



Kidney Biomarkers as clue from Toxicological Studies of Methanol Extract of *Syzygium aromaticum* seed on Wister rats

Usman, H.B., Ndatsu, Y., Abubakar, A. and Abubakar, H.

¹Department of Biochemistry, Ibrahim Badamasi Babangida University, Lapai, Nigeria

ABSTRACT

The toxicological administration of methanol extract of *Syzygium aromaticum* (SYZ) seed, commonly known as clove (which has been locally employed for treating gastrointestinal tract diseases and also used as food spices), on some biochemical indices, such as body weight, kidney functions were studied in adult albino rats. Selected doses of 300 and 600 mg/kg were given orally to groups of animals for a period of 29 days, while the control group received deionized water throughout the duration of study via the same route. Blood samples were collected after therapy. Measuring the body weight of study animals revealed that at 300 mg/kg SYZ exerted no significant ($P < 0.05$) change in body weight gain after the 4th weeks when compared with the control. However, the group of animals that received 600 mg/kg showed significant ($P < 0.05$) decrease in body weight gain in week 4th when compared with the control. Kidney function biomarkers also revealed the extract at doses of 300 and 600 mg/kg b.wt did not produce any significant ($p > 0.05$) difference in the serum creatinine, sodium and potassium of treated rats, when compared to the control. However, it showed dose dependent and significant ($p < 0.05$) increase in serum concentration of urea and chloride when compared to the control group. LD₅₀ for the oral routes of SYZ was 6803.33 mg/kg and 1000 mg/kg as established safe dose. Therefore short term administration SYZ is encourages as against a prolong consumption that could raise hepatic challenges.

Keywords: *Syzygium-aromaticum*, toxicity, kidney Biomarkers

INTRODUCTION

Natural plants products have developed into a sophisticated therapeutics, purposely as medicinal, since the time immemorial and their utilizations are of high demand nowadays (Agbaje, Adeneye, & Daramola, 2009; Calixto, 2000). The used of medicine to treat different diseases had become a world's culture (Dibazar, Fateh, & Daneshmandi, 2014). A vast majority of these users depends on this as treatment option because alternative health care options available are more expensive and their safety and efficacy had remained questionable due to associated side effects (Agbaje et al., 2009). Therefore, despite the growing popularity of the synthetic products; due to its production cost, time effectiveness, easy quality control, stringent regulation and quick effects, 80% of the total population in the developing world depends on the wholly or partly natural products because of its inexpensiveness, time tested safety and efficacy (Veeresham, 2012). In Nigeria and other parts of West Africa, natural product remedies still retained a substantial health care

alternative, even in urban areas. The ease of accessibility, relatively cheap cost, makes it an interesting alternative to synthetic drugs. Current data by WHO indicated that 11% of the 252 drugs considered as basic and essential in this 21st century, were exclusively plant origin. Perhaps, the reason more and more attention is now focused on the use of wholly or partly on natural plants product as alternative treatment options. The used of these natural plants products was characterize in the developing nations, by indiscriminate usage mainly by the local populations for the management of various diseases without proper know-how, nor having adequate information on the safety/toxicity risks associated with their usage (Agbaje et al., 2009). Hence, the need for proper guidance and enlightenment of this populace on proper

Received 24 November, 2018

Accepted 19 December, 2018

Address Correspondence to:

ubhamzat@ibbu.edu.ng

dosage as well as the need for professional documentation on the safety/toxicity dosing acclaimed medicinal plants. Such research could advance the utilization of these indigenous natural plants products orthodox treatment and dietary supplement (Parek & Chanda, 2006)

The indigenous medicinal plants in Nigeria and other West Africa form an important component of untapped natural wealth. Spices, a group of aromatically scented natural (herbal) products was widely in use since ancient times as food flavors and preservatives, perfumes and preparing incenses. They constitute an important class of medicinal plants well-practiced in Indian and Chinese traditional systems of medicine (Vijayasteltar *et al.*, 2016). The term “spice” was said to originate from the word “species,” which was applied to class of exotic foodstuffs in the middle ages (Vijayasteltar *et al.*, 2016). Common spices for instance; cinnamon, thyme mint, oregano and clove, have been employed for centuries as medicinal plants mainly due to its antioxidant and antimicrobial activities and above all served as food preservatives (Diego, Claudia-Regina, & Wanderley, 2014). They are reported to have biological properties such as antibacterial, antifungal, antiviral and anticarcinogenic (Cavaleiro, 2018; Diego *et al.*, 2014; Elmastas & Aboul-enein, 2012; Liu *et al.*, 2017; Maharjan *et al.*, 2011).

