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Tetracarpidium conophorum Müll. Arg modulates sexual behaviour and biochemical parameters relevant to sexual function in male Wistar rats

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ABSTRACT

Walnut (*Tetracarpidium conophorum* Müll. Arg) has been reported to be an essential ingredient in folklore medicine for sexual enhancement with little scientific validation. Hence, this study investigated the effects of walnut supplemented diet on sexual behaviour and biochemical parameters relevant to erection in male Wistar rats. Forty animals used in this study were divided into five groups ($n = 8$); Group 1 – normal control rats fed with basal diet, Group II – rats fed diet supplemented with 10% processed walnut, Group III – rats fed diet supplemented with 10% raw walnut, Group IV – rats fed diet supplemented with 20% processed walnut and Group V – rats fed diet supplemented with 20% raw walnut. Behavioural studies (copulation tendency and anxiety) associated with sexual function, measurement of nitric oxide (NO) levels, adenosine deaminase (ADA), arginase and acetylcholinesterase (AChE) activities in the *Corpus cavernosum* as well as characterization of bioactive components of the nut were evaluated. Marked reductions in ADA and arginase activities and a concomitant increase (% inclusion dependent) in the level of NO as well as enhanced sexual behaviours were observed in rat fed supplemented walnut when compared to the control. Furthermore, analysis of the walnut using high performance liquid chromatography indicated the presence of some polyphenols. From our findings, it showed that walnut improves sexual behaviour and modulates activities of key enzymes relevant to erection in male rats which may justify its used in traditional medicine.

1. Introduction

Erectile dysfunction (ED) or male sexual dysfunction is simply defined as the persistent or repeated inability to achieve and/or maintain an adequate erection to accomplish a complete and satisfactory sexual activity [1]. According to Laumann et al. [2], ED is estimated to affect around 100–150 million men worldwide and projected to increase to 322 million by 2025 [2]. The prevalence rate of ED is estimated to vary according to the age of the population from 39% for 40 years to 75% in men aged 80 years [3,4]. From our previous research, it was established that walnut dietary supplementation (10%) enhanced sexual behaviour [5]. Also, modulatory effect of walnut hydroalcoholic extract on extracellular metabolism of ATP and adenosine through nitric oxide synthase (NOS)/cyclic guanine monophosphate (cGMP)/phosphokinase G (PKG) signalling pathway has been reported [6].

Nitric oxide (NO) is a potent vasodilator and a key mediator of vascular relaxation and has been implicated in endothelial dysfunction which represents a major pathophysiologic process leading to abnormal vaso-reactivity [7]. Thus, reduced NO bioavailability has been linked to the pathophysiology of ED, since NO-mediated relaxation of smooth muscles plays a pivotal role in penile erection. NO is synthesized from L-arginine and plays vital role in smooth muscle relaxation and penile erection [7]. Synthesis of NO from a reaction catalyzed by endothelial nitric oxide synthase (eNOS) favours the binding of NO to soluble guanylate cyclase which in turn increases cyclic guanosine monophosphate (cGMP) levels and stimulates cGMP-dependent protein kinase G (PKG) activity, leading

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to smooth muscle cell relaxation and cavernosal dilation. Subsequent haemodynamic changes such as increased arteriolar shear flow stimulate the phosphatidylinositol-3-kinase/protein kinase B (Akt) pathway leading to activation of eNOS in penile endothelium and further NO release. NO also plays a vital role as a neurotransmitter in the central nervous system (CNS), where it controls sexual behaviour by promoting dopamine release in the medial preoptic area [8] and activates the release of luteinizing hormone-releasing hormone (LHRH) [9] which elevates sexual behaviour and neuroendocrine responses to primer pheromones [10].

Arginase, a binuclear manganese metalloenzyme has been suggested to enhance eNOS activity by sustaining the substrate pool of L-arginine for NO biosynthesis, thereby enhancing NO-dependent smooth muscle relaxation. It catalyzes the hydrolysis of L-arginine to form L-ornithine and urea; and regulates eNOS activity, thereby attenuating NO-dependent physiological processes by depleting the substrate pool of L-arginine [7,11].

Acetycholinesterase (AChE) is the primary cholinesterase in the body; it hydrolyzes the neurotransmitter acetylcholine to acetate and choline. Inhibition of acetycholinesterase makes the acetylcholine available to stimulate the production of NO in the endothelial cells and importance in male erectile function has also been reported [12]. Previous studies have demonstrated that increase AChE activity is associated with low level of NO production and subsequently inhibits NO-dependent smooth muscle relaxation which mediates erectile function [12,13].

Furthermore, adenosine share multiple features with NO, making it an excellent candidate contributing to erectile function. Firstly, both are well-known potent vasodilators and neurotransmitters. Secondly, they both have very short half life (<10 s) [14] and thirdly, both of them induces cyclic nucleotide second messengers and penile erection [15,16]. Specifically, adenosine functions through G protein-coupled receptors to modulate adenylyl cyclase and the synthesis of cAMP. NO functions through guanylyl cyclase to induce the synthesis of cGMP. Adenosine-mediated cAMP induction and NO-mediated cGMP induction are capable of inducing protein kinase A and protein kinase G respectively, resulting in decreased calcium levels and subsequently enhanced smooth muscle relaxation [16].

Plants and its materials have been employed in the treatment and management of male erectile dysfunction for several years and this practice cut across many cultures and traditions of the world. They are commonly prepared as extracts, infusions, teas and nutraceuticals which are mostly taken orally or by topical application as the case may be. Studies have been carried out to investigate and validate the efficacy of medicinal plants/plant materials on erectile function in both human and animal studies [17–19,40].

Walnut, *Tetracarpidium conophorum* Müll. Arg., is a tropical plant of the family *Euphorbiaceae*. *T. conophorum* Müll Arg. is a climbing shrub 10–20 ft long, it is known in the Southern Nigeria as ukpa (igbo), Western Nigeria as awusa or asala (Yoruba) and in the littoral and the western Cameroon as kaso or ngak. It has been reported to contain bioactive compounds such as plant sterols, vitamin C and omega-3 fatty acids [20]. Consumption of walnuts has been shown to reduce serum cholesterol level as well as inhibit initiation and propagation of lipid peroxidation [19]. Walnut leaves and peel have also been reported to have anti-hyperglycemic effect in diabetic rats [21]. Recently, we published from our laboratory the modulatory effect of walnut hydroalcoholic extract on ectonucleotidases [6]. Traditionally, walnut is extensively been used as male sexual stimulant majority in the South Western region of Nigeria. However, the possible mechanism of action is yet to be understood. Hence, this study evaluates the effect of walnut supplemented (10% and 20%) diet on sexual behavioural parameters and some key enzymes activities relevant to erection in male Wistar rats.

