EFFECTS OF QUERCETIN ON ORAL ADMINISTRATION OF CANNABINOL AND ALCOHOL ON REPRODUCTIVE FUNCTIONS IN MALE WISTAR RATS

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ABSTRACT
The male reproductive system ensures the continuity of species; however, several toxicants have demonstrated hampering effects on the structures of male reproductive system, thereby leading to reduced physiological functions. This study was carried out to investigate the effect of quercetin in experimental rats treated with alcohol and/or cannabinol. Forty-five male rats were grouped into the following: Group 1 rats were given distilled water**.** Group 2 rats were administered with methanol**.** Group 3 rats were administered with alcohol (3g/kg bw as 25%v/v)**.** Group 4 rats were administered with cannabinol (10mg/kg bw). Group 5 rats were administered with cannabinol (10mg/kg bw) and alcohol (3g/kg bw as 25%v/v)**.** Group 6 rats were administered with quercetin (30mg/kg bw)**.** Group 7 rats were administered with alcohol (3g/kg bw as 25%v/v) and quercetin (30mg/kg bw). Group 8 rats were administered with cannabinol (10mg/kg bw) and quercetin (30mg/kg bw). Group 9 rats were administered with cannabinol (10mg/kg bw) plus alcohol (3g/kg bw as 25%v/v) plus quercetin (30mg/kg bw). At the end of the experiment, animals were sacrificed and their reproductive organs were harvested and weighed immediately. Results showed that rats treated with alcohol and/or cannabinol had significant decrease ($p<0.05$) in sperm indices, lipid peroxidation, gonadotrophic hormones and male sex hormone (testosterone); however, with quercetin co-treatment with alcohol and/or cannabinol administration the results obtained in the reproductive parameters showed ameliorative potential of quercetin in the present study.

Keywords: Alcohol, cannabinol, quercetin, oxidative stress, male reproductive profile.

INTRODUCTION
The abuse of recreational drug is on the increase at an alarming rate. Among the drugs that are commonly used by youths because of the relative ease with which they are obtained are alcohol, cannabis (cannabinoids), nicotine and so on. However, alcohol is found in various beverages such as beer, gin and so on; while cannabinoids are present in Indian hemp, marijuana including Δ-8-tetrahydrocannabinol (Δ^8-THC), cannabinol (CBN), cannabidiol (CBD), cannabicyclol (CBL), cannabichromene (CBC) and cannabigerol (CBG) (Cacciola *et al*., 2010). Alcohol is not only a popular mind-altering drug but is also regarded in many cultures as food staples, as a so source of emptying calories, an addictive drug, and a toxin (Lieber, 1991).
Alcohol is also known as ethanol and is rapidly absorbed from the digestive tract, about 20% of it is absorbed in the stomach and 80% is absorbed in the proximal small intestine. Carbonation, as in beer and sparkling wines increases its absorption (Kenneth- Saladin, 1998). Chronic alcohol consumption has been reported to cause gastritis, bleeding, ulcer and liver cirrhosis (Limuro *et al*., 2000). The ability of alcohol to suppress reproductive functions in several animals has been demonstrated (Fernandez-Solari *et al.,* 2004). Studies have shown that heavy alcohol consumption adversely affects the leydig cells and results in reduced plasma testosterone Duca *et al*., 2019).
In the pituitary gland, alcohol administration causes a decrease in the production, release and activities of luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Adams and Cicero, 1991, Duca *et al*., 2019). Chronic alcoholics are often associated with impotence, loss of libido, premature or delayed ejaculation, sterility, testicular atrophy and gynaecomastia (Jana *et al*., 2010, Condorelli *et al*., 2015). Both clinical and experimental studies have shown a hampering effect on the hypothalamo-hypophyseal-testicular axis (Duca *et al.*, 2019). In the leydig cells, alcohol inhibits the action of enzymes critical to spermatogenesis such as 3-β-hydroxysteroid dehydrogenase and 17-ketosteroid