Amongst them, *Syzygium aromaticum* (SYZ) formerly *Eugenia caryophyllata*, and commonly called Clove, is of particular interest. It is an evergreen tree in the Myrtle family and grows in warm climates but cultivated commercially in Indonesia, India, Malaysia, Sri-Lanka, Tanzania, Sumatra, Madagascar and Brazil (Agbaje *et al.*, 2009; Diego *et al.*, 2014). It is an indigenous medicinal plant in Nigeria, and is broadly utilized for therapeutic purposes in the treatment of heart, kidney and gastrointestinal diseases. Clove is also a popular remedy for dental and respiratory disorders, headache, and sore throat in traditional medicines of Australia, and in Asian countries (Dibazar *et al.*, 2014). Clove as a medicinal plant is reported in many scientific literature to possess biological activities, such as antioxidant, antidiabetic, anti-inflammatory, antimutagenic, antiulcerogenic, antithrombotic and antiparasitic antithrombotic, anesthetic, pain-

relieving, and insect-repellent properties (Cavaleiro, 2018; Diego *et al.*, 2014; Elmastas & Aboul-enein, 2012; Goñi *et al.*, 2016; Gupta, 2010; Liu *et al.*, 2017). The essential oil extract from buds of clove are used for acne, warts, scars and parasites. Phytochemical composition of clove was documented to constitute 70-90 % eugenol as the most bioactive component and remaining 15% comprise of phenolic compound as major vegetal sources comprising flavonoids, hydroxycinnamic acids hydroxybenzoic acids, and hydroxyphenyl propens.

Kidney is the major organ of excretion of drug and metabolite from the system. Some of the toxic effect of drugs manifest clinically in the kidney by either enhanced or reduced reabsorption of electrolyte by the kidney tubules (Rang *et al.*, 2007). The kidney is one of the organs responsible for the maintenance of constant extracellular environment through the excretion of such purine catabolite as urea; creatinine, blood urea nitrogen (BUN) and uric acid and additionally electrolyte balance (Uboh *et al.*, 2009). Abnormal concentration of this catabolite and some electrolyte in plasma or serum is a reasonable sign of renal capacity disability (Zanna *et al.*, 2008). The kidney function might be evaluated from the level of some electrolyte, (for example, Na⁺, and K⁺,) and metabolite, (for example, creatinine, urea, uric corrosive and BUN) in the serum (Atangwho *et al.*, 2007).

But to the best of our knowledge, scanty information on safety of the Seed of *Syzygium aromaticum* exists. Therefore, it becomes imperative to carry out a research on the effect of methanol extract of *Syzygium aromaticum* on kidney function parameter and LD₅₀.

METHODOLOGY

Reagent and Chemicals

Methanol, Normal saline, and DMSO were obtained from Sigma; USA and all other chemicals used were of analytical grade and used as received. Deionized water was used for all the washing, cleaning and preparation of solutions.

Instruments

Electronic weighing balance, Drying oven, water bath, mortar and pestle and electronic blending machines. All the apparatus used were washed with water and detergents and

also rinsed with deionized water before oven drying.

Plant Sample

Fresh samples of *Syzygium aromaticum* was purchase in a Market in Lapai, Niger State Nigeria. This specie was identified by an authenticated Botanist at the Biological science Department of IBBUL.

Animals

Twenty four (24) healthy Wister rats (Male and Female) of average weight 130 ± 20 g were purchased from Department of Biochemistry, Federal University of Technology (FUT), Minna, Niger State Nigeria. The rats were housed in clean plastic pens and kept up under standard research conditions ($23 \pm 2^\circ\text{C}$ temperature, $60 \pm 5\%$ relative humidity, and 12 h light and dark cycle) in the Natural Chemistry lab, FUT Minna till the coveted weight was achieved. They were enabled unhindered access to rodent pellets and water. The investigation was carried out strictly in accordance with the ethical norms for the Care and Use of Laboratory Animals as approved by the Institute of Laboratory Animal Resources, Commission of Life Sciences, National Research Council, USA (Medina *et al.*, 2007)

Sample preparation and extraction

The collected fresh samples of *Syzygium aromaticum* were washed with clean-water, dried at room temperature and finally grounded using a grinder mill and dried at room temperature. Fifty gram (50 g) of the dried powdered plant material was weighed and extracted with 200 ml of Methanol via Soxhlet Extraction. The resulting extract was concentrated in a water bath.