2. Materials and methods

2.1. Chemicals and equipments

Adenosine, N-(l-naphthyl) ethylenediamine dihydrochloride, sulphanilamide, L-arginine, acetylthiocholine iodide and coomassie brilliant blue G were obtained from Sigma Chemical Co (St. Louis, MO, USA) and bovine serum albumin, nitrate, and Vanadium chloride (VCl_3) were obtained from Reagen (Colombo, Paraná, Brazil). All other reagents were of analytical grade and the water used was glass distilled. High performance liquid chromatography (HPLC-DAD) was performed with a Shimadzu Prominence Auto Sampler (SIL-20A) HPLC system (Shimadzu, Kyoto, Japan), equipped with Shimadzu LC-20AT reciprocating pumps connected to a DGU 20A5 degasser with a CBM 20A integrator, SPD-M20A diode array detector and LC solution 1.22 SP1 software while the spectrophotometric analysis was performed by using UV-visible spectrophotometer (Jenway 6305 model; Jenway, Barlo world Scientific, Dunmow, United Kingdom).

2.2. Plant material and sample preparation

Walnut was purchased from a local market in Akure, South West, Nigeria. Authentication was carried out at the Department of Biology, Federal University of Technology, Akure, Nigeria. A voucher specimen of the sample has been deposited in the University's Botany Departmental Herbarium. The nuts were thoroughly washed under running tap to remove stones and other dirt. Thereafter, a portion of the nuts was cooked (categorized as 'processed') on an electric gas cooker for 30 min at 100 °C and the shells were removed after cooking. For the uncooked (categorized as 'raw') portion, their shells were also removed and oven dried along with the cooked walnut sample at 45 °C to a constant weight. The two processed samples were pulverized, defatted in cold n-Hexane and kept in an air-tight container prior to use. Furthermore, the proximate analysis of the samples was carried out (data not shown) which was considered for diet formulation and the 10% and 20% dietary supplemented was calculated based on the percentage protein of the sample.

2.3. Preparation of extract

Ten (10) grams of the defatted samples was weighed in 100 ml of distilled water (1:10 w/v) and soaked for 24 h at room temperature. Thereafter, the solutions were centrifuged (Model KX3400 C) at 3000 × *g* for 30 min and the supernatants were further filtered through a whatman filter paper (no. 2) to obtain clear filtrates. The filtrates were lyophilized (Lab-Kit FD-10-MR model; Lab-Kits, Utherm International, Xiangtan City, Hunan Province, China) and stored in the refrigerator at −4 °C prior to HPLC analysis.

2.4. Quantification of compounds by HPLC-DAD

Reverse phase chromatographic analyses were carried out under gradient conditions using Phenomenex C_{18} column (4.6 mm × 150 mm) packed with 5 μm diameter particles; the mobile phase was water containing 2% formic acid (A) and acetonitrile (B), and the composition gradient was: 5% of B until 10 min and changed to obtain 20%, 40%, 70%, 20% and 2% B at 20, 30, 40, 50 and 60 min, respectively, following the method described by Pinho et al. [22] with slight modifications. *T. conophorum* Müll. Arg. aqueous extract was analyzed at a concentration of 15 mg/ml. The presence of some antioxidants compounds was investigated and identification of these compounds was performed by comparing their retention time and UV absorption spectrum with those of the commercial standards. The flow rate was 0.6 ml/min and injec-

Table 1
Diet formulation for basal and supplemented diet for control and test groups.

	Groups				
Components	I (g)	II (g)	III (g)	IV (g)	V (g)
Skimmed milk	37.5	29.4	31.9	21.3	26.3
Oil	10.0	10.0	10.0	10.0	10.0
Vitamin mix.	4.0	4.0	4.0	4.0	4.0
Corn Starch	48.5	46.6	44.1	44.7	39.7
Wal 1	–	10.0	–	20.0	–
Wal 2	–	–	10.0	–	20.0
Total	100.0	100.0	100.0	100.0	100.0

Key: Skimmed milk = 32% protein. One gram of vitamin mixture= 3200 IU vitamin A, 600 IU. vitamin D3, 2.8 mg vitamin E, 0.6 mg vitamin K3, 0.8 mg vitamin B1, 1 mg vitamin B2, 6 mg niacin, 2.2 mg pantothenic acid, 0.8 mg vitamin B6, 0.004 mg vitamin B12, 0.2 mg folic acid, 0.1 mg biotin H2, 70 mg choline chloride, 0.08 mg cobalt, 1.2 mg copper, 0.4 mg iodine, 8.4 mg iron, 16 mg manganese, 0.08 mg selenium, 12.4 mg zinc, and 0.5 mg antioxidant.
Group I – normal control rats, fed with basal diet; Group II – rats fed with basal diet plus 10% processed walnut inclusion; Group III – rats fed diet supplemented with 10% raw walnut; Group IV – rats fed diet supplemented with 20% processed walnut; Group V – rats fed diet supplemented with 20% raw walnut. Wal 1, Processed walnut; Wal 2, Raw walnut.

tion volume was 40 μl. The samples and mobile phase were filtered through 0.45 μm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. Stock solutions of standards references were prepared in the HPLC mobile phase at a concentration range of 0.025–0.500 mg/ml. The chromatography peaks were confirmed by comparing its retention time with those of reference standards and by DAD spectra (200–600 nm). All chromatography operations were carried out at ambient temperature and in triplicates.

2.5. In vivo bioassay

2.5.1. Animals

Forty (40) adult male rats (200–250 g) were procured for the study from the Central Animal House of the Afe Babalola University, Ado Ekiti. Nigeria. The animals were handled according to the guidelines of the National Council for Animal Experiments Control (CONCEA) and in accordance with institutional ethical committee. The animals were housed in stainless steel cages and kept in a room where 12 h light/dark cycle was maintained and they were allowed free access to water and diet throughout the period of the experiment. The animals were kept at room temperature.

2.5.2. Experimental protocol and diet formulation

The rats were acclimatized for two weeks and were randomly divided into five groups of eight animals ($n = 8$) each. Group I (normal control rats fed basal diet), Group II (rats fed diet supplemented with 10% (w/w) processed walnut), Group III (rats fed diet supplemented with 10% (w/w) raw walnut), Group IV (rats fed diet supplemented with 20% (w/w) processed walnut) and Group V (rats fed diet supplemented with 20% (w/w) raw walnut) as presented in Table 1. Daily feed intake was monitored throughout the experiment and the experiment lasted for 14 days. The male rats were given diet freshly formulated according to a modified method of Akinyemi et al. [23] while the adult females (40) used for sexual behavioural assay were fed normal standard diet.

