Journal of Anatomical Sciences

Email:anatomicaljournal@gmail.com

J Anat Sci 12 (2)

Histomorphological Evidences of the Mitigating Effect of Aqueous *Citrus Limon* Peels Extract on Alcohol Induced Testicular Injury in Wistar Rats.

[1]Idoko GO, [2]Saalu LC

[1]Department of Anatomy, College of Health Sciences, Benue State University, Makurdi

[2]Department of Anatomy, College of Health Sciences, Benue State University, Makurdi

Corresponding Author: Idoko GO
Tel: +2347065696893/ email: gidoko75@gmail.com

ABSTRACT

Citrus limon products (pulp and peels) have been reported to contain a variety of bio-active ingredients which are believed to have health promoting benefits. This work was carried out with the aim to unmask possible histo-morphological evidences that aqueous extracts of Citrus *limon* peels can mitigate testicular toxicity caused by alcohol. Thirty-Five(35) matured male rats weighing between 180g-200g were divided into seven (7) groups containing five (5) rats each and were treated daily with oral doses of: 4mg/kg of Distilled water (group I), 2100mg/kg of 30% alcohol only (group II), 2100mg/kg of 30% alcohol + 200mg/kg aqueous *Citrus limon* peels extract (group III), 2100mg/kg of 30% alcohol + 400mg/kg aqueous *Citrus limon* peels extract (group IV) for 30 days; a second set of rats were given 2100g/kg of 30% alcohol only (group V), 2100mg/kg of 30% alcohol + 200mg/kg aqueous *Citrus limon* peels extract (group VI), 2100g/kg of 30% alcohol + 400mg/kg aqueous *Citrus limon* peels extract (group VII) for 60 days. The animals were sacrificed 24 hours after the last treatment. The left testicles were removed, the weights were taken and testicular volumes measured before preservation and processing for H&E staining. The histological examination of the sections revealed only a slight incidence of cellular distortion. Moderate desquamation within the seminiferous tubules and normal appearance of interstitial cells within the inter tubular connective tissue of the groups treated with aqueous Citrus *limon* peels extract alongside alcohol. The results were however different in the groups treated with alcohol alone, which showed high levels of cellular distortion , marked tubular desquamation and loss of interstitial cells. The measurement of testicular weights and volumes across the groups however did not show any statistically significant differences ($P< 0.05$). **Conclusion:** Aqueous extract of Citrus limon peels can mitigate testicular injury caused by alcohol in the testes of Wistar rats as portrayed by histological evidence.

Key Words: Alcohol, *Citrus limon,* Histo-morphology, Testes.

INTRODUCTION

Alcohol is widely consumed all over the world.[1,2,3] As a matter of fact, man's history with alcohol dates back to tens of thousands of years ago when people became aware that a strange metamorphosis occurred when honey, fruit, berries, cereals and other plant materials when mixed with water and left in the sun, produced fermented products.[4] Over the years alcohol has become a substance of public health interest and has been the reported cause of many psychosocial and health related problems.[5] Alcohol is toxic to the body and has been confirmed to produce many side effects including injury to the male reproductive system; the principal victim being the testes, in which it is reported to cause damage to the spermatogenic cells, Sertolli cells and the testosterone producing Leydig cells. The excess generation of reactive oxygen species (ROS) and the subsequent precipitation of oxidative stress are known to be responsible for the toxic effects of alcohol on the testes.[6,7,8,9]

Efforts to provide a solution to alcohol mediated toxicity on the human system has been the subject of many studies and several published works exist in this regard.

It is worthy of note that the search for solutions in recent times have in a way tilted towards materials of herbal origin as potent attenuating agents against alcohol induced toxicity because of their rich constituent of bioactive phytochemicals such as polyphenols, flavonoids and ascorbic acid which have proven to have counteracting effects against oxidative stress.[10, 11, 12] A common example of such is the *Citrus limon* plant.

