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Effects of Melatonin and Ethanolic Extract of *Cannabis sativa* leaves on Sexual Behaviour Parameters of Female Wistar Rats

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ABSTRACT: Cannabis is the most commonly abused illicit drugs with medicinal values worldwide. However, there have neither been studies to explain the contribution of oxidative stress on cannabis-related female gonadotoxicity nor the possible ameliorative potential of melatonin on gonadotoxicity in females consuming cannabis. This study investigated the ameliorative property of melatonin on gonadotoxic effects of *cannabis sativa* in female rats. Twenty female rats ($156\text{g} \pm 1.05$) and four male rats ($192\text{g} \pm 1.42$) were assigned into four groups of six animals each (female to male ratio, 5:1), such that the rats in groups I, II, III and IV received orally 1mL of distilled water, 2mg/kg of ethanolic extract of *cannabis sativa* (EECS), 2mg/kg of EECS plus 4mg/kg of melatonin and 4mg/kg of melatonin, respectively. The female sexual behaviour parameters were monitored on days 7 and 14 respectively. Gonadotropin releasing hormone, Luteinizing hormone, follicle stimulating hormone, estradiol, progesterone and prolactin were determined. EECS significantly ($p < 0.05$) reduced darting frequency, hopping frequency, lordosis frequency, licking behavior, GnRH, FSH, LH, estradiol, progesterone and prolactin respectively. However, it increased the darting latency, hopping latency and lordosis latency significantly ($p < 0.05$). All these effects were significantly reduced ($p < 0.05$) when combined with melatonin. The study revealed that EECS decreased sexual behaviour parameters which was ameliorated by melatonin.

Key words: *Cannabis-sativa*, melatonin, reproductive hormones, sexual behaviour

Introduction

According to the International Programme on Chemical Safety (IPCS), endocrine disruptor is exogenous substances that affect the function(s) of the endocrine system (IPCS, 2002). *Cannabis sativa* has been identified as critical signals of the intricate network that control male and female reproduction, at different stages, with direct effects on gonads, and having target on both the hypothalamus and the pituitary (Schuel and Burkman, 2016). There have neither been convincing studies to fully explain the contribution of oxidative stress on cannabis-related female gonadotoxicity, nor has there been an attempt to investigate the possible ameliorative or preventive potential of well-known anti-oxidant supplement

(e.g. melatonin) on gonadotoxicity, reproductive hormonal toxicity, and oxidative stress in human or female animals consuming cannabis.

Cannabis is obtained from the flowering tops, leaves and resin of the female plant of *Cannabis sativa* L. (family *Cannabidaceae*). It is the most commonly abused illicit drugs worldwide (Abdel-Salam, 2016). with medicinal uses (Mechoulam, 1986). The active component, Δ^9 -tetrahydrocannabinol (Δ^9 -THC), has been used for treating migraine headache, glaucoma, nausea, and anorexia (Mechoulam, 1986). However, its detrimental effects on reproductive system have been reported. For instance, it has been shown to be spermatotoxic in male (Adamson *et al.*, 2015) and ovotoxic in female (Alagboni and Olayaki, 2017). It is easily abused by women of childbearing age (Wang *et al.*, 2006). Statistically, it has been reported that about 3.2% of females are cannabis smokers in Nigeria (Adamson *et al.*, 2015). It has also been reported that about 64-79% of female are cannabis users nationwide (Wang *et al.*, 2006) which can lead to pregnancy loss (NSDUH, 2013), low birth weight (Wang *et al.*, 2003), prematurity (Fried *et al.*, 1984), intrauterine growth retardation, presence of congenital abnormalities, prenatal death and delay in the time of commencement of respiration (Sherwood *et al.*, 1999). Cannabinoids have also been reported to have negative effects on the activity of gonadotropin releasing hormone (GnRH)-secreting neurons by direct and indirect mechanisms (Gibson *et al.*, 1983)(Murphy *et al.*, 1994). It also has direct effect on the pituitary gland through its receptors (Gammon *et al.*, 2005). Moreover, it has been shown to have direct oestrogenic effect on the uterus (Wenger *et al.*, 1999) leading to the binding of 3β -estradiol to oestrogen receptors (Solomon *et al.*, 1977). It also has direct effect on the ovary (Tsafiriri *et al.*, 172) leading to the inhibition of ovarian prostaglandin synthesis which is implicated in the mechanism of follicle rupture at ovulation (Rawitch *et al.*, 1977).