reductase which catalyse the conversion from pregnenelone to progesterone and from androstenedione to testosterone (Duca *et al.*, 2019). It is worth noting that progesterone is a precursor for testosterone so a decrease in its production could adversely affect the biosynthetic pathway for testosterone (Muthusami and Chinnaswamy, 2005). Studies on semen samples of alcoholics have decreased sperm count, impaired forward motility of morphologically normal spermatozoa and increased number of teratozoospermia
(Muthusami and Chinnaswamy, 2005), which are indicative of impaired reproductive functions. Cannabinol (CBN), is a weak psychoactive cannabinoid found in cannabis sativa and cannabis indica (Karniol *et al.*, 1975). It is also a metabolite of tetrahydrocannabinol (THC) (McCallum *et al.*, 1975). Cannabinol acts as a weak agonist of CB1 receptors but has a higher affinity to CB2 receptors, with lower affinity in comparison to THC (Mahaderan *et al.*, 2000). The use of alcohol and cannabinoid seem to remain a board public health concern since millions use alcohol and some engage in the use of cannabinols for a prolong period of time. Antioxidants terminate oxidation reactions by removing free radical intermediates, and inhibit other oxidation reactions. They do this by being oxidized themselves, so antioxidants are often reducing agents. Quercetin is one of the most widely spread naturally occurring flavonoids, found in onions, garlic, cabbage, leek, broccoli, apples, blueberries, tea and red wine. It also may be used as an ingredient in supplements, beverages or foods (Manach *et al.*, 2004). One study found that organically grown tomatoes had 79% more quercetin than "conventionally grown" fruit (Mitchell *et al.*, 2007). Quercetin is present in various kinds of honey from different plant sources (Mitchell *et al.*, 2007). It is known that quercetin may exhibit anti-oxidant properties due to its chemical structure, particularly the presence and location of the hydroxyl (-OH) substitutions (Tan *et al.*, 2003, Hardwood *et al.*, 2007). However, Individual studies on alcohol and cannabinoids have been reported to affect reproductive activities both in humans and animals resulting in poor spermatozoa leading to infertility. This study therefore investigates whether quercetin will ameliorate the effect of cannabinol and alcohol on reproductive parameters in male adult rats.

