Technology, Yola, Nigeria.
[3]Department of Biochemistry, Modibbo Adama University of Technology, Yola, Nigeria.

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The toxicity of the mixture of neem and moringa seed oils in the ratio of 1:3 was evaluated based on some biomarkers of liver and kidney functions of Wistar Albino rats. Thirty male Wistar albino rats were randomly divided into six groups of five rats each. Group A served as control. Groups B, C, D, E and F received doses of 100, 1000, 1600, 2900 and 5000 mg/kg body weight of ratio 1:3 neem-moringa seed oil, respectively. The albino rats were observed for any changes for seven days; during this period, they were allowed free access to food and water *ad-libitum.* The rats were weighed and made to fast overnight. The serum obtained was used to determine the serum level of alanine transaminase (ALT), alkaline phosphatase (ALP) and aspartate aminotransferase (AST). Similarly, liver and kidney tissues were removed and homogenized separately in a normal saline in ratio of 1:10 w/v. The homogenate of liver was centrifuged and the supernatant was used to determine total protein and billirubine while that of kidney was used for determining creatinine and urea. The results of all the biochemical parameters tested did not show any significant difference (P>0.05) from the control up to the dose of 5000 mg/kg body weight and did not produce any visible toxic effect. The dosage of 1:3 mixtures of neem-moringa seed oils appeared to be safe for humans.

Key words: Wistar albino rats, acute toxicity, neem-moringa seed oil, safety, biomarker.

INTRODUCTION

Insecticides are classified according to their mammalian toxicity, chemical origin or composition, mode of entry, formulation and their usage in stored grains (Van-Valkenburg, 2000; Ware, 1995). Based on mammalian toxicity, Van-Valkenburg (2000) reported that toxicological studies are conducted to determine the threshold limit

*Corresponding author. E-mail: yemisi_akinyele@yahoo.com.

of a chemical which an animal or a human being is capable of building without significant biological effects. The usual beginning in any toxicological evaluation is the assessment of the acute toxicity that is the effects of a single dosage of the chemical. Botanical insecticides have eco-toxicological advantages compared to traditional synthetic insecticides because they can have favourable eco-toxicological properties (low human toxicity), rapid degradation and reduced environmental impact which make them suitable insecticides for organic agriculture (Zanuncio et al., 2016).

According to a report by the United Nations Environment Program (UNEP) and the World Health Organization (WHO), pesticides are responsible for poisoning around 3 million people and causing about 200 thousand deaths each year world-wide. Such cases of poisoning are reported more in developing countries (95%) than in developed countries (World Health Organization, 1990; Yadav et al., 2015).

Neem oil is a natural occurring pesticide found in the seed of neem tree. It is yellow to brown in colour, has a bitter taste, and a garlic/sulphur smell. Azadirachtin is the most active component and is used for repelling and killing pests. It works as an effective non-toxic insect control agent in agriculture. Neem is considered harmless to humans, animals, birds, beneficial insects and earthworms and has been approved by the United States Environmental Protection Agency (USEPA) for use on food crops and bed bugs. USEPA also reported that based on the data available, it has been determined that there were no unreasonable adverse effects to the American population and the environment when labelled instructions are followed and good agricultural practices are employed. Laboratory studies indicate that the active ingredient is not toxic, following the oral inhalation or dermal exposure (http://draxe.com>neemoil).

Neem oil contains more than a dozen azadirachtin analogs but the major contributor to the insecticidal activity is azadirachtin. The remaining triterpenoids including nimbin, salannin, and their derivatives contribute little to the efficacy (Isman, 2006). Interestingly, neem oil is non-toxic to mammals, birds and fishes and exhibit fewer chances of resistance due to its multiple mode of actions on insect (Chaudhary et al., 2017).