Melatonin (N-acetyl-5-methoxytryptamine), is expressed in the darkness because its highest level always coincides with the dark phase of light/dark cycle (Freudenthal *et al.*, 1972), and is secreted in the pineal gland and other extra-pineal sources like retina, gut, skin, bone marrow, lymphocytes, and ovaries (Reiter *et al.*, 2000). Its ability to scavenge free radicals like hydroxyl radical ($\cdot\text{OH}$), singlet oxygen ($^1\text{O}_2$), hydrogen peroxide (H_2O_2), superoxide anion ($\text{O}_2^{\cdot-}$), hypochlorous acid (HOCl), peroxy nitrite anion (ONOO^-), nitric oxide (NO^{\cdot}), and others in many conditions (Slominski *et al.*, 2005) by free radical scavenging actions (Aydogen *et al.*, 2006). Its role in reproduction has been contradictory, as both detrimental and beneficial effects have been reported (Reiter *et al.*, 2003).

One of the important aspects of well being and quality of life for human beings is to have adequate sexual performance (Chemineau *et al.*, 2008). In some cases, sexual function in females may be hindered and sexual performance unsatisfactory due inadequate functioning of the reproductive system. Failure to have satisfactory sexual performance in females is referred to as female sexual dysfunction (FSD) (Walton and Thorton, 2003). FSD is expressed as chronic (3 months or more) lack of desire in sexual activity, persistent or inability to attain or maintain sexual excitement, chronic difficulty in attaining orgasm following sufficient arousal, involuntary vagina spasm that interferes with penetration, pain during intercourse and genital pain following stimulation during foreplay that occur can during the reproductive age of 21–45 years (González *et al.*, 2006). Thus, there is need to educate people on the plant which can cause FSD. This study therefore, investigated the gonadotoxic effects of *cannabis sativa* and melatonin on libido, pregnancy and reproductive hormones in female rats.

Materials and Methods

Animals

Twenty four albino rats were used for this experiment. Twenty (20) were female rats ($156\text{g} \pm 1.05$) and Four (4) were males ($192\text{g} \pm 1.42\text{g}$). They were obtained from the Department of Biochemistry, University of Ilorin, Ilorin, Kwara State, Nigeria and were housed in cages at the animal holding of the faculty of Basic Medical Sciences, University of Ilorin, at room temperature with unrestricted access to diet and water and maintained on a daily light/dark cycle. Principles of laboratory animal care (NIH publication No. 85-23, revised 1985) were followed.

Drug, Plant sample and assay kits

Cannabis sativa(CS), was kindly donated by National Drug Law Enforcement Agency (NDLEA), Nigeria, for research purpose only, Melatonin was a product of Sigma Aldrich Company, Mannheim, Germany. The gonadotropin releasing hormone (GnRH), luteinizing hormone (LH), follicle stimulating hormone (FSH), estradiol (E), progesterone and prolactin assay kits were products of Monobind Inc., Lake Forest, California, USA. All other chemicals used were products of Sigma Aldrich Company, Mannheim, Germany.

Extraction of Cannabis sativa leaves

Extraction of *Cannabis sativa* (CS) was done with Soxhlet apparatus by soaking 600 grams of CS in 98% ethanol for 48 hours. It was filtered and the filtrate was poured into a round bottom conical flask which was fixed with a rotary evaporator. It was then evaporated and cooled. The dried yield of the extract was 40.3%.

Screening of secondary metabolites

A preliminary screening of secondary metabolites in the CS leaves was carried out for alkaloids, tannins, flavonoids and phenolics according to the method described by Kokate, (1997), saponins, steroids and terpenoids according to the method described by Sofowora, (1993). The detected secondary metabolites were quantified by adopting standard procedures described by Hudson and El- Difrawi 1979 and Ying and Wan, 2012, for saponins, flavonoids, phenolics, tannins, steroids and alkaloids respectively.