Toxicity Studies

Acute toxicity (LD50)

Acute toxicity study was according to Lorke's method (Chinedu, Arome, & Ameh, 2013). Clove's acute toxicity studies were initially performed to observe the scope of harmfulness with the aim of developing the appropriate dosage. The methanol extract was suspended in deionized water and orally administered as a single dose at specified concentration in a sequential manner. The investigation were directed in two phase. In the principal phase, twelve rats were partitioned into four (4) groups, with each group containing three (3) animals with similar weight (130 ± 20 g).

Group I, II, and III were administered with the extract concentrate at 10, 100 and 1000 mg/kg b.wt respectively, to potentially settled the scope of measurement delivering any poisonous impact, while group IV was the control (untreated). In the second phase, the trial was set up as in the first phase, however, with a higher oral administration of the extract concentrate at 1600, 2900 and 5000 mg/kg b.wt respectively per group.

Sub-chronic study

This investigation was in accordance with the ethics and norms of the Organization for Economic Cooperation and Development (OECD) (Oecd, 1995). Fifteen (15) rats were partitioned into three (3) groups containing five (5) animals with similar weight (130 ± 20 g). Group I and II, were treated with the extract concentrate at dosages of 300 mg/kg and 600 mg/kg b.wt respectively, while group III was the control (untreated). The initial weight of the animals was taken before treatment. The post treatment weights were also measured week by week all through the period 30 days.

Organs and blood Samples collection.

On the 29th day, after treatment with the extracted concentrates of seed of *SYZ*, the animals were sacrificed under light ether anesthesia. They were bleed as grouped via cardiovascular cut into heparinized and EDTA bottles. Plasma was obtained by centrifugation at 3000 rpm and were collected and kept in a bio-cooler (-20°C) for further investigation on the biochemical indices of toxicity. All the organs (kidney, liver, heart and spleen) were separated and weighed after washing in normal saline. The relative organ weight was likewise recorded and calculated.

Body Weight and Body Weight Gain

Singular body weights were recorded at study initiation (day 0), and week by week from that point. Mean body weight gains were calculated for each group at each interval and for the overall (days 0–28) testing interval. Animals were likewise weighed instantly before relinquish (fasted body weight) for calculation of relative organ weight.

Determination of Organ-to-Body Weight Ratio

The organ-to-body weight ratio was determined by taking the weight of the organ

and comparing it with the final body weight of each rat according to the formula:

Organ-to-body weight ratio =

Weight of the organs

Final body weight of animal

Kidney Function Test (Urea, creatinine and electrolyte)

Urea Concentration

A urea test kit (Randox Laboratories Ltd., Crumlin, UK) was used for this investigation. Urease catalyzes the hydrolysis of serum/plasma urea to ammonia. The ammonia is measurable photometrically by Berthelot's reaction (Weatherburn, 1967). The content of the kit is R1_a (Sodium nitroprusside and urease solution), R2_a (40 ml of R2 (phenol) in 280 mL of dH₂O), R3_a (10 mL of R3 (Sodium hypochlorite) in 340.9 mL of dH₂O) and standard. 10 µL of each sample was mixed with 100 µL of R1_a, while 10 µL of standard and 10 µL of dH₂O with 100 µL were mixed for standard and blank respectively, these were incubated for 10 min at 37°C. Thereafter, 2.50 ml of R2_a and 2.50 ml of R3_a was added and incubated for additional 15 minutes. Absorbance of standard A and sample A were read against the negative control. The concentration of Urea was calculated as follows:

$$\text{Urea Conc.} = \frac{\text{Sample A} \times \text{Standard Conc.}}{\text{Standard A}}$$

(Urea standard conc. = 80.5 mg/dl)