Moringa oil is a nice cooking oil. It is used for deep frying sautéing. Its smoke point is about 200°C which is good for deep frying purpose. Moringa seed oil is preferred as salad oil in many places. Moringa oil is generally safe to use and it can also be consumed internally. Moringa oil is made up of monounsaturated fatty acid (MUFA) and saturated fat. It is exceptionally high in oleic acid (Omega-A). About 70% of the oil is oleic acid. This may lead to blood pressure lowering effect which is similar to what is seen while using olive oil (http://oilhealthbenefits.com/moringa-oil-ben-oil/).

According to O'Brien (2005), the general technique for acute toxicity evaluation is the determination of the LD_{50} (the dosage necessary to produce death or reproducible effect in 50% of the animal population tested). The compound is administered on a weight/weight basis (milligram or gram of compound per kg of body weight of test animals) in a suitable solvent and suspension system. This is evaluated by acute tests, orally (AO) or dermally (AD), chronic oral tests (CO), vapour toxicity tests (VA), chronic vapour tests (VC) and inhalation test (IT) (O'Brien, 2005).

According to Davis and Freed (2000), the insecticides used in stored product treatment is supposed to be of low mammalian toxicity in a formulation that is likely to be effective against the species involved, persistent for the required period of time under given storage conditions and will not alter the flavour, colour and odour of the stored commodity. It was reported on the Nigerian Television Authority (NTA) Channel-7 (2006) that a particular insecticide used in the preservation of cowpea led to the death of some individuals in Lagos, Nigeria which scared people away from buying and consuming cowpea at that period. Also in August 2011 in Gombi Local Government Area of Adamawa state Nigeria, it was reported on the NTA and Adamawa Television (ATV) Yola (local television stations) that a whole family was wiped out after consuming cowpea due to the chemical used in storing the produce. Some people blamed these unfortunate incidences on the fact that the chemical was not allowed to expire before the cowpea was cooked and consumed but the crux of the matter is that the people died of some certain insecticides.

The research conducted by Ilesanmi and Gungula (2010) established that mixture of neem and moringa seed oils in ratio 1:3 at a concentration of 0.5 ml/200 g cowpea recorded over 90% success when used for cowpea storage. Also, the result of cookability and palatability test conducted by Ilesanmi and Gungula (2011) at the end of 180 days of storage of cowpea using 0.5 ml/200 g cowpea revealed that there is no significant difference between the control (untreated) cowpea and the mixture of neem-moringa seed oils 1:2 and 1:3 treated cowpea, suggesting that it was well accepted. But it is not clear yet whether the oil has any toxic effect on mammals, this is therefore the focus of this research. Thus, the objective is to assess the acute toxicity of mixture of neem and moringa seed oils in 1:3 using rat models.

MATERIALS AND METHODS

Plant materials

The neem seeds used for the oil extraction were handpicked in Modibbo Adama University of Technology, Yola, Adamawa State, Nigeria while the *Moringa oleifera* seeds were partly purchased

Table 1. Effect of mixture of neem and moringa seed oils (1:3) administration on the body weights of Wistar strain albino rats.

Treatment (mg/kgbwt)	Body weight (in g)		
	Initial weight	Final weight	% Difference in body weight
Control	64.60±2.17^{d}	80.90±2.07^{c}	25.17±2.04^{a}
100	67.00±1.90^{d}	81.00±2.40^{c}	20.82±3.72^{ab}
1000	72.40±2.30^{c}	83.10±3.00^{c}	14.83±2.27^{c}
1600	85.40±3.07^{b}	99.40±2.20^{b}	16.35±2.75^{bc}
2900	88.60±1.55^{b}	103.40±4.20^{b}	16.75±0.46^{bc}
5000	111.80±4.50^{a}	131.90±4.00^{a}	14.73±2.30^{c}

Results are presented as means ± SEM of five replicates.

from Girei market in Girei Local Government Area of Adamawa State and partly from Kaltungo in Kaltungo Local Government of Gombe State, Nigeria.