Citrus limon (commonly known as Lemon) is a fruit

bearing plant which belongs to the family Rutacae.[13] The lemon plant, which is cultivated on a large scale in many countries has also gained a level of popularity in Nigeria due to the increasing knowledge of its nutraceutic benefits.[14] The lemon peel is the outer pericarp of the ripe *Citrus limon* fruit. Phytochemical studies have shown that Citrus limon products, especially the peel contain an abundance of polyphenols and Vitamin C (ascorbic acid) which are known to have profound anti-oxidant and anti-inflammatory properties.[15]

MATERIALS AND METHODS

Chemical: Analytical grade of ethanol manufactured by BDH chemicals limited, Poole, England with product number: 38304 was procured from World Corsica Laboratory Services Limited, Benue Crescent, Makurdi. This was reconstituted to 30% ethanol by diluting with the appropriate ratio of distilled water.

Plant Material: Fresh fruits of *Citrus limon* also known as *Alemu o'hono* in the local Idoma language was obtained in September, 2018 from a fruit shop at the modern market, Makurdi, Nigeria. The fruits were authenticated at the herbarium of the botanical unit, Department of Biological Sciences, Benue State University, Makurdi by Mr. Joshua Waya.

***Citrus limon peels* and Aqueous Extraction Procedure:** The fruits collected were washed with distilled water then peeled and the rinds collected. The *Citrus limon* peels were sliced and air dried over a period of 2 weeks at room temperature. The dried peel was milled into fine powder and aqueous extraction of product carried out according to the method described by Hegazy and Ibrahim, (2012) with little modification. Briefly; 15 g of plant powder was soaked in 200ml of distilled water at room temperature for 24 hours with constant shaking. The mixture was filtered using Whatman filter paper No.1 then concentrated by evaporation to dryness in a water bath at 70˚C. The yield was weighed on a weighing balance. The extract was stored in a dark bottle and kept in a refrigerator at a temperature of about 4˚C. This was later reconstituted in water for subsequent administration.

Percentage (%) Yield: The percentage yield of aqueous *Citrus limon* peels extract was determined by applying the formula proposed by John *et al (*2017):

$$\% \text{ Yield of Extract} = \frac{\text{Extract weight}}{\text{Dry weight of powder}} \times 100$$

Consequently, 41.5 g of extract was obtained from 60 g of dry powder amounting to 69.2% yield.

The aqueous solution administered was hence prepared by dissolving 41.5g of dried extract sample in 415 ml of distilled water to yield a stock solution of 0.1 g/ml (100 mg/ml).

Experimental Animals: Thirty-five (35) matured male Wistar rats weighing about 180-200 g were used for the study. The animals were obtained from the animal breeding facility of the Institute of Veterinary Research, Vom, Plateau State, Nigeria. The animals were kept and maintained in the research laboratory at the Animal House of College of Health Sciences, Benue State University, for the duration of the experiment. The animals were housed in polypropylene cages and kept in a well-ventilated room where they were acclimatized over a period of two weeks. During this period the animals were subjected to standard atmospheric temperature (25±5˚C) and 12/12-hour light-dark cycle. They were also adequately fed with feed pellet and allowed access to water *ad libitum*. Prior the commencement of the experiment the animals were certified to be reproductively viable as well as disease free by the lead animal scientist of the animal house.

Ethical Considerations: All experimental protocols were in compliance with the guidelines on animal experiment prescribed by the Ethics Committee of the College of Health Sciences, Benue State University, Makurdi, Nigeria.

Animal Grouping and Treatment: The animals were randomly divided into seven (7) groups; I, II, III, IV, V, VI and VII each consisting of 5 animals in the following order.

Group I: Served as the control group and were administered distilled Water (4 ml/kg) for a period of 30 days using an Oesophago-gastric cannula.

Group II: Were administered 30% alcohol orally, (2100mg/kg) body weight, once a day for 30 days using an Oesophago-gastric cannula.

Group III: Received 30% alcohol orally (2100mg/kg) body weight along with aqueous lemon peel extract *(200 mg/kg) body weight,* once a day for 30 days by means of an Oesophago-gastric cannula.

Group IV: Received 30% alcohol orally (2100mg/kg) body weight along with twice (2x) the dosage of lemon peel extract *(400 mg/kg) body weight*, once a day for 30 days using an Oesophago-gastric cannula.

Group V: Received 30% alcohol orally (2100mg/kg) body weight, once a day for 60 days using an Oesophago-gastric cannula.

Group VI: Received 30% alcohol orally (2100mg/kg) body weight along with lemon peel extract *(200 mg/kg) body weight*, once a day for 60 days using an Oesophago-gastric cannula.