Creatinine Concentration

Standard creatinine test kit (Randox Laboratories Ltd., Crumlin, UK) was involved in this investigation. The formation of coloured complex when creatinine (in alkaline solution), reacts with picric acid serves as the bases of this investigation. The intensity of the coloured complex formed is directly proportional to the creatinine concentration (Bartels, Böhmer, & Heierli, 1972). The content of kit is R1_a (picric acid), R1_b (sodium hydroxide) and creatinine standard. The reagent for experiment contained equal volumes of R1_a and R1_b. 100 µL of plasma, 100 µL of creatinine standard and 100 µL of distil water were separately placed in different test tubes and 1000 µL of working reagent was added to each tube. The content of each tube were mixed gently and absorbance A₁ sample

and A₁ standard were read at 492 nm against blank after 30 sec. Thereafter, 2 min of initial reading, Absorbance A₂ sample and A₂ standard were also read. The concentration of creatinine was calculated as follows:

$$\text{Creatinine Conc.} = \frac{\Delta A \text{ sample} \times \text{Standard conc.}}{\Delta A \text{ standard}}$$

(Standard Creatinine Conc. = 1.97 mg/dL)

Potassium

Turbid suspension of potassium tetraphenylboron is produced when Potassium ions react with sodium tetraphenylboron in a protein-free alkaline medium. The intensity of turbidity is proportional to the potassium concentration. Fifty (50) µL of serum sample was mixed with 500 µL of the precipitating reagent. The solution was centrifuged at 4000 rpm for 10 min. The supernatant was carefully decanted and kept. 100 µL of every serum sample was transferred into a pre-labeled corresponding test tube to which 1.0ml of the working reagent was already added. 1.0 mL of the working reagent (equal amount of sodium tetraphenylboron and sodium hydroxide) was also added to each of the standard and blank tubes. 100 µL of potassium standard was added into the test tube labeled standard; while a corresponding volume of water was added to the blank. The content of each tube was vortexed and kept for 5 min (room temperature). The absorbance (A) was read at 600 nm wavelength against reagent blank.

$$\text{Potassium conc.} = \frac{A \text{ sample} \times 5.0 \text{ Mmol/L}}{A \text{ standard Chloride}}$$

Chloride ions

Mercurious thiocyanate reacts with Chloride ions to form thiocyanate and mercury perchlorate. The thiocyanate then forms a red colored complex together with ferric chloride in the presence of nitric acid. The intensity of the color complex is directly proportional to the chloride concentration. Test tubes were arranged corresponding to the number of serum samples. Other Two test tubes were set for the standard and blank. 10 µL of each sample was pipette into the appropriate test tube. Correspondingly, 10 µL of chloride standard (100 mmol) was added into the standard tube while the blank contained 10 µL of distilled water. At the end, 1000 µL of the working reagent (thiocyanate reagent containing a mix together of ferric III nitrate,

nitric acid, ammonium chloride, mercury nitrate and ammonium thiocyanate) was added to each tube. The contents of each test tube were mixed together and incubated for 5 min at 37°C in a water bath. The absorbance of the sample and standard were measured against reagent blank at 460 nm wavelength.

$$\text{Urea Conc.} = \frac{\text{Sample A} \times \text{Standard Conc.}}{\text{Standard A}}$$

(Urea standard conc. = 80.5 mg/dl)

Statistical Analysis

Values were analyzed using statistical package for social science (SPSS) version 16 and presented as means \pm SE of the mean. Comparisons between different groups were carried out by one way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT). The level of significance was set at $P < 0.05$ (Adamu & Johnson, 1997).

RESULTS AND DISCUSSION

Table 1: Acute Oral Toxicity report of methanol extract of *Syzygium aromaticum*

Group	Dosage (mg/kg)	Mortality/Toxicity
1	10	ND, NST
2	100	ND, NST
3	1000	ND, NST
4	1600	No death, animals were
5	2900	Restless
6	5000	Animals were restless/ hypercardia. One death, other animals were very weak/erythremia

Note: ND = no death NST = no sign of toxicity

From the equation $Y = 0.009x - 11.23$. (At $Y=50$, $LD_{50} (X) = 6803.33$ mg/kg b.wt)
Selected safe dose = 1000 mg/kg b.wt

Historically plants have given a wellspring of motivation to novel medication mixed as plant inferred prescriptions have made expansive commitment to human health and prosperity. The oral LD_{50} *Syzygium aromaticum* was observed to be > 5000 mg/kg. Scientifically plants are examined for LD_{50} values to establish their safety. The outcome for this study showed that the oral LD_{50} are within safety margins. This recommends the extract is safe for utilization.