Experimental animal

The Wistar strain albino rats for the toxicity experiment were purchased from the Nigerian Institute for Trypanosomiasis Research, Vom, Jos, Nigeria and the cages to house these Wistar strain albino rats were obtained from the Department of Biochemistry, Modibbo Adama University of Technology, Yola, Adamawa State, Nigeria. All the rats were allowed free access to commercially formulated rat feed and water *ad-libitum*. The rats were then allowed to acclimatize for one week.

Extraction of neem seed oil

The processing of neem seed for oil extraction involved cleaning, de-hulling and oil extraction. The cleaning process was dry-cleaning. The seeds were de-hulled with a mortar and pestle and then winnowed. The de-hulled seeds were milled using hammer mill and 1000 g of the resultant powder was used for extraction. The oil was extracted manually using hands to knead the paste with occasional addition of cold water until the oil exuded out. The extracted oil was sieved to remove impurities.

Extraction of moringa seed oil

The dried *M. oleifera* seeds were de-hulled using mortar and pestle, the kernels were then milled using hammer mill and 1000 g of the powder obtained was used for the extraction. The extraction was done manually by kneading the moringa paste with hand with occasional mixing of the paste with water. The extracted oil was then sieved to remove impurities.

Experimental design

The experimental design here was completely randomized design (CRD). Thirty male Wistar strain albino rats aged 6 to 8 weeks weighing 80 ± 20 g were randomly divided into six groups of five rats each. Group A served as the normal control. Groups B, C, D, E, and F received doses of 100, 1000, 1600, 2900, and 5000 mg/kg body weight of ratio 1:3 neem-moringa seed oil, respectively as described by Dietrich (1983). Group A, which is the control were administered water. All dosages were administered orally (intragastically). All the experimental rats were allowed free access to food (commercially formulated rat feed) and water *ad-libitum* after the oil was administered. Their cages were cleaned daily and food and water changed daily. The experimental Wistar strain albino rats were allowed to stay for a period of seven days and were observed frequently from the day of treatment. The nature and time of adverse effect was noted. Observations were carried out for seven days and the experiment terminated. All rats were then weighed.

The Wistar strain albino rats were allowed to fast overnight then blood samples were collected by heart puncture under diethyl ether anesthesia. The blood was left for 15 min and was centrifuged at 3000 rpm for 15 min. The serum obtained was used to determine the serum levels of alanine aminotransferase (ALT), aspartate amino transferase (AST) as described by Reitman and Frankel (1957) and alkaline phosphatase (ALP) as described by Rec. Gscc (DGKC) (1972) using commercial kits (Ramdox Co. Atrium UK). Creatinine was determined according to the method described by Henry (1974), bilirubin was determined according to the method described by Malloy and Evelyn (1937), the serum was also used to determine the serum level of urea and total protein as described by Weatherburn (1967) and Reinhold (1953), respectively using commercial kits (Ramdox Co. Atrium UK). Similarly, liver and kidney tissues were removed and homogenized separately in normal saline in the ratio of 1:10 w/v. The homogenate of liver was centrifuged and the supernatant was used to determine total protein (Reinhold, 1953) and billirubin (Malloy and Everlyn, 1937), while that of the kidney was used for determining creatinine (Henry, 1974) and urea (Weatherburn, 1967). All these were carried out using commercial kits (Ramdox Co. Atrium UK).

Statistical analysis

Analysis of variance was used to ascertain the significant differences between means. Least significant differences (LSD) test was used to compare means that were significantly different at $p<0.05$ using generalized linear model procedure of the SAS/STAT® software Release 9.2.