Experimental protocol

After 2 weeks of acclimatization, animals were randomized into four groups (I–IV) of six animals each, female to male ratio (5:1). Animals in Groups I, II, III and IV were given orally 1mL of distilled water, 2mg/kg body weight (BW) of EECS, 2mg/kg BW of EECS plus 4mg/kg BW of melatonin and 4mg/kg BW of melatonin, respectively. The doses were administered once daily for 14 days. The animals were sacrificed 24hrs after the 14th daily dose.

Mating behaviour test

The procedure described by Giuliano *et al.*, (1999) was adopted for the assessment of mating behaviour in the female rats. The mating behaviour was carried out on all the female rats in all the groups by placing each in an opened plastic cage with the male in that group and this was done between 5:30am-7:30am local time. The following female sexual behaviour parameters were monitored on days 7 and 14:

- i. Proceptive sexual behaviour of (1) darting latency (DL, a short run that is characterized by sudden stoppage and exposure of the posterior parts to the male rat), (2) darting frequency (DF, number of darting runs during the period of observation), (3) hopping latency (HL, a short jump with stiff legs followed by immobility and a presenting behaviour) and (4) hopping frequency (HF, number of hops during the period of observation).
- ii. Receptive sexual behaviour of (1) lordosis latency (LL, time taken to exhibit posture that allows mounting by the male) and (2) lordosis frequency (LF, number of lordosis postures during the period of observation).
- iii. Orientational activity of (1) licking behaviour (LB, number of time the female rat lick its private part to show sign of sexual arousal. The experimental protocol was approved by Ethical Committee of the University of Ilorin, Ilorin, Kwara State, Nigeria (Ref. UERC/ASN/2018/1152), University of Ilorin, Ilorin, Nigeria.

Preparation of serum

The procedure described by Yakubu *et al.*, (2005) was adopted for the preparation of the serum samples. The female rats were sacrificed under ketamine anaesthesia and blood was collected by cardiac puncturing into sample bottles. The blood was left for 30 min to clot and thereafter centrifuged at 625×g for 10 min using a Uniscope Laboratory Centrifuge (Model SM800B, Surgifield Medicals, Essex, England). The serum was collected into plain bottles with the aid of a Pasteur pipette. Sera were stored in a freezer maintained at -4 °C and used within 12 hours of preparation.

Quantification of reproductive hormones

The serum hormone concentrations of GnRH, FSH, LH, E, progesterone and prolactin were quantified according to the instruction provided by assay kit manufacturers, using microplate immunoenzymometric (EMA/ELISA) assays. The serum hormone concentrations were then interpolated from their respective calibration curves. The analyzer was calibrated and validated for use with rat sera.

Statistical analyses

Results were expressed as the mean ± standard error of mean. Data were analyzed using a one-way analysis of variance, followed by the LSD post-hoc test to determine significant differences in all the parameters with Students Package for Social Science, version 20.0 (SPSS Inc., Chicago, USA). Differences with values of $p < 0.05$ were considered statistically significant.

Results

Secondary metabolites

The secondary plant metabolites content of the aqueous extract of CS leaves revealed, in order of decreasing abundance, terpenoids (14.62 mg/kg), flavonoids, phenols, tannins, alkaloids saponins and steroids (Table 1).

Table 1: Secondary metabolites constituents of *Cannabis sativa* leaves

| Secondary metabolites | Concentration (mg/kg) |
|------------------------------|------------------------------|
| Flavonoids | 4.38 |
| Alkaloids | 5.10 |
| Terpenoids | 14.62 |
| Phenolics | 6.34 |
| Saponins | 1.37 |
| Steroids | 0.48 |
| Tannins | 5.32 |

Female sexual behaviour

The administration of 2 mg/kg body weight (b.w) of the CS extract significantly ($p < 0.05$) decreased the darting frequency (DF), hopping frequency (HF), lordosis frequency (LF), and licking behaviour (LB) when compared with the distilled water-treated, cannabis plus melatonin treated and melatonin-treated female rats (Tables 2–5). In contrast, the darting latency (DL), hopping latency (HL), and lordosis latency (LL) following the administration of 2 mg/kg b.w of CS extract increased significantly ($p < 0.05$) compared with the distilled water-treated, cannabis plus melatonin treated and melatonin-treated female rats (Tables 2–5).