Group VII: Received 30% alcohol orally (2100mg/kg) body weight along with twice (2x) the dosage of aqueous lemon peel *extract (400 mg/kg) body weight*, once a day for 60 days using an Oesophago-gastric cannula.

Determination of Acute Oral Toxicity: Determination of acute oral toxicity was carried out; the safe dose of oral toxicity for aqueous lemon peel extract was calculated according to the organization for economic co-operation and development (OECD) guidelines 423 (adopted in 2001). Six (6) female Wistar rats were administered lemon peel extract at a dosage of 2000mg/kg body weight and were monitored for 7 days to observe any immediate toxic effects. Animals were closely monitored for unusual behavioral changes and for symptoms such as loss of appetite, diarrhea, weight loss, fur discoloration, sedation, irritation and convulsion. The result of the toxicity study suggested that the LD_{50} cut off must be greater than 2000mg/kg body weight of animals. Hence it was considered safe to administer lemon peel extract at a concentration of up to 400mg/kg body weight of animals.

Histological Study: Preparation of tissue for histological study was carried out as described by Mohamed *et al*., 2014. Testicular tissue was collected and immediately fixed in 10% buffered formalin, these were allowed to fix for 48 hours. The tissues were then dehydrated in ascending grades of ethanol for 1 hour each beginning with 70%, followed by 90% and terminating finally in 2 changes of absolute ethanol each lasting for the same period. Following treatment with ethanol, the tissue was cleared in three changes of xylene lasting 15 minutes each. Impregnation with molten paraffin at 60ºC was done overnight before embedding in paraffin blocks. The blocks were trimmed and Sections (5µm) were taken using a Rotary Microtome. The sections were floated in a warm water bath at 28ºC and then collected on glass slides smeared with albumin and air dried. The slides were stained with hematoxylin and eosin dye for Light microscopic observation.

Statistical Analysis: The results obtained were expressed as Mean ± Standard deviation. Statistical comparisons of the data obtained for the different groups were made using The Analysis of Variance (ANOVA) test and Bonferroni's post hoc test. The level of statistical significance was taken as $P < 0.05$.

All the analyses were carried out using the Version 17.0; SPSS software package program.

RESULTS

Testicular Morphology: It was observed at the end of the experiment that there were similarities in the mean testicular weights and volumes recorded across the groups. The animals in Group I recorded a mean testicular weight of 1.04 ± 0.19 with a mean volume of 1.12 ± 0.08. This is compared with Group II which recorded a mean weight of 1.10 ± 0.20 and a mean volume of 1.02 ± 0.04. Animals in Group III recorded a mean testicular weight of 1.02 ± 0.08 and a mean volume of 1.06 ± 0.09 whereas group IV had a mean testicular weight of 1.10 ± 0.07 and a mean volume of 1.18 ± 0.05. The animals which were treated for an extended period of Sixty (60) days maintained the same pattern; with group V recording a mean testicular weight of 1.10 ± 0.07 and a mean testicular volume of 1.04 ± 0.09. This is compared to groups VI and VII which recorded mean weights and volumes of 1.20 ± 0.12, 1.18 ± 0.13 and 1.14 ± 0.09, 1.08 ± 0.13 respectively. Statistical comparison of the results showed that only animals in groups III and IV recorded significant increases in the testicular volumes of the rats relative to the animals treated with only alcohol ($P < 0.05$), whereas the differences in the testicular weights across the groups were not considered to be statistically significant ($P < 0.05$) Table 1.

Table 1: The Value of Testicular Weights and Volumes of the Treated Rats across Different Groups.

Groups	Testicular Weight (g)	P value	Testicular Volume (cm^3)	P value
1	1.04± 0.19	0.471	1.12± 0.08	0.101
II	1.10± 0.20		1.02± 0.04	
III	1.02± 0.08	0.338	1.06± 0.09	0.504
IV	1.10± 0.07	1.000	1.18± 0.05	0.011#
V	1.10± 0.07		1.04± 0.09	
VI	1.20±0.12	0.233	1.18± 0.13	0.025ǂ
VII	1.14± 0.09	0.630	1.08± 0.13	0.101

Values are Mean ± SD (n=5)
 # = significant relative to 30% alcohol (30 days) at P < 0.05), ǂ = significant compared with 30% Alcohol (60 days) at P < 0.05

Histological Profile: At the end of the experiment rats in group I showed no histological abnormalities in their testicular sections when viewed with the light microscope. The sections showed normal arrangements of the seminiferous tubules, spermatogenic cells and interstitial connective tissues containing Leydig cells (Plate 1).