RESULTS AND DISCUSSION

After the mixture of neem-moringa seed oils (1:3) was administered to the Wistar strain albino rats, it was observed that in the first 2 h of administration, the experimental rats were weak and sleepy. Thereafter, they became very active throughout the experimental period. The effect of mixture of neem and moringa (1:3) seed oils on the body weight of Wistar strain albino rats is presented on Table 1. The percentage average final body

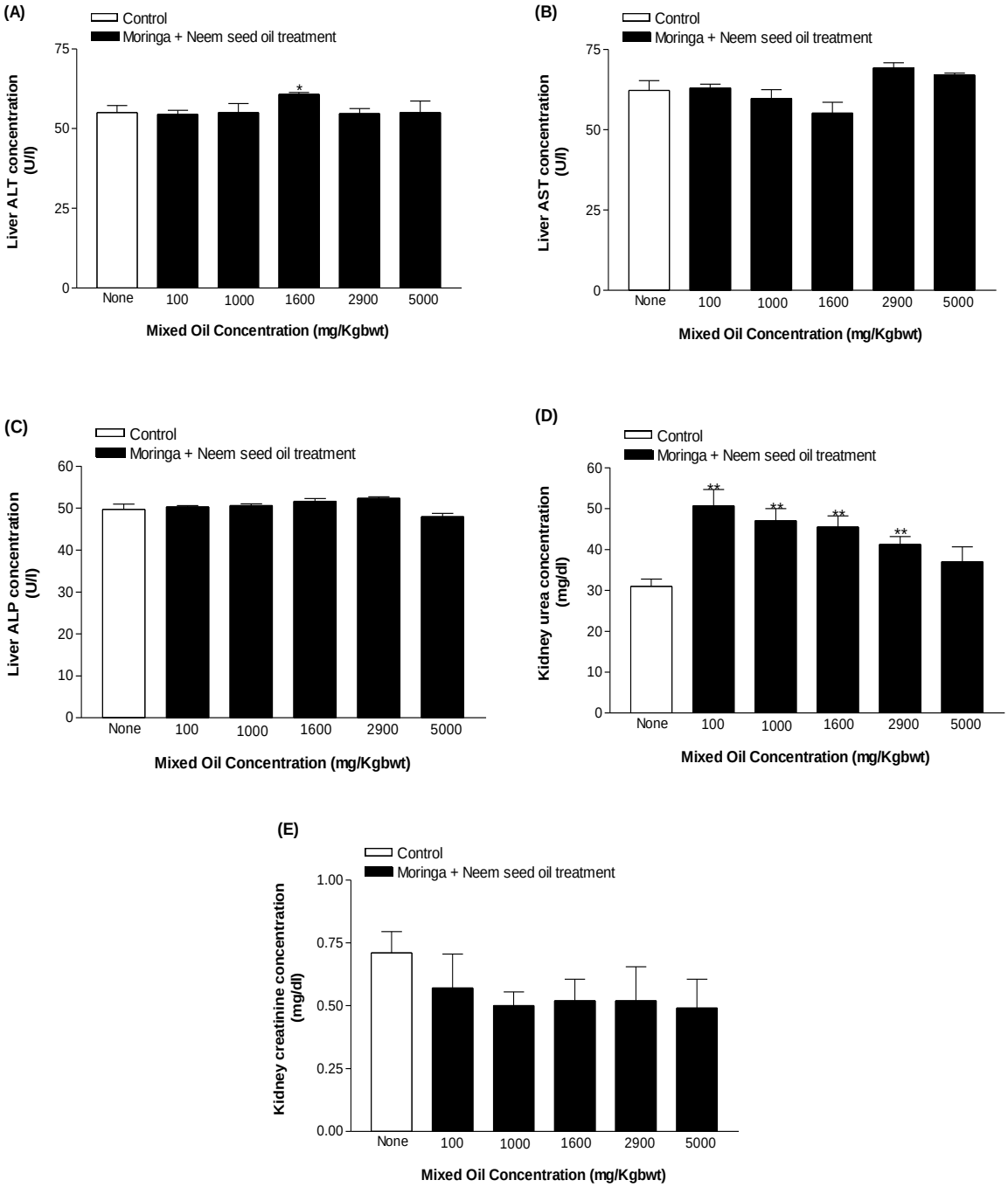

Figure 1. Level of some biochemical parameters in liver and kidney of Wistar albino rats administered with ratio 1:3 neem - moringa seed oils. Values are mean ± SEM with n = 5. *p<0.05 compared to control.

weight gain of the treated albino rats ranged between 14.73 to 25.17%. The highest percentage average body weight gain (25.17%) was recorded for the saline-treated group (control) while the group treated with 5000 mg/kg body weight recorded the lowest percentage of average body weight gain (14.83%). There was a drop in percentage body weight gain in all the groups treated with the mixture of the oils when compared with the control group.