MATERIALS AND METHODS

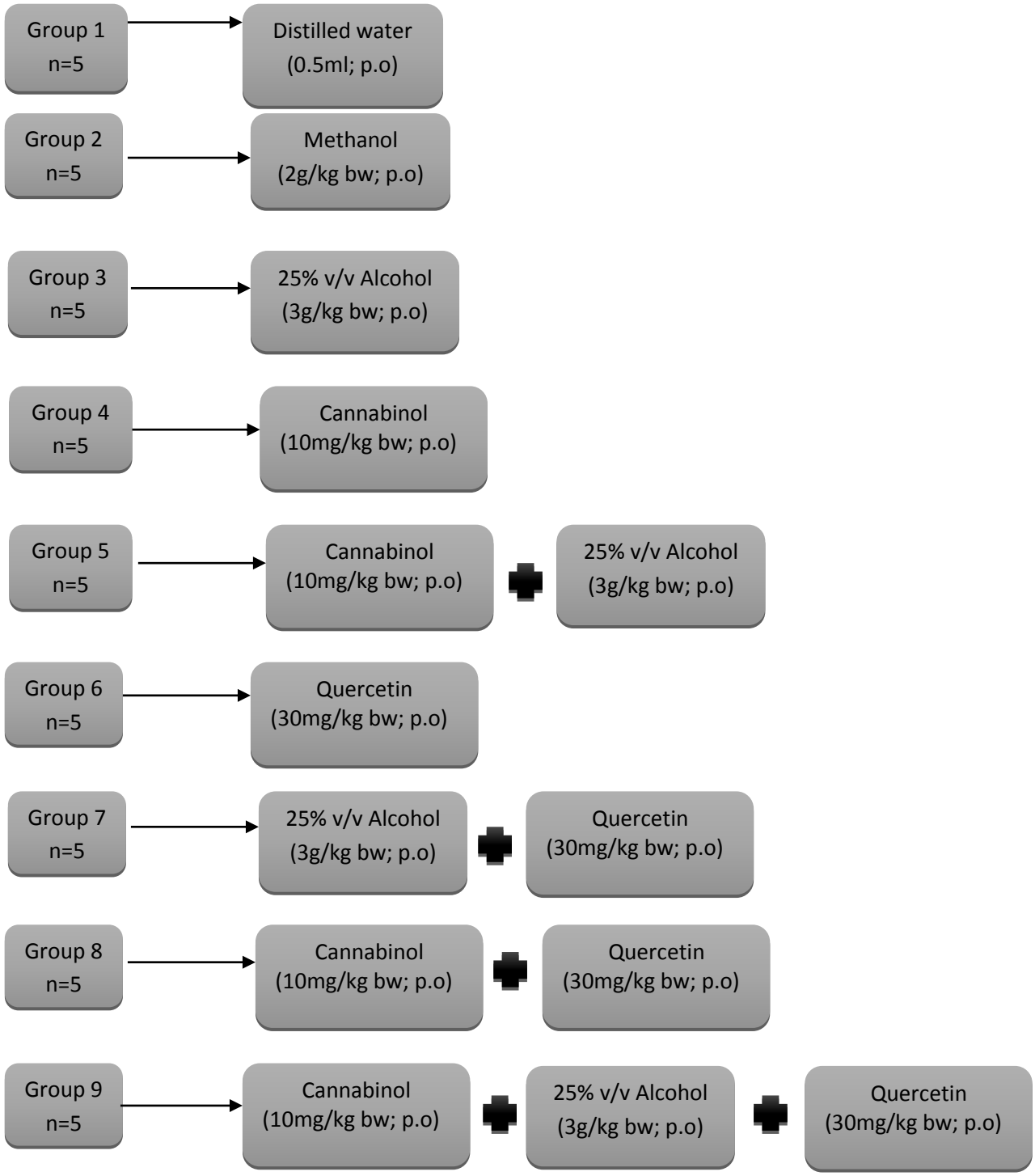
Experimental animals and drugs

Male wistar strain albino rats weighing between 175-210g obtained from National Institute for Pharmaceutical Research and Development (NIPRD) Iju-Abuja, Nigeria, were used for this study. Standard diet and water were provided ad libitum. The rats were divided into groups and each rat was housed separately in a wire mesh cage. Cannabinol solution (cat no: C6520) was purchased from sigma and ethanol was obtained from BDH Poole company for the study. The rats were raised in the animal house of the department of physiology of Basic Medical Sciences of Bingham University, Nassarawa state, Nigeria and fed on growers' pellet bought from Masaka, Nassarawa state and they were also given clean water. The animals were kept in cages at room temperature with 12 hours light and dark cycle. The animals were allowed a 3 weeks period of acclimatization before the commencement of the experiment.

Experimental Design

Forty-five (45) male albino (5 per group) wistar rats were randomly allotted into experimental groups as follows

Forty-two rats (150-250) were randomly divided into seven groups consisting of six rats per group. The groups are as follows



Drugs and vehicle administration were done orally using an oral cannula for 48 days. Drug administered to each rat was based on its body weight (in grams). The animals were weighed before and after the treatment periods. The drug treated groups were sacrificed after the experimental period (48 days). The organs were harvested and weighed. Blood samples were collected via retro-orbital sinus with heparinized capillary tube for gonadotrophin and testosterone hormone estimations. The left epididymis of each rat was immediately excised and semen was withdrawn from the caudal portion for sperm characteristics analysis.

Sperm characteristics analysis

Immediately after excision, an incision was made into the caudal epididymis and the semen was squeezed on the pre-warmed microscope slide. Two drops of warm 2-9% sodium citrate were added to the semen. The slide was examined under the microscope using the ×40 objective. The spermatozoa were seen to move in a wave-like fashion and different wave patterns ranging from an absence of waves (10%) to the appearance of prominent waves in very rapid motion (100%) were recorded and scored for mass spermatozoal motility.

Sperm viability

Sperm viability was done using nigrosin/ eosin stain. The stain is an isotonic mixture of 100% nigrosin and 4% eosin. Semen was squeezed on the microscope slide and 2 drops of the stain were added. A solution of the mixture was sneered on a clean microscope slide and air dried. The slide was examined under microscope using the ×40 objective. The live spermatozoa were seen as clear and the dead ones as pink-stained cells against a blue background. The numbers of the stained and unstained sperm cells in the field were counted and the percentage viability was calculated. After assessing the sperm viability, the slide was viewed under the microscope using ×100 objectives. A random observation of at least 200 spermatozoa was made and the average was taken for each form of sperm observation. The right epididymis was placed in a tube containing 5ml of normal saline and its volume was measured by displacement. The content of the tube was then poured into a ceramic mortar in which the epididymis was macloated, to release the spermatozoa. Then the Neubauer haematocytometer was used to determine the sperm count. A clean cover slip was placed firmly on the slide and after thorough mixing, a drop of the diluted semen sample was introduced under the cover slip by capillary action. The ruled area was located at the centre of the microscope field of view by means of the ×10 objective and the cells were counted under the ×40 objective.