This research work assessed the sub-acute effect of methanol extract of *SYZ* on the serum creatinine, urea and electrolytes levels in treated rats. The measurements utilized as a part of this investigation depended on the acute toxicity results obtained in this research. The result of the research showed that the extract has no antagonistic impact on kidney at the doses used and for the period of the experiment, when compared to the negative control. Increasing the dosage may inspire some unfavorable impacts. Toxicity relies upon the dosage of the intoxicant (Braide & Anika, 2007).

Body Weight Changes

Table 2 showed the changed in body weight of animals investigation of *SYZ*. At 300 mg/kg *SYZ* exerted no significant ($P < 0.05$) change in body weight gain after the 4th weeks when compared with the control. However, the group of rat that received 600 mg/kg shows significant ($P < 0.05$) decrease in body weight gain in week 4th when compared with the control. Rat administered. *SYZ* at 300 and 600 mg/kg had weight gain of 22.63 g and 13.04 g respectively, against the higher weight gain of 24.25 g observed in the control group.

Table 2: Change in body weight of rats administered methanol extract of *Syzygium aromaticum*

Groups	Doses (mg/kg)	Week 1 (g)	Week 2 (g)	Week 3 (g)	Week 4 (g)	Weight Gain
I	300	133.06 \pm 4.08	140.03 \pm 4.21	149.31 \pm 2.64	155.32 \pm 3.42	22.63
II	600	126.56 \pm 3.45	130.13 \pm 3.46	138.57 \pm 3.46	139.60 \pm 4.57	13.04
Control	0.0	125.67 \pm 2.34	133.46 \pm 2.45	149.92 \pm 3.16	149.92 \pm 2.79 ^b	24.25

Values are mean \pm SEM of 3 determinations.

Organs Body Weight Ratios

Table 3 shows the effect of sub chronic administration of methanol extract of SYZ on

organ body weight ratios in rats. The kidney, liver and heart body weight ratios were not altered by all the doses of the extracts tested.

Table 3: Sub-chronic effect of methanol extract of SYZ on organs weight/body ratios in rats

Dosage Groups	Kidney	Liver	Heart	Spleen
Control	0.0062±0.00 ^a	0.0032±0.00 ^a	0.0038±0.00 ^a	0.0034±0.00 ^a
300mg/kg	0.0064±0.00 ^a	0.033±0.00 ^a	0.0039±0.00 ^a	0.0039±0.00 ^a
600mg/kg	0.0067±0.00 ^a	0.033±0.00 ^a	0.0037±0.00 ^a	0.0037±0.00 ^a

Values are mean ± SEM of 3 determinations. The Values at same column with different superscripts are significantly different ($p < 0.05$).