The biochemical parameters in liver and kidney of Wistar strain albino rats treated with the mixture of neem and moringa (1:3) seed oil is presented in Figure 1A to E.

There were no significant differences ($p > 0.05$) in organ ALT, AST and ALP activities of the treated Wistar strain albino rats compared to saline-treated (control) rat at different treatment doses. In the same vein, the creatinine level of the Wistar strain albino rats treated with different doses of ratio 1:3 neem and moringa seed oils are significantly ($p<0.05$) the same. The urea concentration of the group treated with 100, 1000, 1600 and 2900 mg/kg body weight of the oil mixture had no significant difference ($p > 0.05$) from the control. The group treated with 5000 mg/kg body weight of the oil mixture was not significantly different ($p > 0.05$) from the saline-treated group (control). There was also no death observed even at 5000 mg/kg body weight of the experimental rats.

Figure 2A to G presents the results of acute toxicity of mixture of neem and moringa seed oils on the serum biochemical parameters of Wistar strain albino rats. There were no significant differences ($p > 0.05$) in the serum ALT, AST and ALP activities of treated rats and control rats, but there was an insignificant reduction in the serum level of ALT, AST, and ALP, no significant ($p > 0.05$) difference was observed in the level of total protein, bilirubin, urea and creatinine of ratio 1:3 neem-moringa oil-treated albino rats compared to saline-treated Wistar strain albino rats. There was a slight increase in the enzyme activities in serum ALT of groups administered with 100, 2900 and 5000 mg/kg body weight of ratio 1:3 neem-moringa oil but did not differ significantly from that of the control rats.

DISCUSSION

The observation made in the first 2 h of administration of the ratio 1:3 mixture of neem and moringa seed oil to the experimental albino rats is in agreement with the findings in organic facts (http://www.organicfacts.net>oil) that moringa oil aids sleep when taken internally because of its soothing properties. It is known to relax the body and promote a sense of calmness. The finding opined that moringa oil can be used for aroma therapy treatment or topically applied to the chest or temples for sedative effect. It appeared that the percentage weight gain is age dependent, as the rats that are six weeks old (64 to 67 g) appeared to have higher percentage weight gain (20 to 25%) while rats that are 7 to 8 weeks old (72 to 111 g) has lower percentage weight gain (14 to 16%). This low percentage weight gain may be due to their age, as younger mammals tend to grow more rapidly than the older ones. The observation is in accordance with those of Whiteny and Rolfes (1993) who observed that infant rapid growth and metabolism demands an ample supply of all needed nutrients. They also observed that because infants are small, they need smaller total amount of the nutrients than adults do, but when comparison are based on body weights, infants need over twice as much nutrients. With this, they have greater percentage weight gain. It is therefore not clear if the drop in percentage body weight gain is due to the oil administered or the age and weight.

The changes in weight of the Wistar strain albino rats during the period of observation also suggest that the mixture of the oils might have caused an interference in the absorption of nutrients making them unavailable or the intake of the oil might have made them to feel satisfied for some moment thereby reducing their feed intake as it is generally noted that oil or oily foods intake makes one to be satisfied even when less quantity of meal is consumed. This may be due to the fact that 1 g of fat gives 9 kcalories of energy while a gram of protein or carbohydrate gives 4 kcalories of energy (Whitney and Rolfes, 1993). Besides, the percentage weight gain as observed from the study was acceptable over that period of one week. According to Graziela et al. (2011), moringa oil is a monounsaturated oil which is liquid at room temperature. Monounsaturated oil may help lower cholesterol level when used in place of saturated fat and nutritionally its consumption is associated with healthier serum lipid and low cholesterol content of the blood (A Calorie Counter, 2016, www.acaloriencounter.com>diet>mono).