2.6. Sexual behavioural assay

2.6.1. Copulatory behaviour

At the end of the 14-day experimental period, in order to assess the sexual behaviours, estrous female rats were paired with males from experimental groups as described by Thawatchai et al. [17] with slight modifications. Female rats were induced to estrous (estradiol benzoate at a dose of 2 μg kg^{-1} body weight and progesterone, 500 μg kg^{-1} body weight at 48 h and 6 h respectively via subcutaneous route by injection) before the determination of copulatory behaviours. Sexual behaviours were monitored in a separate room for 1 h in a clear plastic box (60 × 60 × 80 cm) and recorded by digital video recording. The assessed sexual parameters include mounting number (the number of mounts without intromission from the time of introduction of the female to the male), mounting latency (the time interval between introduction of the female to the first mount by the male), intromission number (the number of intromissions from the time of introduction of the female until the end of the experiment), and intromission latency (the interval from the time of introduction of the female to the first intromission by the male).

2.6.2. Anxiety behaviour

Using an elevated plus maze (EPM), anxiety linked behaviours were assessed in the male animals ($n = 8$) for 5 min each. The behaviours assessed included the closed arm duration (CAD), open arm duration (OAD), closed arm entries (CAE), open arm entries (OAE), number of transition (T) and frequency of head dip (HD).

2.7. Biochemical analyses

The rats were sacrificed under mild diethyl ether anaesthesia and rapidly dissected. The *corpus cavernosum* was carefully dissected, washed in ice-cold saline, weighed and thereafter, homogenized in phosphate buffer (pH 6.9). The homogenate was centrifuged for 10 min at 5000 × *g* to yield a pellet that was discarded, and a low-speed supernatant (S1) was kept for subsequent analysis. The supernatant so obtained from the homogenized *corpus cavernosum* was used to assay for the in vivo ADA according to Giusti and Gaski [24], AChE [25], arginase [26] activities as well as NO [27] levels. The total protein content of the tissue was also determined by the method of Bradford [28].

2.8. Statistical analysis

Results are presented as means ± SEM or SD of the number of experiments indicated. Statistical significance was assessed by one-way analysis of variance followed by Tukey's test. $p < 0.05$ was considered to represent a significant difference in all experiments. The statistical analyses were performed using the software package GraphPad Prism 5.

3. Results

3.1. Chemical characterization of walnut extracts

In order to identify the phenolic compounds, we characterized walnut (raw, A and processed, B) with reversed-phase HPLC using their retention time and UV spectra in comparison with standard phenolic compounds. HPLC phenolic profile of the walnut extracts revealed the presence of A (raw walnut) to include; gallic acid (t_R = 9.85 min; peak 1), caffeic acid (t_R = 20.57 min; peak 3), *p*-coumaric (t_R = 27.31 min; peak 4), quercetin (t_R = 43.68 min; peak 5), kaempferol (t_R = 45.97 min; peak 6) and luteolin (t_R = 48.25 min; peak 7); while B (processed walnut) revealed the presence of gallic acid (t_R = 9.98 min; peak 1), caffeic acid (t_R = 28.19 min; peak 4) and quercetin (t_R = 49.82 min; peak 7) (Figs. 1 and 5, Table 2).

3.2. Effect of dietary supplementation of walnut (10% and 20%) on ADA, Arginase, AChE activities and NO levels in male rats

The results obtained for the ADA activity in the *corpus cavernosum* of male rats with dietary supplementation of walnut (10%

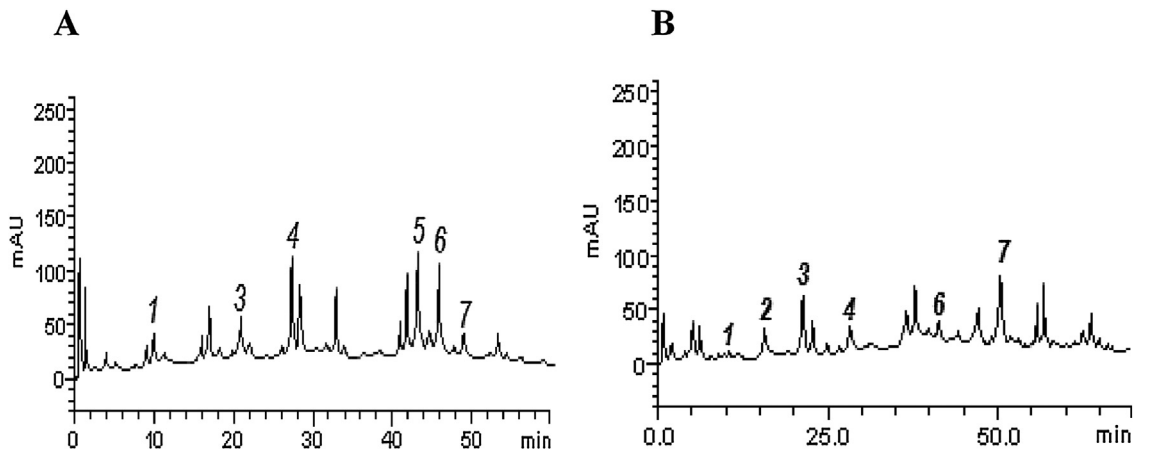

Fig. 1. Representative high performance liquid chromatography profile of *Tetracarpidium conophorum* extracts. A (raw)-Gallic acid (peak 1), caffeic acid (peak 3), *p*-coumaric acid (peak 4), quercetin (peak 5), kaempferol (peak 6) and luteolin (peak 7). B (processed) - Gallic acid (peak 1), caffeic acid (peak 4) and quercetin (peak 7).