Table 2: DF and DL of rats following the administration of ethanolic extract of Cannabis sativa (EECS) and/or melatonin

| Group | Darting Frequency (DF) | | Darting Latency (DL) | |
|----------------------|---------------------------|--------------------------|-----------------------------|-----------------------------|
| | Day 7 | Day 14 | Day 7 | Day 14 |
| Control | 4.40 ± 0.40 | 2.40 ± 0.25 | 125.26 ± 1.83 | 123.78 ± 1.61 |
| Cannabis | 1.80 ± 0.37 [*] | 1.60 ± 0.26 [*] | 148.40 ± 3.49 [*] | 151.22 ± 2.90 [*] |
| Cannabis + Melatonin | 3.40 ± 0.51 ^{*#} | 2.40 ± 0.32 | 132.10 ± 1.49 [#] | 132.66 ± 2.29 ^{*#} |
| Melatonin | 2.20 ± 0.30 [*] | 1.60 ± 0.25 [*] | 139.00 ± 3.32 ^{*#} | 139.80 ± 2.67 ^{*#} |

Table 3: HF and HL of rats following the administration of EECS and/or melatonin

| Group | Hopping Frequency (HF) | | Hopping Latency (HL) | |
|----------------------|--------------------------|--------------------------|------------------------------|-----------------------------|
| | Day 7 | Day 14 | Day 7 | Day 14 |
| Control | 2.80 ± 0.49 | 2.00 ± 0.32 | 50.14 ± 3.06 | 45.54 ± 2.71 |
| Cannabis | 1.00 ± 0.00 [*] | 1.00 ± 0.00 [*] | 173.60 ± 0.93 [*] | 163.00 ± 1.61 [*] |
| Cannabis + Melatonin | 2.80 ± 0.37 [#] | 1.80 ± 0.20 | 87.46 ± 7.66 ^{*#} | 60.67 ± 15.09 |
| Melatonin | 1.60 ± 0.25 [*] | 1.40 ± 0.25 | 128.98 ± 17.78 ^{*#} | 119.38 ± 17.67 [*] |

Table 4: LF and LL of rats following the administration of EECS and/or melatonin

| Group | Lordosis Frequency (LF) | | Lordosis Latency (LL) | |
|----------------------|--------------------------|---------------------------|-----------------------------|-----------------------------|
| | Day 7 | Day 14 | Day 7 | Day 14 |
| Control | 1.60 ± 0.25 | 1.60 ± 0.25 | 164.20 ± 2.20 | 157.80 ± 2.22 |
| Cannabis | 0.20 ± 0.25 [*] | 0.60 ± 0.25 [*] | 131.06 ± 2.96 [*] | 127.64 ± 2.17 [*] |
| Cannabis + Melatonin | 1.60 ± 0.20 [#] | 1.00 ± 0.00 ^{*#} | 123.24 ± 1.44 ^{*#} | 120.00 ± 1.37 ^{*#} |
| Melatonin | 1.00 ± 1.23 [*] | 0.80 ± 0.20 [*] | 156.80 ± 1.39 ^{*#} | 151.00 ± 1.00 ^{*#} |

Table 5: Licking Behaviour (LB) of rats following the administration of EECS and/or melatonin

| Group | Licking Behaviour (LB) | |
|----------------------|--------------------------|--------------------------|
| | Day 7 | Day 14 |
| Control | 2.20 ± 0.37 | 1.40 ± 0.25 |
| Cannabis | 0.60 ± 0.25 [*] | 0.60 ± 0.25 [*] |
| Cannabis + Melatonin | 1.60 ± 0.25 [#] | 1.40 ± 0.25 [#] |
| Melatonin | 1.20 ± 0.20 [*] | 1.00 ± 0.00 |