Acute toxicity test gives clues on the range of doses that could be toxic to the animals; it could also be used to estimate the therapeutic index (LD_{50}/ED_{50}) of drugs and xenobiotics (Maikai et al., 2008). The range of ALT (54.5 to 60.8 U/l), AST (55.3 to 69.2 U/l), ALP (48.01 to 52.39 U/l), urea (31.0 to 50.6 m/dl) and creatinine (0.485 to 0.708 mg/dl) activities in the Wistar strain albino rats' organs were observed in the study. The result was all within the range of normal laboratory values reported by Johnson-Delaney (1996): AST (45.7 to 80 Ul), ALP (56.8 to 128 u/l), creatinine (0.2 to 0.8 mg/dl) but for ALT and urea that are not within this range they still showed no significant variation from the control. Davies and Freed (2000) classified insecticides according to their toxicity based on the LD_{50} and said that an insecticide is relatively not toxic if there is absence of lethal death at 5000 mg/kg body weight. $AOLD_{50}$ = 5000 mg/kg body weight, this ratio 1:3 mixture of neem and moringa seed oil can be classified as relatively non-toxic to mammals.

The concentration ranges for ALT (14.60 to 22.60 u/l), AST (20.20 to 41.0 U/l), ALP (48.18 to 51.26 u/l), total protein (3.72 to 5.03 u/l), creatinine (0.30 to 1.09 mg/dl) from this study are all in the range reported by Johnson-Delaney (1996) as normal laboratory values for Wistar strain albino rats biochemical reference range. The results showed no significant difference ($p > 0.05$) between the serum level of ALT, AST and ALP of treated Wistar strain albino rats and the normal. The serum urea level range of (19.20 to 35.60 mg/dl) and that of Billirubin (0.94 to 1.58 mg/dl) are slightly above those reported by Johnson Delaney (1996) but however not significantly different from group treated with normal saline (control).