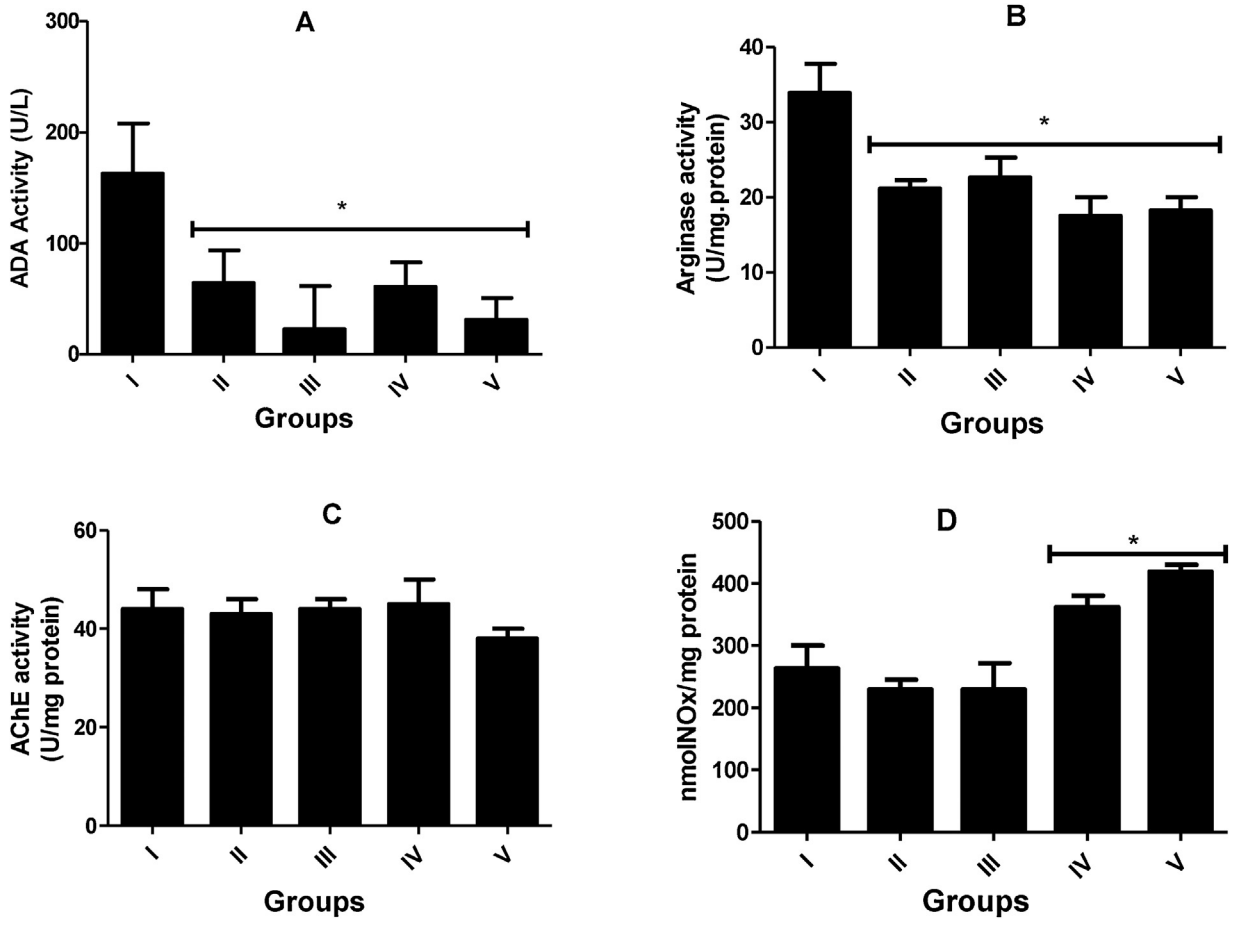

Fig. 2. Adenosine deaminase (A), arginase (B), acetylcholinesterase (C) activities and nitric oxide (D) level in *corpus cavernosum* of rats treated with dietary supplementation with walnut (raw and processed). Data are presented as mean ± SEM ($n = 8$). *Values are significantly ($p < 0.05$) different from control group.
Group I – normal control rats, fed with basal diet; **Group II** – rats fed with basal diet plus 10% processed walnut inclusion; **Group III** – rats fed diet supplemented with 10% raw walnut; **Group IV** – rats fed diet supplemented with 20% processed walnut; **Group V** – rats fed diet supplemented with 20% raw walnut.

and 20%) was significantly different from the control group. A decrease in the activity of ADA (Fig. 2A) was observed. Likewise, arginase activity increased in the control group. However, pre-treated group with dietary supplemented walnut (10% and 20%) caused a decrease in the arginase activity dose dependently when compared to the control group (Fig. 2B). Furthermore, the result of AChE is presented in Fig. 2C. The result revealed that walnut (20%) supplemented groups also had a reduction in the activity of AChE but not significantly different ($p < 0.05$) when compared to the control group. NO level in the *Corpus cavernosum* was increased in walnut dietary supplemented group (20%) with a significant difference ($p < 0.05$) compared to the control group as presented in Fig. 2D. Taking together, raw walnut fed diet showed a better reduc-

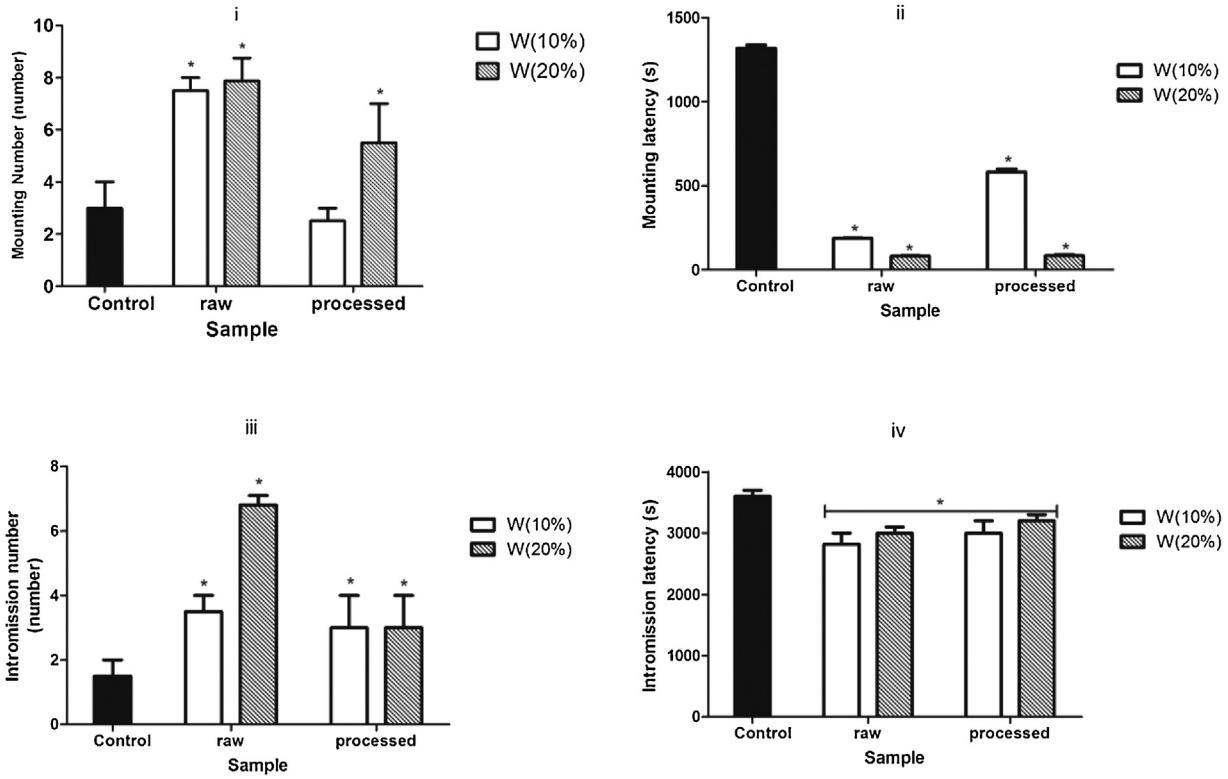

Fig. 3. Effect of dietary supplementation of walnut (10% and 20%) diet fed rats on sexual behaviour (Mounting frequency [i], Mounting latency [ii], Intromission frequency [iii] and Intromission latency [iv]). Data are presented as mean ± SEM ($n = 8$). *Values are significantly ($p < 0.05$) different from control group.