Blood sample collection

Blood was collected from each animal via the retro-orbital sinus with 70µl, non-heparinized capillary tube (Ezzai, 1995) and put into plain sample bottle for gonadotrophins (FSH and LH) and testosterone analysis. The samples were allowed to clot and serum was drawn into plain bottles for hormonal analysis.

Organ collection

The animals were dissected and organs of interest; Testes, seminal vesicle, prostate, epididymis, liver, heart, brain, lungs and kidneys were removed, cleared of adherent tissues and weighed immediately with an electronic weighing balance, model DT 1000 into a capacity of 0.1 to 1000g.

Lipid Peroxidation Assay

The level of lipid peroxidation was estimated as the concentration of thioubabituric acid reactive product malondialdehyde (MDA) as described by Olkawa *et al.*, (1979). 1ml of the tissue (testes) homogenate was thoroughly mixed with 2ml of TCA-TBA-HCL solution and heated for about 15 minutes in water bath. After cooling, the precipitate was removed by centrifugation and the absorbance measured at 523nm using spectrophotometer.

Statistical Analysis

Data analysis was carried out using one way analysis of variance (ANOVA) supported by Newman-Keuls test when followed by Turkey's multiple comparison pair with comparison was done between groups. Results were presented as mean ± SEM, and the differences were considered significant at $p < 0.05$.

RESULTS

Table 1. The effects of Quercetin on mean body weights of rats treated with alcohol and cannabinol

Treatment	Initial body weight(g)	Final Body weight(g)	Weight difference (%)
Control	200±5.01	235±5.00	14.89
Methanol	205±12.25	228±9.82	10.08 [ab]
Alcohol	185±10.00	211±16.80	12.32[ab]
Cannabinol	193±4.90	198±8.60	2.52 [b]
Alcohol+cannabinol	192±10.68	197±23.11	2.54 [b]
Quercetin	200±0.00	230±5.00	13.04 [ab]
Alcohol+Quercetin	195±5.00	221±14.27	11.76 [ab]
Cannabinol+Quercetin	200±0.00	210±6.12	4.76 [a]
Alcohol+Cannabinol+Quercetin	190±10.00	198±9.67	4.04 [a]

Values are expressed as mean±S.E.M. n=5. Values with different superscript in the same column are significantly different at $p<0.05$. n=5.

Table 2. The effects of Quercetin on viscera organ weights of rats treated with alcohol and cannabinol

Treatment	Heart(g)	Lungs(g)	Spleen(g)	Kidney(g)	Brain (g)	Liver (g)
Control	0.80 ± 0.01	1.94 ±0.03	0.80±0.06	0.79± 0.09	1.85± 0.06	7.67±0.41
Methanol	0.75 ± 0.07	1.91 ±0.28	0.73 ±0.08	0.69 ± 0.05	1.54 ±0.06	6.86±0.99
Alcohol	0.78 ± 0.04	1.72 ±0.47	0.82 ±0.10	0.61 ± 0.07	1.67± 0.13	5.82±0.58
Cannabinol	0.78 ± 0.04	2.06± 0.53	0.65 ± 0.09	0.69 ± 0.09	1.50± 0.08	5.67±0.99
Alcohol+cannabinol	0.87 ± 0.10	1.48 ±0.27	0.96 ± 0.04	0.64 ± 0.05	1.67± 0.04	5.74±0.20
Quercetin	0.76 ± 0.04	1.20 ± 0.16	0.65 ± 0.04	0.57 ±0.01	1.67 ±0.06	6.07±0.46
Alcohol + quercetin	0.75 ± 0.05	2.00±0.56	0.88 ± 0.08	0.53 ± 0.01	1.52 ±0.04	5.83±0.35
Cannabinol+quercetin	0.70 ± 0.03	1.40 ±0.06	0.74 ± 0.05	0.66 ± 0.03	1.54± 0.07	6.66±0.67
Alcohol +Cannabinol +quercetin	0.68 ± 0.06	1.19 ±0.11	0.75±0.09	0.71 ± 0.05	1.65± 0.09	6.26±0.78

Data are represented as mean±S.E.M.. n=5.