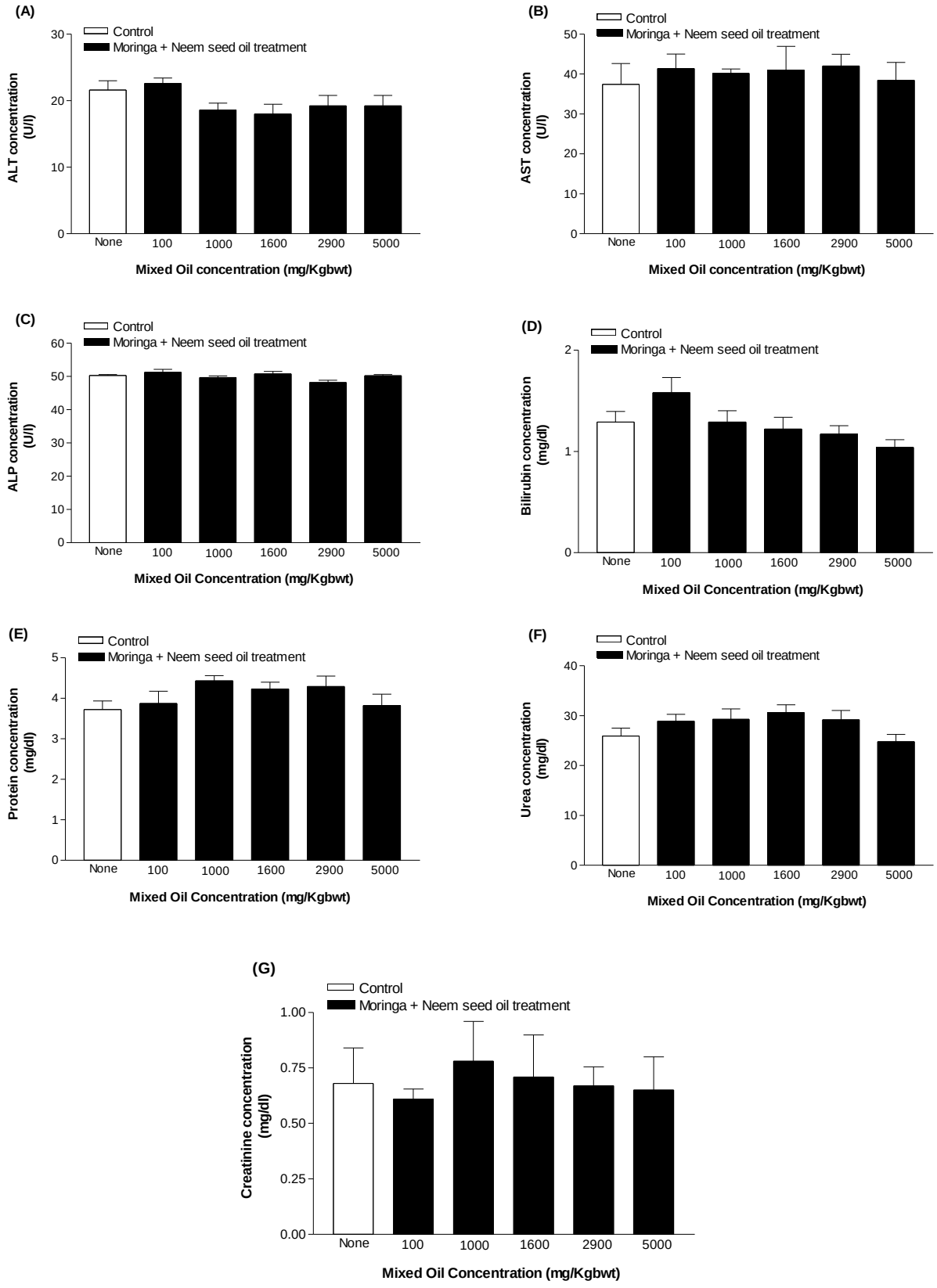

Figure 2. Level of some biochemical parameters in serum of Wistar albino rats administered with ratio 1:3 neem - moringa seed oils. Values are mean ± SEM with n = 5.

The slight reduction in the level of serum AST, ALT and ALP as the dosage increased as shown in the study is in agreement with the finding of Olatosin et al. (2013); that moringa oil helps to lower enzymes markers of liver damage (ALT, AST and ALP) in the serum even at a single dose.

Renal or hepatic injuries as well as major alteration in serum proteins were not induced by the tested oils. This is in line with the result of Graziela et al. (2011) who reported on biological evaluation of crude and degummed oil from *M. oleifera* seeds.

Conclusion

From the observation taken after the administration of the mixture of neem and moringa seed oils to the Wistar albino rats, it was concluded that the mixture of the oils has sedative effects. It is suggested that further investigation be carried out to ascertain if the nature of these oils in ratio 1:3 can be used to treat insomnia. The percentage gain in body weight of experimental rats may not be due to the single dose of the oil administered but the age range of the experimental rats. Since the serum and organ biochemical analysis showed no significant differences in any of the parameter tested even at 5000 mg/kg body weight dosage, then the mixture of these oils may not be toxic. Finally, there was no death, no renal or hepatic injury recorded even at 5000 mg/kg body weight dosage, it is therefore concluded that the mixture of neem and moringa seed oils in ratio 1:3 may not have any adverse effect on humans, and it is suggested that further investigation be carried out to ascertain if a dosage above 5000 mg/kg body weight will be disastrous to albino rats. It is also suggested that the mixture of these oils be used to treat cowpea grains meant for storage against infestation because of its non-toxic characteristics and its insecticidal activities.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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