Rats in group II had sections of their testis showing marked desquamation within the seminiferous tubules, along with visible cellular distortion (plate 2).

Rats in group III showed only slight cellular distortion, moderate desquamation within the seminiferous tubules and normal appearance of interstitial cells within the connective tissue (plate 3).

The sections taken from rats in group IV showed that there was improvement in the architecture of the seminiferous tubules, there was also an improved population of spermatogenic cells and the presence of connective tissue containing moderate number of interstitial cells (plate 4).

The testicular sections from rats in group V showed moderate tubular degeneration and cellular distortion (plate 5).

Rats in group VI showed sections of the testis with normal seminiferous tubules containing spermatogenic cells and the connective tissues which contained interstitial cells (plate 6).

The testicular sections from rats in group VII also displayed normal architecture of seminiferous tubules which contained spermatogenic cells and the connective tissues containing interstitial cells (plate 7).

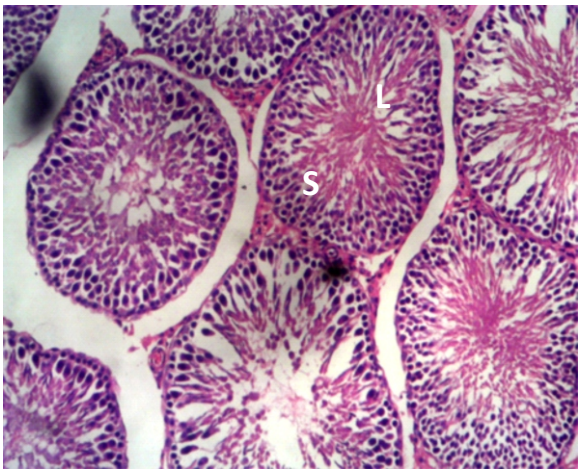
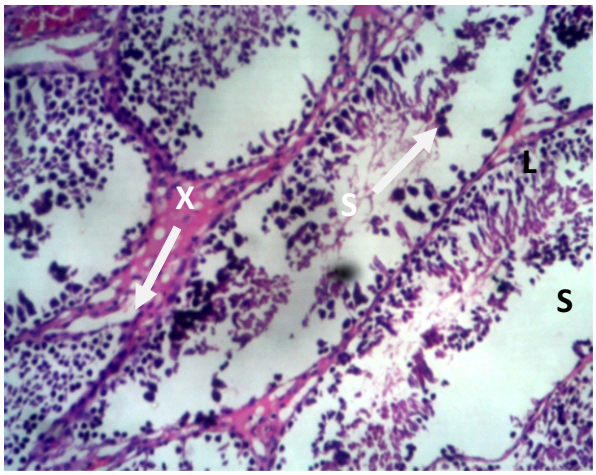


Plate 1: Testis of Group I showing the lumen **L**, normal seminiferous tubules **ST**,
Spermatogenic cells **S** and connective tissue containing interstitial cells **X**.
Magnification ×200. Stain: H&E



SPlate 2: Testis of Group II, showing marked desquamation within the seminiferous
tubules **ST**, along with visible cellular distortion. Obliteration of interstitial spaces and
consequent loss of interstitial cells **X**. Magnification ×200. Stain: H&E**L**

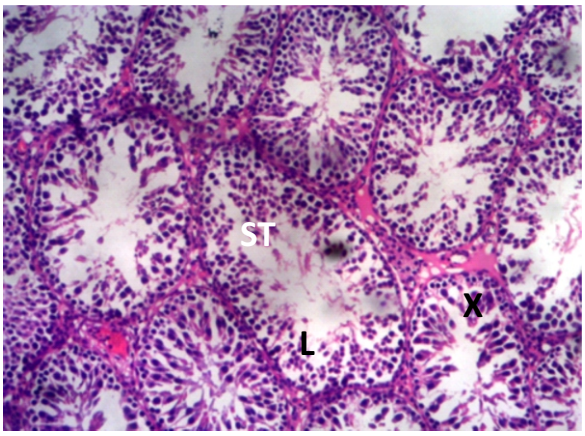


Plate 3: Testis Group III, showing moderate desquamation within the
seminiferous tubules **ST** and normal appearance of interstitial cells **X** within
the connective tissue. Magnification ×200. Stain: H&E