Table 2
Composition of walnut (*Tetracarpidium conophorum* Müll. Arg.) aqueous extract.

Compounds	Raw (mg/g)	Processed (mg/g)
Gallic acid	1.04 ± 0.02^{a}	0.14 ± 0.01^{a}
Caffeic acid	1.19 ± 0.01^{a}	2.09 ± 0.03^{b}
p-Coumaric acid	3.87 ± 0.05^{b}	–
Quercetin	3.91 ± 0.03^{b}	5.38 ± 0.03^{e}
Keampferol	3.75 ± 0.01^{b}	–
Luteolin	0.98 ± 0.01^{a}	–

Results are expressed as mean ± standard deviations (SD) of three determinations. Averages followed by different letters differ by Tukey test at $p < 0.05$. Values with different alphabet superscripts along the column are significantly ($p < 0.05$) different.

tive activities in these enzymes (ADA, AChE and arginase) as well increased NO levels when compared to the processed.

3.3. *Effect of dietary supplementation of walnut (10% and 20%) on sexual behaviours and anxiety in male rats*

Copulatory behaviour of rats after diet supplementation with walnut (10% and 20%) is presented in Fig. 3. The result revealed a significant ($p < 0.05$) increase in both mounting number and intromission number compared to the control group. Mounting latency and intromission latency were also decreased significantly compared to the control ($p < 0.05$). Furthermore, the walnut (10% and 20%) dietary supplemented groups showed significant decrease in anxiety when compared to the control group ($p < 0.05$). Fig. 4 (i–vi) showed an increase in the number of entries in the open and closed arms, time spent in the open arm, transition time and frequency of head dip in rats treated with dietary supplemented walnut (10% and 20%) in the elevated plus maze task when compared to the control group rats ($p < 0.05$).

4. Discussion

The present study investigated the effects of walnut (*T. conophorum* Müll. Arg.) on sexual behaviour and some key enzymes relevant to erection in male rats. HPLC of the walnut extracts revealed its major component to be quercetin. In addition to quercetin, the uncooked walnut was also rich in phenolic compounds such as *p*-coumaric, kaempferol, gallic acid, caffeic acid and luteolin (Figs. 1 and 5, Table 2). It is note worthy that *p*-coumaric, kaempferol and luteolin absence in the processed walnut extract may be attributed to cooking of the walnut sample, of which a report from our laboratory supported processing to affect the polyphenolic profile of nut sample [29]. Quercetin, a dietary flavonoid had been reported by Zhang et al. [30] to ameliorate erectile dysfunction in diabetic rats by inhibiting oxidative stress and moreover, a recent study also revealed quercetin treatment improves arterial erectile dysfunction by up-regulating intra cavernous pressure in Wistar rats [31].

Erectile dysfunction (ED) does not only limit the quality of life but also is a predictor of cardiovascular health and diabetes [32]. ED has been postulated to be caused by a change in a precise balance between endothelium-derived contractile and relaxant factors, which is characterized by decreased nitric oxide (NO)-mediated neurogenic and endothelium-dependent relaxation of *Corpus cavernosum* [33] and negatively influences the daily social interactions and quality of life in men. In our present study, as presented in Fig. 2A, we observed a significant reduction in adenosine deaminase (ADA) in the groups treated with 10% and 20% walnut dietary supplementation as compared to the control group. Investigation by Takahashi et al. [34] found that adenosine increased arterial blood flow and intracavernous pressure, thereby inducing penile erection which indicate that adenosine is capable of inducing penile erection via adenosine receptor signalling. Wu et al. [35] reported that adenosine induced relaxation of the *Corpus cavernosum* of

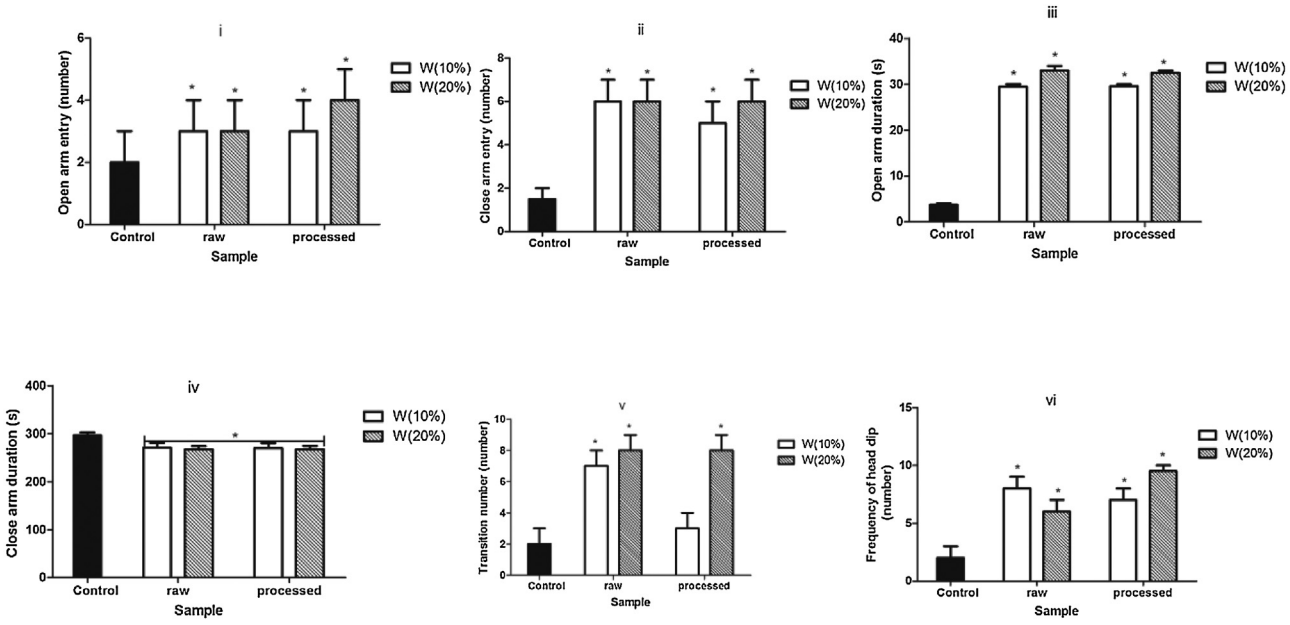

Fig. 4. Effect of dietary supplementation of walnut (10% and 20%) diet fed rats on anxiety behaviour (Open arm [i] and Close arm [ii] entries; Open arm [iii] and Close arm [iv] durations; Transition number [v] and frequency of Head dip [vi]). Data are presented as mean ± SEM ($n = 8$). *Values are significantly ($p < 0.05$) different from control group.