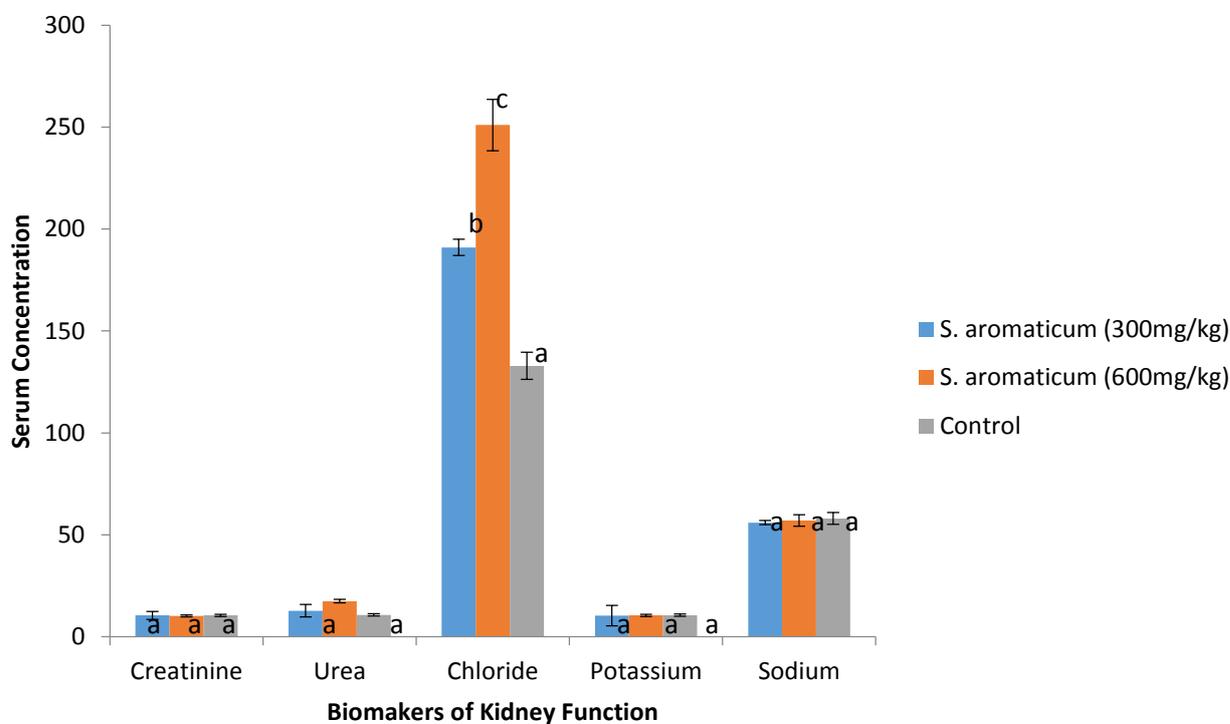


Figure 1: Effect of sub-chronic administration methanol extract of SYZ on kidney biomarkers

Organ body weight ratio was regularly investigated to check changes in the organ weight in relation to the weight of the entire animal (Shittu *et al.*, 2015). The nonappearance of an impact on the computed kidney, liver and heart/body weight ratio may suggest that the extract did not cause any type of swelling, decay and hypertrophy on the organs (Berinyuy *et al.*, 2015). This finding concurs with the investigations by Olaniyan, *et.al.*, (2015) who detailed that administer of crude aqueous extract of *N. campestris* at

dosage of 100, 300 and 600 mg/kg for 28-days did not inspire any pernicious impact on the weight of kidney, liver and heart.

Kidney function Biomarkers

The effect of methanol extract of SYZ on the urea, creatinine, serum, and electrolytes of the treated animals was also investigated (figure 1). The extract at doses of 300 and 600 mg/kg b.wt did not produce any significant ($p > 0.05$) difference in the serum creatinine, sodium and potassium of treated rats, when compared to

the normal control. However, it showed dose dependent and significant ($p < 0.05$) increase in serum concentration of urea and chloride when compared to the normal untreated animals.

The serum creatinine, blood urea nitrogen and electrolyte are used to assess the function of the kidney (Sunheimer & Graves, 2011). Creatinine is metabolic waste product of creatine and creatine phosphate. Its endogenous production is constant and directly proportional to muscle mass of the individual. Serum level is an index of renal function and measures the glomerular filtration rate (Onoja *et al.*, 2015). Creatinine is promptly filtered and does not experience any huge tubular reabsorption. The after effect of the impact of methanol extract of *Syzygium aromaticum* on serum creatinine may recommend that the extract has impact on the kidney function and glomerular filtration rate, thus the formation or excretion of urine is not impaired (Brar, Sandhu, & Singh, 2011). Urea is the real nitrogen-containing metabolic waste product of protein catabolism in mammals. It is formed from exogenous or endogenous protein from the breakdown of cell in the body (Brar *et al.*, 2011). Over 90% of the urea is excreted by the kidneys and the rest is lost through the gastrointestinal tract and skin. The consequence of the impact of methanol extract of *Syzygium aromaticum* on serum blood urea nitrogen level of the treated rats may recommend that the extract has impact on the kidney function in regard of protein catabolism.