Table 3. The effects of Quercetin on male sex organ weights of rats treated with alcohol and cannabinol

Treatment	Testes(g)	Epididymis(g)	Prostate(g)	Seminal vesicle(g)
Control	1.45 ± 0.10	0.86 ± 0.12	0.59 ± 0.23	0.45 ± 0.03
Methanol	0.84 ± 0.15	0.80 ± 0.18	0.36 ±0.09	0.43 ± 0.05
Alcohol	1.27 ± 0.10	0.63 ±0.04	0.27 ±0.03	0.35 ± 0.03
Cannabinol	0.96 ± 0.15	0.49 ±0.05	0.26 ±0.05	0.25 ± 0.05
Alcohol+cannabinol	0.72 ± 0.13	0.48 ± 0.03	0.24 ±0.03	0.21 ± 0.03
Quercetin	1.01 ± 0.11	0.49 ± 0.03	0.30 ±0.03	0.27 ± 0.02
Alcohol + quercetin	0.87 ± 0.15	0.53 ± 0.07	0.25 ± 0.04	0.42 ± 0.22
Cannabinol+quercetin	0.91 ± 0.22	0.76 ±0.12	0.31 ±0.01	0.29 ± 0.03
Alcohol+Cannabinol+quercetin	1.04 ± 0.07	0.66 ± 0.15	0.28 ±0.02	0.31 ± 0.02

Data are expressed as mean±S.E.M. n=5.

Table 4. Effect of Quercetin on alcohol and cannabinol altered sperm profile in rats

Treatment	Sperm motility(%)	Sperm count ($x10^6$/ml)	Abnormal sperm morphology(%)
Control	93.60±1.56^{ab}	17.00±1.16^{a}	6.29^{a}
Methanol	85.00±2.23^{ab}	20.00±0.97^{a}	7.43^{a}
Alcohol	40.00±1.58^{a}	4.40±0.51^{b}	23.10^{ab}
Cannabinol	35.00±1.87^{a}	4.80±0.37^{b}	26.95^{ab}
Alcohol+cannabinol	37.00±1.12^{a}	4.20±0.85^{b}	26.17^{ab}
Quercetin	96.20±0.73^{ab}	19.00±1.87^{a}	4.92^{ac}
Alcohol+Quercetin	79.00±1.87^{ab}	13.00±1.22^{ab}	7.31^{a}
Cannabinol+Quercetin	72.00±2.54^{ac}	13.80±1.16^{ab}	6.86^{a}
Alcohol+Cannabinol+Quercetin	67.00±1.00^{ac}	12.86±1.05^{ab}	8.00^{a}

Values are expressed as mean±S.E.M. Values with different superscript in the same column are significantly different at $p<0.05$. n=5.

Table 5. Effect of Quercetin on alcohol and cannabinol induced lipid peroxidation

Treatment	MDA
Control	2.91 ±0.22
Methanol	6.12±0.19^{a}
Alcohol	11.11±0.74^{ab}
Cannabinol	9.89±1.15b
Alcohol + Cannabinol	11.75±0.43^{ab}
Quercetin	3.65±0.26
Alcohol+Quercetin	3.94±0.31
Cannabinol+Quercetin	3.60±0.45
Alcohol+Cannabinol+Quercetin	4.48±0.37

Values are expressed as mean±S.E.M. Values with different superscript in the same column are significantly different at $p<0.05$. n=5.

DISCUSSION

Traditionally, the black Kwao Krua (*Mucuna macrocarpa* or *M. colletti*) has been used by Asian men for maintenance of reproductive functions (Ladachart *et al.,*2008). Quercetin, one of the bioactive constituents isolated from the black Kwao Krua, proved in the present study to have ameliorative effect on infertility. The results of the present study showed that there was no significant reduction ($p>0.05$) in body weight of rats in the experimental groups when compared with the control rats (***Table1***). No significant difference ($p>0.05$) was also observed in the mean weight of visceral organs of the experimental rats co-treated with quercetin compared to those treated with alcohol and cannabinol as shown in table 2. Also, there was an insignificant difference ($p>0.05$) in the mean weight of sex organs of rats co-treated with quercetin compared to those treated with only alcohol or/and cannabinol (***Table 3***).