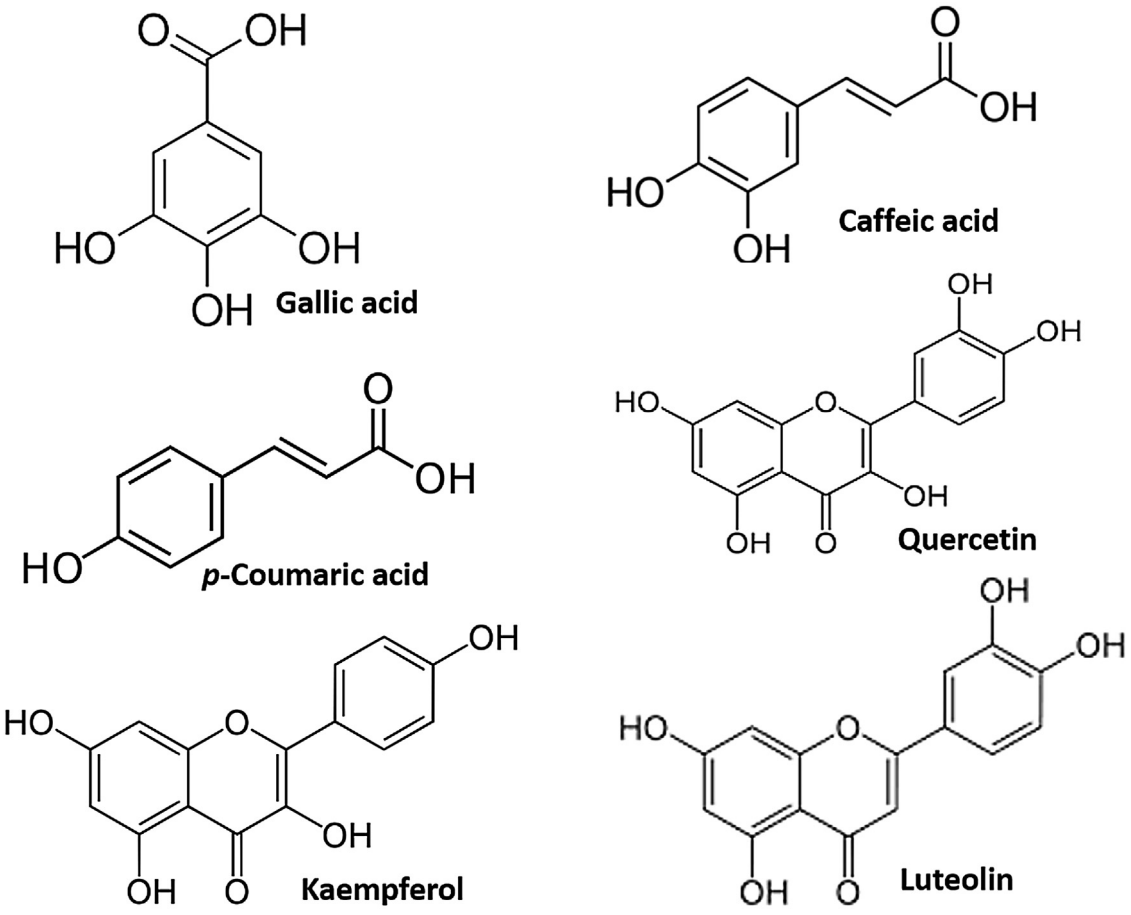

Fig. 5. Graphic for chemical structures of compounds detected using high performance liquid chromatography as highlighted in Table 2 (https://pubchem.ncbi.nlm.nih.gov/compound).

rabbits, and more importantly, Chiang et al. [36] observed dose-dependent inhibition of nerve stimulated contraction of corpus cavernosal strips of rabbits by adenosine and its analogues (5-*N*-ethyl-carboxamidoadenosine or *R*-phenylisopropyl adenosine). Filippi et al. [37] observed that adenosine relaxed the precontracted human corpus cavernous strips in a dose-dependent manner and

at high concentrations almost completely relaxed the tissue. Similarly, adenosine and its analogues induced relaxation in corpus cavernous strips from mice [12,37,38]. Taken together, this study demonstrated the capability of adenosine to induce corpus cavernosal relaxation and subsequently penile erection. We presume by our study that reduction of adenosine deaminase activity might have a direct relaxation effect on the *Corpus cavernosum*. In this sense, its reduction would favour the availability of adenosine and subsequent stimulation of adenosine receptors leading to relaxation of the *Corpus cavernosum* which favours penile erection. Arginase competes with NOS for arginine and therefore decreases the level of cGMP, and arginase inhibition increases cGMP level in penile smooth muscle. From our study, the observed decrease in arginase activity in the diet supplemented group with walnut 10% and 20% is an indicator of NO release causing relaxation of the smooth muscle. This finding is consistence with what was reported by Goswami et al. [39].

Furthermore, the reduction in ADA activity as well as arginase in the rats' *Corpus cavernosum* may be associated with the action of certain phytoconstituents. Reports have shown that phytochemicals could modulate activities of critical enzymes of physiological relevance and has formed the basis of major therapeutic drugs discoveries [40,41]. Chief among these phytochemicals are the polyphenols with potent antioxidant properties and diverse biological activities. Hence, the observed reduction in the ADA and arginase activities in animals fed diet supplemented with walnut may be attributed to the inhibitory effect of its constituent polyphenolics. The result of the present study demonstrated no alterations in acetylcholinesterase activities in *Corpus cavernosum* of the diet supplemented group compared to the control group (Fig. 2). Earlier study in dog showed that intracavernous injection of adenosine induced a full erection, which was independent of acetylcholine [42]. This infers that at reduced level of acetylcholine bioavailability, maximum stimulation of the *corpus cavernosum* can occur resulting in relaxation of the smooth muscle. Evaluating the level of nitric oxide revealed a significant increase ($p < 0.05$) in the group supplemented with 20% compared to that of 10% and the control group taking together.