In the present investigation, the concentrate produced no significant ($p > 0.05$) difference in all the serum potassium and sodium levels as when contrasted with the control. This may be an indication that the concentrate might not have any electrolyte reabsorption, secretion and diuretic impact in the kidney tubule. These findings are in concurrence with the investigations by Onoja *et al.*, (2015), who detailed that administration of spice (Alligator pepper) at dose of 100, 200 and 400mg/kg significant ($p > 0.05$) difference in serum creatinine, BUN and electrolytes level in the treated animals when compared with the control. However, the significant increment in the serum concentration of chloride following administration of concentrate of SYZ indicated that the normal function of kidney with respect to it is metabolite had been traded off.

Conclusion

The methanol extract of *Syzygium aromaticum* do not show any undesirable effect on the kidney at the dosage utilized for the time of research, yet may show toxic impact at higher dosage or potentially more drawn out period treatment. Therefore, there is need for further investigation on long-term effect of *Syzygium aromaticum*. Prolonged consumption of *Syzygium aromaticum* at higher dosage should be discouraged, since it may be toxic to the kidney..

REFERENCES

- Adamu, S. O., & Johnson, T. L. (1997). *Statistics for Beginners*. Ibadan, Nigeria,: SAAL Publication.
- Agbaje, E. O., Adeneye, A. A., & Daramola, A. O. (2009). Biochemical and Toxicological studies of aqueous extract of *Syzygium Aromaticum* (L.) Merr. & Perry (Myrtaceae) in rodents. *African Journal of Traditional, Complementary and Alternative Medicines*, 6(3), 241–254. Retrieved from www.africanethnomedicines.net
- Atangwho, I. J., Ebong, P. E., Eteng, M. U., Eyong, E. U., & A.U. Obi. (2007). Effect of *Vernonia amygdalina* Del leaf on kidney function of diabetic rats. *International Journal of Pharmacology*, 3, 143–148.
- Bartels, H., Böhmer, M., & Heierli, C. (1972). Serum creatinine determination without protein precipitation. *Analytica Chimica Acta*, 37, 193–7.
- Berinyuy, E. B., Lawal, B., Olalekan, A. A., Yusuf, A. A., Sakpe, S., & Ossai, P. C. (2015). Hematological Status and Organs/Body-weight Parameters in Wistar Rats during Chronic Administration of *Cassia occidentalis*. *International Blood Research and Review*, 4(3), 1–7.
- Braide, V. ., & S.M. Anika. (2007). *Environmental toxicology. 1st ed. Enugu: SNAAP press Ltd.*
- Brar, R. ., Sandhu, H. ., & Singh, A. (2011). *Veterinary clinical diagnosis by*