Table 6: Serum reproductive hormones of female Wistar rats following oral administration of EECS and/or melatonin

| | GnRH (pg/ml) | FSH (mIU/ml) | LH (mIU/ml) | Oestradiol (pg/ml) | Progesterone (ng/ml) | Prolactin (ng/ml) |
|----------------------|----------------|---------------|---------------|--------------------|----------------------|-------------------|
| Control | 32.24 ± 3.93 | 7.83 ± 1.5 | 27.00 ± 1.97 | 75.00 ± 11.07 | 13.00 ± 0.63 | 16.82 ± 2.39 |
| Cannabis | 11.38 ± 0.77* | 5.04 ± 0.95* | 4.17 ± 0.20* | 38.32 ± 11.2* | 2.78 ± 0.36* | 7.82 ± 0.59* |
| Cannabis + Melatonin | 21.75 ± 0.96*a | 6.93 ± 1.14*a | 6.59 ± 1.02*a | 63.32 ± 9.42*a | 7.92 ± 1.17*a | 9.18 ± 1.12*a |
| Melatonin | 29.43 ± 1.32* | 7.17 ± 0.79* | 6.86 ± 1.04a | 71.68 ± 6.39a | 8.41 ± 1.09*a | 9.50 ± 0.88*a |

n=5

*p<0.05 vs control, ap < 0.05 vs cannabis

Discussion

Studies have shown that secondary plant metabolites like alkaloids, flavonoids, phenolics and saponins have aphrodisiac activities (Modelska and Milián, 2004) while some may stimulate the level of sex hormones, others might enhance the action of these sex hormones in the body. Thus, the decrease in the quantity of alkaloids, flavonoids, phenolic and saponin observed in the cannabis-treated group revealed that cannabis decreases aphrodisiac activities whereas, when it was administered in combination with melatonin, it ameliorated it, suggesting that melatonin may be stimulatory to aphrodisiac activities. Results from this study shows a significant decrease ($p < 0.05$) in DF, HF, LF and LB, however, DL, HL and LL increased significantly ($p < 0.05$) in the cannabis-treated female rats compared with other groups. This may be due to low level of alkaloids, flavonoids, phenolics and saponins compared with *Anthonotha macrophylla* leaves which has high aphrodisiac activity (alkaloids- 13, flavonoids-6, saponins-4 and phenolics-1.3mg/dl) (da Silva *et al.*, 2012). However, when it was combined with melatonin, it reversed the effects ($p < 0.05$) possibly by acting on the hypothalamic pituitary gonads axis to stimulate LH and FSH secretion which can stimulate sexual behaviour (Yakubu *et al.*, 2016). The decreased in the oestradiol level observed in the cannabis-treated group may be linked to the inhibition of hormone synthesis by the granulosa cells of the growing follicles in the ovary which may be due to decreased FSH level, which decreased the secretion of the hormone and was ameliorated when combined with melatonin. A previous study has shown that suppression of oestrogen receptors in the ventromedial nucleus of the hypothalamus in female rats could also be used to explain reduced proceptivity and receptivity in female rats (Olivier *et al.*, 2011). Therefore, the reduction in these sexual behaviour parameters by cannabis-treated group may be due to the possible decline in the oestradiol content.

Normal female reproductive functions depend on the secretion of LH and FSH by the pituitary gland under the influence of hypothalamic gonadotropin-releasing hormone (GnRH). In females, LH stimulates the theca cells of the ovaries to secrete progesterone while FSH induces the granulosa cells of the growing follicles to produce oestradiol. Therefore, the decreased in the levels of GnRH, LH, FSH and progesterone observed in the cannabis-treated group may be ascribed to an inhibitory effect on the hypothalamic-pituitary axis which was ameliorated when combined with melatonin. The lowering of serum prolactin levels observed in the cannabis-treated group could be due to the antioestrogenic nature of 'THC' (Spiteri *et al.*, 2010) which was ameliorated when combined with melatonin.

Conclusion

This study concluded that EECS decreased female sexual behaviour parameters which could be stimulated by low level of its secondary metabolites and endocrine disruption. However, these effects may be ameliorated by melatonin. Since the consumption of *Cannabis sativa* is increasing globally because of its medical and health benefits that has led to legalization in some parts of the world, thus, the use of melatonin as supplement is recommended for *Cannabis sativa* users to prevent its gonadotoxic effects.

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