Moreover, there was a significant reduction ($p<0.05$) in sperm profile (sperm count, sperm motility) of rats treated with alcohol alone, cannabinol alone, alcohol plus cannabinol when compared with the control group. However, the rats treated with quercetin plus alcohol and cannabinol showed a significant increase ($p<0.05$) in sperm profile (sperm count, sperm motility) when compared with the alcohol alone, cannabinol alone, alcohol plus cannabinol treated groups, and which is in agreement with the study by Ma *et al*. (2004), who revealed that quercetin might acts through the hypothalamic pituitary-testicular axis thereby stimulating the secretion of testosterone. Quercetin caused an increase in sperm count and sperm motility when compared with the control. Sperm count and sperm motility were increased when quercetin was co-administered with alcohol or/and cannabinol than when it was not (Table 4).

Abnormalities of sperm morphology was also reduced in groups which had quercetin as part of their treatment. A study by Aravindashan *et al.,* (1985) showed that treatment with high dose of quercetin (300mg/kg body weight) reduced sperm profile which affects fertility rate of male rats during the first two mating with the female rats and thereafter the fertility rate was recovered to be comparable to the control group. The increase in sperm motility could be due to the antioxidant property of quercetin on the epididymis (Amália, 2007). Quercetin could also act to increase sperm count and motility by stimulating the testis or other sex organs in males (Ma *et al*., 2004). The reduced sperm content in the alcohol and cannabinol treated rats implies an adverse effect of alcohol and cannabinol on spermatogenesis. In support of this, a number of clinical and experimental studies have shown impaired spermatogenesis under chronic alcohol consumption (Muthusami and Chinnaswamy, 2005). Anderson *et al.,* 1983 also observed low sperm content in caudal epididymis of alcohol (5% for 20 weeks, and 6% for 5 weeks) consumed rats. However, the impaired sperm motility in alcohol or/and cannabinol treated rats is indicative of a defect in the acquisition or maintenance of motility. This is evidenced by a study that reported a decrease in average sperm velocity in alcohol-consumed C57B1/6J mice (Anderson *et al.* 1983). After spermatozoa leave the testis in an immature state, they undergo maturational changes and acquire the potential for progressive motility during passage through the caput and corpus epididymis. Therefore, the reversal of sperm content to normal level in groups treated with alcohol or/and cannabinol plus quercetin suggest that quercetin possesses antioxidant scavenging property. Supportive evidence can be drawn from the works of Muthusami and Chinnaswamy, (2005) and Sermondade *et al*. (2010) who have shown that alcohol exposure to human spermatozoa in vitro decreased the percentage of motile spermatozoa and their average speed. However, the results of this study showed that there was an increase in the level of lipid Peroxidation in the groups treated with alcohol alone, cannabinol alone, alcohol plus cannabinol when compared with the control group. But, the level of lipid Peroxidation was decreased in the groups treated with

alcohol or/and cannabinol plus quercetin than when it was not (***Table 5***) and this is in support of the work by Morel *et al.,* 1993 and Tan *et al.*, 2003, who showed that quercetin was responsible for the potent inhibitory action on lipid peroxidation, and this is as a result of the presence of the hydroxyl group at C-3 position of the skeleton of quercetin. Moreover, sperm membranes are rich in polyunsaturated fatty acid and they may be susceptible to oxygen induced damage mediated by lipid Peroxidation (Sikka, 2001). Oxidative stress is an important factor in the etiology of poor sperm function including morphological alterations (Aziz *et al*., 2004) and oxidative damage to DNA membranes and proteins (Stadtman and Levine, 2003).

In addition, excess of reactive oxygen species and free radical generation have frequently been detected in the seminal plasma and sperm of infertile men (Baker and Aitken, 2005). Quercetin has been attributed having the ability of scavenging reactive oxygen species (Celik and Arinc, 2010). Quercetin has been found by Ma *et al.* (2004) to stimulate an increase in testosterone level. Moreover, the results of this study showed that the LH, FSH and testosterone levels were increased significantly ($p<0.05$) in rats co-treated with quercetin plus alcohol and/or cannabinol compared with those treated with only alcohol and/or cannabinol.

CONCLUSION

The results of the present study established the gonadotoxic effects of alcohol and cannabinol on male Wistar rats which are supported by previous findings indicating alcohol and/or cannabinol-related infertility. However, quercetin was shown to ameliorate the pathophysiological effects produced by alcohol and/or cannabinol by improving the levels of gonadotrophins and testosterone thereby enhancing a positive outcome on reproductive functions in this study. Thus, quercetin may be used as a supplement in the treatment of alcohol and/or cannabinol related infertility.

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