- laboratory methods*. New Delhi: Kalyani Publishers.
- Calixto, J. B. (2000). Efficacy, Safety, Quality control, marketing and regulatory guidelines for herbal medicines (Phytotherapeutic agents). *Brazilian Journal. Medicine. & Biological Research.*, 33(2), 179–189.
- Cavaleiro, C. (2018). Antifungal activity of the clove essential oil from *Syzygium aromaticum* on *Candida*, *Aspergillus* and dermatophyte species, (2009), 1454–1462.
<https://doi.org/10.1099/jmm.0.010538-0>
- Chinedu, E., Arome, D., & Ameh, F. S. (2013). A New Method for Determining Acute Toxicity in Animal Models. *Toxicology International*, 20(3), 224–226.
<https://doi.org/10.4103/0971-6580.121674>
- Dibazar, S. P., Fateh, S., & Daneshmandi, S. (2014). Clove (*Syzygium aromaticum*) ingredients affect lymphocyte subtypes expansion and cytokine profile responses: An in vitro evaluation. *Journal of Food and Drug Analysis*, 22(4), 448–454.
<https://doi.org/10.1016/j.jfda.2014.04.005>
- Diego, F. C., Claudia-Regina, F. S., & Wanderley, P. O. (2014). Clove (*Syzygium aromaticum*): a precious spice. *Asian Pacific Journal of Tropical Medicine*, 4(2), 90–96.
[https://doi.org/10.1016/S2221-1691\(14\)60215-X](https://doi.org/10.1016/S2221-1691(14)60215-X)
- Elmastas, M., & Aboul-enein, H. Y. (2012). Antioxidant activity of clove oil – A powerful antioxidant source, 489–499.
<https://doi.org/10.1016/j.arabjc.2010.09.016>
- Goñi, M. G., Roura, S. I., Ponce, A. G., & Moreira, M. R. (2016). *Clove (Syzygium aromaticum) Oils. Essential Oils in Food Preservation, Flavor and Safety*. Elsevier Inc. <https://doi.org/10.1016/B978-0-12-416641-7.00039-0>
- Gupta, M. (2010). Pharmacological Properties and Traditional Therapeutic Uses of Important Indian Spices: A Review. *International Journal of Food Properties*, 13(5), 1092–1116.
<https://doi.org/10.1080/10942910902963271>
- Liu, Q., Meng, X., Li, Y., Zhao, C., Tang, G., & Li, H. (2017). Antibacterial and Antifungal Activities of Spices, 1–62.
<https://doi.org/10.3390/ijms18061283>
- Maharjan, D., Singh, A., Lekhak, B., & Basnyat, S. (2011). Study on Antibacterial Activity of Common Spices, 12, 312–317.
- Medina, L. V., Hrapkiewicz, K., Tear, M., & Anderson, L. C. (2007). Fundamental training for individuals involved in the care and use of laboratory animals: a review and update of the 1991 NRC Core Training Module. *ILAR Journal*, 48(2), 96–108.
- Oecd. (1995). *Recommendation of the Council of the Oecd on Improving the Quality of Government Regulation*. Paris.
- Olaniyan, J. M., Muhammad, H. M., Makun, H. A., Busari, M. M. B., & Abdullah, A. S. (2015). Acute and sub-acute toxicity studies of aqueous and methanol extracts of *Nelsonia campestris* in rats. *Journal of Acute Disease*, 5(1), 62–70.
- Onoja, S. O., Ezejal, M. I., Omeh, Y. N., & Ekwulu, J. T. (2015). Evaluation of the Effects of Methanolic Extract of Alligator Pepper (*Aframomum melegueta*) on Serum creatinine, Blood Urea Nitrogen and Electrolytes in Wistar Albino Rats. *International Journal of Biochemistry Research and Review*, 6(1), 11–17.
- Parek, J., & Chanda, S. (2006). In vitro antimicrobial activities of extract of *launarea procumbens roxb* (labiatae), *Vitis vinifera* L. (Vitaceae) and *Cyprenus rotundus* L. (cyperaceae). *African Journal of Biomedical Research*, 9, 89–93.
- Rang, H. P., Dale, M. M., Ritter, J. M., & Moore, R. K. (2007). *Pharmacology* (6th editio). Edinburgh: Churchill livingstone.
- Shittu, O. K., Lawal, B., Abubakar, N. A., Berinyuy, B. E., Busari, M. B., & Ibrahim, A. O. (2015). Toxicological Implications of Methanol Extract from Nigerian Bee Propolis on Some Selected

- Rat Tissues. *Journal of Pharmaceutical Biomedical Science*, 5(7), 524.
- Sunheimer, R. L., & Graves, L. (2011). *Clinical Laboratory Chemistry*. New York: Pearson Education.
- Uboh, F. E., Akpanabiatu, M. I., Ndem, J. I., Alozie, Y., & Ebong, P. E. (2009). Comparative nephrotoxic effect associated with exposure to diesel and gasoline vapours in rats. *Journal Toxicological and Environmental Health Science*, 1, 68–74.
- Veeresham, C. (2012). Natural products derived from plants as a source of drugs. *Journal of Advanced Pharmaceutical Technology & Research*, 3(4), 200–201.
- Vijayasteltar, L., Gopinathan, G., Maliakel, B., & Kuttan, R. (2016). Safety assessment of a standardized polyphenolic extract of clove buds: Subchronic toxicity and mutagenicity studies. *Toxicology Reports*, 3, 439–449. <https://doi.org/10.1016/j.toxrep.2016.04.001>
- Weatherburn, M. W. (1967). Phenol-hypochlorite reaction for determination of ammonia. *Analytical Chemistry*, 39(8), 971–974. JOUR. <https://doi.org/10.1021/ac60252a045>
- Zanna, H. S., Adeniji, B. B., Shehu, S., & Modu, G. M. I. (2008). Effect of aqueous suspension of the root of *Hyphaenethebaica* (L) mart on some indicators of liver and kidney function in rats. *Journal of Pharmacology and Toxicology*, 3(330–333).