Sexual behaviour (copulation) increased significantly in the groups fed with walnut supplemented diet (raw and processed) as it was evident by decrease in mounting latency and intromission latency as well as increased mounting frequency and intromission frequency (Fig. 3). Mounting latency and frequency are measures of sexual motivation, whereas intromission depends on relaxation of penile smooth muscle as reported by Andersson [13]. Another behavioural aspect that can measure ED status is anxiety [43]. The elevated plus maze task which is commonly used in the evaluation of anxiety revealed a reduced anxiety-like behaviour in rat fed walnut dietary supplement when compared to the control group. Several studies have found that the prevalence of anxiety disorders varies from 2.5% to 37% in males affected with ED [44–46]. Various aspects of anxiety are historically considered in arousal disorders, particularly the vicious circle of anxiety/dysfunction/performance anxiety [47]. The reduction in anxiety-like behaviour in these animals reflected in the decrease of time spent in the open arms compared to the closed arms as well as the increase in the frequency of transition ($p < 0.05$) (Fig. 4). This is an indication that the supplemented diet may have anxiolytic effect. Adenosine bioavailability had been reported to be involved in the regulation of behavioural state [48,49]. It is also worthy of note that phenolic compounds have shown anxiolytic effects in experimental model associated with increase in the anxiety behaviour [50,51]. Therefore, erectile function ability exhibited by walnut in male rats might have been influenced via NO bioavailability through the inhibition of ADA and arginase activities as well as enhanced sexual behaviour. Nevertheless, raw walnut supplement showed a better biological activities dose dependently when compared to the processed. The bioactive phenolic constituents revealed may not be unconnected with the observed biological activities.

5. Conclusion

In conclusion, erectogenic effect of walnut (*T. conophorum* Müll. Arg.) can be attributed to its potential to down-regulate adenosine deaminase and arginase activity as well as increase NO levels which might have been responsible for sexual behaviour enhancement in male rats thereby enabling NO-dependent smooth muscle relaxation. Further studies should be conducted to know the efficacy of walnut in ED-induced animal model.

Declaration of interest

None.

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References

[1] H.A. Feldman, I. Goldstein, D.G. Hatzichristou, et al., Impotence and its medical and psychosocial correlates: results of the Massachusetts Male Aging Study, J. Urol. 151 (1994) 54–61.
[2] E.O. Laumann, A. Paik, R.C. Rosen, Sexual dysfunction in the United States: prevalence and Predictors, J. Am. Medic. Assoc. 281 (1999) 537–544.
[3] J.E. Morley, Impotence, Am. J. Med. 80 (1986) 897–905.
[4] I.A. Ayta, J.B. Mckinlay, R.J. Krane, The likely worldwide increase in erectile dysfunction between 1995 and 2025 and some possible policy consequences, BJU Inter. 84 (1999) 50–56.
[5] A.A. Olabiyi, G. Oboh, S.A. Adefegha, Effect of dietary supplementation of tiger nut (*Cyperus esculentus* L.) and walnut (*Tetracarpidium conophorum* Müll. Arg.) on Sexual behavior, Hormonal level and Antioxidant status in normal male rats, J. Food Biochem. 14 (3) (2016) 1–9.
[6] A.A. Olabiyi, F.B. Carvalho, N.B. Bottari, V.M. Morsch, A.F. Morel, G. Oboh, M.R. Schetinger, Tiger nut and walnut extracts modulate extracellular metabolism of ATP and adenosine through the NOS/cGMP/PKG signalling pathway in kidney slices, Phytomed. 43 (2018) 140–149.
[7] W.W. Wang, C.P. Jenkinson, J.M. Griscavage, et al., Co-induction of Arginase and nitric oxide synthase murine macrophages activated by lipopolysaccharide, Biochem. Biophys. Res. Commun. 210 (1995) 1009–1016.
[8] G. Cirino, F. Fusco, C. Imbimbo, et al., Pharmacology of erectile dysfunction in man, Pharmacol. Ther. 111 (2006) 400–423.
[9] S.M. McCann, C. Mastronatfi, A. Walczewska, et al., The role of nitric oxide in reproduction, Braz. J. Med. Biol. Res. 32 (1999) 1367–1379.
[10] R.E. Brown, An Introduction to Endocrinology, Cambridge University Press, New York, 2002, pp. 408.
[11] R.C. Cavalli, C.J. Burke, S. Kawamoto, et al., Mutagenesis of rat liver arginase expressed in *Esherichia coli*: role of conserved histidines, Biochemistry 33 (1994) 10652–10657.
[12] K.E. Andersson, Pharmacology of penile erection, Pharmacol. Rev. 53 (2001) 417–450.
[13] R.C. Dean, T.F. Lue, Physiology of penile erection and pathophysiology of erectile dysfunction, Urol. Cli. North Am. 32 (2005) 379–395.
[14] S. Patole, J. Lee, P. Buettner, et al., Improved oxygenation following adenosine infusion in persistent pulmonary hypertension of the newborn, Biol. Neonate 74 (1998) 345–350.
[15] S. Filippi, M. Mancini, S. Amerini, et al., Functional adenosine receptors in human corpora Cavernosa, Int. J. Androl. 23 (2000) 210–217.
[16] C.S. Lin, G. Lin, T.F. Lue, Cyclic nucleotide signaling in cavernous smooth muscle, J. Sex. Med. 2 (2005) 478–491.
[17] P. Thawatchai, W. Jintanaporn, I. Sitthachai, et al., Moringa Oleifera leaves extract attenuates male sexual dysfunction, Am. J. Neuro. 3 (2012) 17–24.
[18] A. Cassidy, M. Franz, E.B. Rimm, Dietary flavonoid intake and incidence of erectile dysfunction, Am. J. Clin. Nutr. (2016), http://dx.doi.org/10.3945/ajcn.115.122010.
[19] S.F. Akomolafe, G. Oboh, S.I. Oyeleye, et al., Aqueous extract from *Ficus capensis* leaves inhibits key enzymes linked to erectile dysfunction and prevent oxidative stress in rats' penile tissue, NFS J. 4 (2016) 15–21.
[20] A.M. Kanu, J.E. Kalu, A.C. Okorie, Nutritional and health values of African Walnut (*Tetracarpidium conophorum*), Int. J. Scientific Technol. Res. 4 (2015) 215–220.
[21] S. Javidanpour, S.R.F. Tabtabaei, A. Siahpoosh, et al., Comparison of the effects of fresh leaf and peel extracts of walnut (*Juglans regia* L.) on blood glucose and

