



Melatonin Mitigates Oxidative stress in Ethanolic Extract of Cannabis-Treated Female Wistar Rats

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Abstract: *Cannabis sativa* (CS) is one of the commonly abused substances by women of childbearing age. About 3.2% females are cannabis smokers in Nigeria, with varied consequences of congenital abnormalities due to interplay between oxidants, oxidants and antioxidants. This study investigated the effects of melatonin and cannabis on oxidative stress in female rats. Twenty female rats were assigned into four groups of five animals each. Groups I, II, III and IV received orally 1ml distilled water, 2mg/kg body weight (bw) of ethanolic extract of *C. sativa* (EECS), 2mg/kg bw of EECS plus 4mg/kg bw of melatonin and 4mg/kg bw of melatonin, respectively. Serum level of Superoxide dismutase (SOD), catalase, glutathione peroxidase, glutathione reductase, total antioxidant capacity (TAC), lactate dehydrogenase and Malondialdehyde (MDA) were assayed using standard methods. CS significantly ($p < 0.05$) decreased catalase, SOD, glutathione peroxidase, glutathione reductase and TAC levels. But it significantly ($p < 0.05$) increased lactate dehydrogenase and MDA levels. All these effects were ameliorated to the level comparable to the control when the extract was administered in combination with melatonin. CS showed alterations of biochemical parameters which was probably mediated by oxidative stress. However, these effects were ameliorated by melatonin. Since the consumption of CS is increasing globally because of its medical uses, thus, consumption of melatonin as supplement may be recommended.

KEYWORDS: *Cannabis-sativa*, Gonadotoxicity, Melatonin, Oxidative stress

1.0 Introduction

Cannabis sativa (CS) is one of the commonly abused substances by women of childbearing age (Mechoulam, 1986). About 3.2% of females are cannabis smokers in Nigeria (Adamson *et al.*, 2015) which can cause congenital abnormalities, intrauterine growth retardation, prenatal death, miscarriage (Wang *et al.*, 2003), prematurity (Fried *et al.*, 1984) and low birth weight (Gibson *et al.*, 1983; Sherwood *et al.*, 1999).

Oxidative stress (OS) is precipitated by an imbalance between pro-oxidants and antioxidants (Al-Gubory *et al.*, 2010). This ratio can be altered by increased levels of reactive oxygen species (ROS) or a decrease in antioxidant defense mechanisms (Burton and Jauniaux, 2010). Each month, a cohort of

oocytes begins to grow and develop in the ovary, but meiosis I resumes in only one of them, the dominant oocyte. This process is targeted by an increase in ROS and inhibited by antioxidants. In contrast, antioxidants promote the progression of meiosis II (Behrman *et al.*, 2001), suggesting that there is a complex relationship between ROS and antioxidants in the ovary.

Melatonin (N-acetyl-5-methoxytryptamine), is derived from tryptophan (Slominski *et al.*, 2002), and it is secreted by the pineal gland (Yu *et al.*, 1981) and other extra-pineal sources like retina, gut, skin, bone marrow, lymphocytes, and ovaries (Slominski *et al.*, 2008). It is also referred to as a chemical expression of darkness because its peak in the blood of vertebrates always coincides with the dark phase of light/dark cycle. It has the scavenging ability for free radicals like hydroxyl radical ($\text{OH}^\cdot$), singlet oxygen ($^1\text{O}_2$), hydrogen peroxide (H_2O_2), superoxide anion ($\text{O}_2^{\cdot-}$), nitric oxide ($\text{NO}^\cdot$), and

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others in many conditions by acting directly as free radical scavenger (Allegra *et al.*, 2003), and indirectly by stimulating the activities of anti-oxidative enzymes (Tomas- and Coto-Montes, 2005). Thus, reducing the leakage of electrons from the mitochondrial electron transport chain (Leon *et al.*, 2005) along with stimulating the synthesis of glutathione (Winiarska *et al.*, 2006), and synergistically interacting with other anti-oxidants (Lopez-Burillo *et al.*, 2003).

Available information on its role in reproduction is contradictory, as both detrimental and beneficial effects have been reported (Reiter *et al.*, 2003). This study thus investigated the effects of ethanolic extract of *C.sativa* on oxidative stress together with the ameliorative effects of melatonin in female rats.

2.0 Materials and Methods

2.1 Materials

2.1.1 Plant material

Cannabis sativa was a donation from National Drug Law Enforcement Agency (NDLEA) for research purpose only.

2.1.2 Experimental animals

Twenty (20) female Wistar rats (150-160 g) were used for this experiment. They were obtained from the Department of Biochemistry, University of Ilorin, Ilorin, Kwara State, Nigeria; and were housed at room temperature with unrestricted access to diet and water and maintained on 12hr light/dark cycle. Principles of laboratory animal care (NIH publication No. 85-23, revised 1985) were followed. The experimental protocol was approved by Ethical Committee of the University of Ilorin, Ilorin, Kwara State, Nigeria (Ref. UERC/ASN/2018/1152).