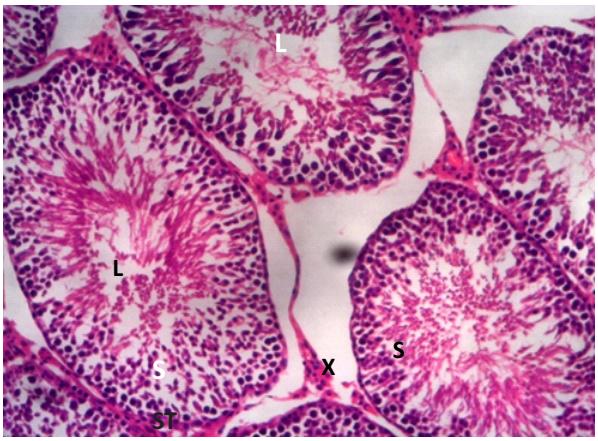


Plate 4: Testis of Group IV showing improvement in the architecture of the seminiferous tubules **ST**,improved population of spermatogenic cells and the presence of connective tissue containing moderate number of interstitial cells **X.**
Magnification ×200. Stain: H&E**.**

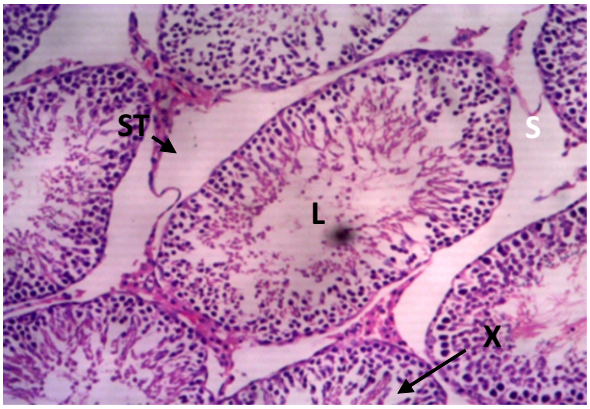


Plate 5: Testis of Group V showing moderate tubular degeneration and cellular distortion within the seminiferous tubules **ST** as well only a few sperm cells within the lumen **L**. Magnification ×200. Stain: H&E S

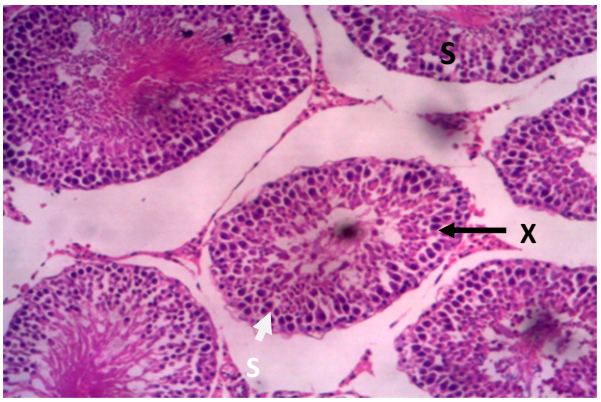


Plate 6: Testis of Group VI showing normal seminiferous tubules **ST**, spermatogenic cells **S** and connective tissue containing interstitial cells **X.** Lumen **L** also has rich population of sperm cells**.**. Magnification ×200. Stain: H&E

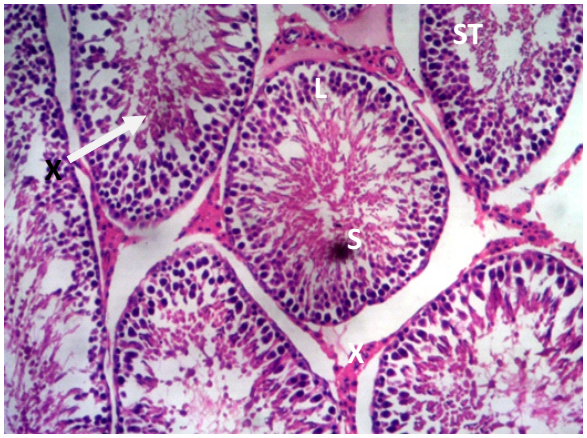


Plate 7: Testis of Group VII showing normal seminiferous tubules **ST** containing spermatogenic cells **S** and the connective tissues which containing interstitial cells **X**.
Lumen has a rich population of sperm cells. Magnification ×200. Stain: H&E.