β-cells of streptozotocin-induced diabetic rats, Vet. Res. Forum 3 (2012) 251–255.
[22] F.V.S.A. Pinho, L.C. da Cruz, N.R. Rodrigues, et al., Phytochemical composition antifungal and antioxidant activity of *Duguetia furfuracea* A. St. -Hill, Oxidative Med. Cellul. Longev. (2016) 1–9.
[23] A.J. Akinyemi, A.O. Ademiluyi, G. Oboh, Inhibition of angiotensin-1-converting enzyme activity by two varieties of ginger (*Zingiber officinale*) in rats fed a high cholesterol diet, J. Med. Food 17 (2014) 317–323.
[24] G. Guisti, B. Galanti, Colorimetric method, in: H.U. Bergmeyer (Ed.), Methods of Enzymatic Analysis, Verlag Chemie, Weinheim, 1984, pp. 315–323.
[25] G.L. Ellman, K.D. Courtney, V. Andres, et al., A new and rapid colorimetric determination of acetylcholinesterase activity, Biochem. Pharmacol. 7 (1961) 88–95.
[26] C. Zhang, T.W. Hein, W. Wang, et al., Constitutive expression of arginase in microvascular endothelial cells counteracts nitric oxide mediated vasodilatory function, FASEB J. 15 (2001) 1264–1266.
[27] K.M. Miranda, M.G. Espay, D.A. Wink, A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite, Nitric Oxide Biol. Chem. 5 (2001) 62–71.
[28] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[29] A.A. Olabiyi, F.B. Carvalho, N.B. Bottari, et al., Dietary supplementation of tiger nut alters biochemical parameters relevant to erectile function in L-NAME treated rats, Food Res. Int. 109 (2018) 358–367.
[30] W. Zhang, Y. Wang, Z. Yang, et al., Antioxidant treatment with quercetin ameliorates erectile dysfunction in streptozotocin induced diabetic rats, J. Biosci. Bioeng. 112 (2011) 215–218.
[31] Y. Zhang, C. Huang, S. Liu, et al., Effects of quercetin on intracavernous pressure and expression of nitrogen synthase isoforms in arterial erectile dysfunction rat model, Inter. J. Cli. Exp. Med. 8 (2015) 7599–7605.
[32] S. Dalaklioglu, N. Kuscu, C. Celik-Ozenci, et al., Chronic treatment with taurine ameliorates diabetes-induced dysfunction of nitric oxide-mediated neurogenic and endothelium-dependent corpus cavernosum relaxation in rats, Fundam. Clin. Pharmacol. 28 (2014) 394–404.
[33] T. Yamada, K. Hara, H. Umematsu, et al., Erectile dysfunction and cardiovascular events in diabetic men: a meta-analysis of observational studies, PLoS One 7 (2012) 436–473.
[34] Y. Takahashi, N. Ishii, T.F. Lue, et al., Effects of adenosine on canine penile erection, J. Urol. 148 (1992) 1323–1325.
[35] H.Y. Wu, G.A. Broderick, J.K. Suh, et al., Effects of purines on rabbit corpus cavernosum contractile activity, Int. J. Impot. Res. 5 (1993) 161–167.
[36] P.H. Chiang, N. Wu, E.M. Tsai, et al., Adenosine modulation of neurotransmission in penile erection, Br. J. Clin. Pharmacol. 38 (1994) 357–362.
[37] S. Filippi, S. Amerini, M. Maggi, et al., Studies on the mechanisms involved in the ATP-induced relaxation in human and rabbit *corpus cavernosum*, J. Urol. 161 (1999) 326–331.
[38] M. Faria, T. Magalhaes-Cardoso, J.M. Lafuente-de-Carvalho, et al., Corpus cavernosum from men with vasculogenic impotence is partially resistant to adenosine relaxation due to endothelial A(2B) receptor dysfunction, J. Pharmacol. Exp. Ther. 319 (2006) 405–413.
[39] S.K. Goswami, M.N. Inamdar, R. Jamwal, et al., Effect of *Cinnamomum cassia* methanol extract and sildenafil on arginase and sexual function of young male wistar rats, J. Sex. Med. 11 (2014) 1475–1483.
[40] L. Chuang, P.L. Albert, Enzyme Inhibition in Drug Discovery and Development: The Good and the Bad, John Wiley & Sons Inc, 2010.
[41] A.A. Olabiyi, G. Oboh, A.J. Akinyemi, et al., Tiger nut (*Cyperus esculentus* L.) supplemented diet modulates key biochemical indices relevant to erectile function in male rats, J. Funct. Food. 34 (2017) 152–158.
[42] Y. Takahashi, N. Ishii, T.F. Lue, et al., Pharmacological effects of adenosine on canine penile erection, Tohoku J. Exp. Med. 165 (1991) 49–58.
[43] J.H. Medina, H. Viola, C. Wolfman, et al., Overview–flavonoids: a new family of benzodiazepine receptor ligands, Neurochem. Res. 22 (1997) 419–425.
[44] D. Mallis, K. Moysidis, E. Nakopoulou, S. Papaharitou, K. Hatzimouratidis, D. Hatzichristou, Psychiatric morbidity is frequently undetected in patients with erectile dysfunction, J. Urol. 174 (2005) 1913–1916.
[45] J.M. Farre, F. Fora, M.G. Lasheras, Specific aspects of erectile dysfunction in psychiatry, Int J. Impot. Res. 16 (2) (2004) S46–S49.
[46] I.C. Lee, D. Surridge, A. Morales, J.P. Heaton, The prevalence and influence of significant psychiatric abnormalities in men undergoing comprehensive management of organic erectile dysfunction, Int. J. Impot. Res. 12 (2000) 47–51.
[47] W.H. Masters, V.E. Johnson, Human Sexual Inadequacy, Little Brown & Co, Boston, 1970.
[48] D.G. Rainnie, H.C.R. Grunze, R.W. McCarley, et al., Adenosine inhibition of mesopontine cholinergic neurons: implications for EEG arousal, Science 263 (1994) 689–692.
[49] C.M. Portas, M. Thakkar, D.G. Rainnie, et al., Role of adenosine in behavioral state modulation: a microdialysis study in the freely moving cat, Neuroscience 79 (1997) 225–235.
[50] J.M. Gutierres, F.B. Carvalho, M.R. Schetinger, et al., Neuroprotective effect of anthocyanins on acetylcholinesterase activity and attenuation of scopolamine-induced amnesia in rats, Int. J. Dev. Neurosci. 33 (2014) 88–97.
[51] R.M. Maciel, F.B. Carvalho, A.A. Olabiyi, et al., Neuroprotective effects of quercetin on memory and anxiogenic-like behavior in diabetic rats: role of ectonucleotidases and acetylcholinesterase activities, Biomed. Pharmacother. 84 (2016) 559–568.