DISCUSSION

This current study exposes the capacity of aqueous extract from citrus limon peels to ameliorate alcohol mediated testicular damage. Though reports from other researchers such as Akomolafe et al. (2015) shows a significant decrease in testicular weight sequel to the ingestion of alcohol alone for 21 days, our findings do not show any significant increase ($P \leq 0.05$) in the testicular weight. However, we noticed significant increases in the testicular volumes of animals in group IV which received 400mg of aqueous *Citrus limon* peels extract for 30 days. Another significant increase in testicular volume was also noticed in group VI, which received 200mg of aqueous *Citrus limon* peels extract alongside alcohol for 60 days, suggestive of the fact that the extract could have influenced the difference in testicular size. The effect of alcohol on the testis is clearly observed upon histological examination of the testis of the alcohol treated animals which reveals marked desquamation within the seminiferous tubules, along with visible cellular distortion and necrosis (plate 2). This result corroborates the fact that alcohol is a testicular toxin whose continuous use may cause testicular atrophy which results primarily from the loss of sperm cells and decreased diameter of the seminiferous tubules occasioned by the damage to the testosterone producing Leydig cells and the Sertolli cells (Emmanuele and Emmanuele, 1998 and Muralidhar *et al.,* 2015). The observed effect may be explained by the fact that free radicals released by alcohol attack germ cells within the seminiferous tubules leading to extensive necrosis and disruption of spermatogenesis (Atkin and Roman, 2007).

Observations from the histological sections of the testis of rats in Group III however demonstrated an improved outlook in the testicular tissue revealing only moderate desquamation within the seminiferous tubules and a normal appearance of interstitial cells within the connective tissue (plate 3).

Similarly, the sections taken from rats in Group IV showed that there was improvement in the architecture of the seminiferous tubules, improved population of spermatogenic cells and the presence of connective tissue containing moderate number of interstitial cells. This result presents a conspicuously improved cellular outlook relative to what is seen in animals administered with alcohol alone. Though alcohol is known to exert cellular damage by inducing the generation of reactive oxygen species (ROS) within the testicular tissue (oxidative stress) there however appears to be a counteracting action by the antioxidant and cyto-protective effects of aqueous *Citrus limon* (lemon) peel extracts which may be attributed to the activities of its bioactive phytochemical constituents which are reported to have profound ROS scavenging properties due to the presence of considerable amounts of vitamin C, phenolic compounds and flavonoids (Mathew *et al.,* 2012).

CONCLUSION

The results from this experiment show that oral administration of aqueous extract of *Citrus limon* peels mitigates testicular injury induced by alcohol administered at a dosage of 2100 mg/kg of 30% v/v in rats. However when 200 mg/kg and 400 mg/kg of the extract were given shortly after alcohol administration, significant positive differences were observed in the level of testicular damage caused by alcohol after 30days and 60days of administration. The efficacy of aqueous extract of *Citrus limon* peels in exerting its protective effect is attributed to its abundant content of bioactive polyphenolic compounds as well ascorbic acid. These have been reported by a few other authors to have free radical scavenging properties; hence offer adequate protection against oxidative stress induced by reactive oxygen species (ROS) generated in the testis by high tissue concentration of alcohol.

RECOMMENDATION

In view of these promising results from rats, we wish to recommend that further studies be carried out on higher animals and possibly human subject to determine if similar results will be observed.

ACKNOWLEDGEMENT

The authors wish to acknowledge the staff of the Animal House, College of Health Sciences, Benue State University, Makurdi, for their contributions to the success of this research.

CONFLICT OF INTEREST

We hereby declare that there was no conflict of interest in performing or reporting this research.

REFERENCES

1. Dosumu OO, Akinola OB and Akang EN. Alcohol Induced testicular Oxidation Stress and Cholesterol homeostasis in rats- the therapeutic Potential of Virgin Coconut oil. *Middle East Fertility Society Journal* 2012; 17:122-128.
2. La Vinegra S, Condorelli RA, Balercia G, Vicari E and Calogero AE. Does alcohol have any effect on the male reproductive function? *Asian Journal of Andrology* 2013; 15: 221-225.
3. Kliczak N. Alcohol for the Ancient: The Oldest Drinks in the World. *Ancient origins.net*
4. Davis K. What Effect Does Alcohol Have on Health? *Medical News Today*. 2018; Retrieved from https:www.medicalnews today.com/articles/305062.php.
5. Emanuele MA and Emanuele NV. Alcohol's Effect on Male Reproduction. *Alcohol Health and Research World* 1998; 22 (3): 195-201.
6. Maneesh M, Jayalekshmi H, Dutta S, Chakrabart A and Vasudevan DM. Effect of Chronic antioxidant System and Steroidogenic Enzyme activity in rats. *Indian Journal of Experimental Biology* 2005; 43:445-9.
7. Taati, M, Alirezaei, M, Measkatal Sadat, MH, Rasoulian B, Kheradmand A and Neamati SH. Antioxidant Effects of aqueous Fruit extract of *ZiziphusJujuba* On ethanol-induced Oxidative stress in rat tests. *Iranian Journal of veterinary Research, Shiraz University* 2011; 12(1):39
8. Oremosu AA and Akang EN. Impact of alcohol on male reproductive hormones, oxidative stress and semen parameters in sprague- dawley rats. *Middle East Fertility Journal* 2014; DOI:10.1016/j.mefs.2014.07.001.
9. Abdel-Moneim AE. Citrus peel extract attenuates acute cyanide poisoning-induced seizures and oxidative stress in rats. *Journal of CNS, Neurological Disorders and Drug Targets,* 2016; 13(4): 638-646.
10. Mohamed NAE, Amany AT, Basmal E and Ahmed EA. Ameliorative effect of citrus peel extract on castration-induced oxidative stress in liver and kidney of rats. *Journal of Applied Pharmaceutical Sciences* 2014; 4 (07): 064-068.
11. Prasad VS, Ghongane BB, Chourishi A, Vakade K and Kunkulol RR. Role of antioxidants in male reproduction. *Andrologia* 2018; 2(4): 1-6.
12. Li S, Lo, CY and Ho CT. Hydroxylated Polymethoxy-Flavones and Flavonoids in Sweet Orange (*Citrus Sinensis*) Peel. *Journal of Agriculture and Food Chemistry* 2006; 54: 4176-4185
13. Acham IO, Ahemen S, Ukeyima MT and Girgih AT. Tropical fruits: Bioactive properties and health promoting benefits in chronic disease prevention and management. *Asian Food Science Journal* 2018; 1- 3. Tropical fruits:

Bioactive properties and health promoting benefits in chronic disease prevention and management. *Asian Food Science Journal*. 1-13
14. Reddy BA, Priya VV and Gyathri R. Comparative phytochemical analysis and total phenolic content of Citrus seed extract (*Citrus sinensis and Citrus limon*). *Drug Intervention Today* 2018; 10(10): 2038-2040.
15. Hegazy A E and Ibrahim MI. Antioxidant Activities of Orange Peel Extracts. *World Applied Science Journal* 2012;18(5): 684-688.
16. John S, Monica SJ, Priyandashin IS and Arumudam P. Investigation on Phytochemical Profile of *Citrus limonum* Peel Extract. *Food Science Journal* 2017; 2(1): 65-67.
17. Muralindhar TS, Anilkumar HV, Javed A, Das J and Reddy CC. Effects of alcohol on sexual dysfunction and hormonal profiles in male Wistar rats. *World Journal of Pharmaceutical Research* 2015; 4(7):455-468.
18. D'Cruz M. Effect of Piperine on the epididymis of adult male rats. *Asian Journal of Andrology* 2005; 7(4): 363 – 8.
19. Mathew BB, Jatawa SK and Tawari A. Phytochemical Analysis of *Citrus limonum* Pulp and Peel. *International Journal of Pharmacy and Pharmacological Sciences* 2012; 4(2): 369-371.