2.2 Methods

2.2.1 Extraction of *Cannabis sativa*

Extraction of *Cannabis sativa* was done with Soxhlet apparatus by soaking 500 grams of CS

in 98% ethanol for 48 hours. It was filtered and the filtrate was collected into a conical flask which was fixed with a rotary evaporator. Following evaporation and cooling, the dried yield of the extract was 51%.

2.2.2 Animal grouping

Following 2 weeks of acclimatization, the animals were randomly divided into four groups (I–IV) of five animals each. Group I was administered 1ml of distilled water orally, Group II was administered 2mg/kg body weight (bw) of ethanolic extract of *Cannabis sativa* (EECS), Group III was administered 2mg/kg bw of EECS plus 4mg/kg bw of melatonin and Group IV was administered 4mg/kg bw of melatonin respectively. The agent was administered once daily for a duration of 14 days after which the animals were sacrificed under ketamine anaesthesia. The melatonin was a product of Sigma Aldrich Company, Mannheim, Germany.

2.2.3 Determination of lethal dose

The LD₅₀, was obtained using twenty-four rats that were divided into 4 groups of six each (n=6). Groups 1 to 4 were administered 10 mg/kg, 20 mg/kg, 50 mg/kg, and 100 mg/kg of EECS respectively. The activities of the animals were observed for 5 days post-treatment for signs of toxicity including mortality. The dose of 20 mg/kg was arrived at as the LD₅₀. The dose of 2 mg/kg of CS was arrived at as the tenth of its LD₅₀. The dose of 4 mg/kg of melatonin (Bulk supplements, Henderson, Nevada, USA) was adopted as previously used by She *et al.* (2009).

2.2.4 Preparation of serum

Blood sample was collected from the right ventricle by cardiac puncture, and then centrifuged at 3000 revolution for 10 min using a Uniscope Laboratory Centrifuge (Model SM800B, Surgifield Medicals, Essex, England). The serum was collected into plain bottles with a Pasteur pipette and stored at -5 °C ahead of analysis.

2.2.5 Determination of oxidative stress markers

2.2.5.1 Lactate dehydrogenase

Lactate dehydrogenase (LDH) activity was assayed spectrophotometrically (Spectramax Plus; Molecular Devices, Sunnyvale, CA, USA) following the instructions of the manufacturer of the kit. (product code BXC0243; Fortress Diagnostics, UK). LDH catalyses the conversion of pyruvate to lactate and oxidises NADH to NAD during this process. The rate of decrease in NADH was directly proportional to the LDH activity, and was determined by measurement of rate of absorbance change at 340 nm due to the reduction. The working reagent was prepared by mixing 5 volumes of buffer containing 50 mmol/l of Tris buffer (pH 7.5) and 0.6 mmol/l of pyruvate with 0.18 mmol/l of NADH. Then, 1000 μ L of the working reagent was pipetted into a cuvette containing 20 μ L each of the animal sera. The contents were mixed and incubated for 1 minute. Absorbance was read at 0, 1, 2, and 3 minutes at 340 nm.

LDH activity was calculated as:

$$\text{LDH (U/L)} = 8095 \times \Delta A$$

2.2.5.2 Superoxide dismutase

The determination of serum superoxide dismutase (SOD) concentration was done by colorimetry using SOD (Fortress Diagnostics Ltd., Antrim, UK; Product code: BXC0531) as instructed by the manufacturer. The assay employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals which reacted with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T.) to form a red formazan dye. The SOD activity was then determined by the degree of inhibition of this reaction. The mixed substrate (1.7 ml) was added to microplates containing 50 μ L each of the sera, SOD standards of known concentration, and sample diluent. After mixing, 250 μ L of xanthine oxidase was added, mixed again, and incubated for 30 s at room temperature. Absorbances were read at 0, 1, 2, and 3 min with microplate reader (Spectramax Plus; Molecular Devices, Sunnyvale, CA, USA). The mean absorbance changes per minute (ΔA) were

determined and used in the calculation as follows:

% inhibition of the standards =

$$100 \times \Delta A_{\text{standard}} / \Delta A_{\text{sample diluent}}$$

% inhibition of the serum =

$$100 \times \Delta A_{\text{serum}} / \Delta A_{\text{sample diluent}}$$

The percentage inhibition of each standard was plotted against the Log_{10} of its concentration. Finally, the percentage inhibition of the serum was used to get its SOD concentration from the standard curve.

2.2.5.3 Glutathione peroxidase activity

The determination of serum glutathione peroxidase (GPx) activity was done with GPx colorimetric assay kit (BioVision Inc., Milpitas, CA, USA), by following the manufacturer's instructions. The assay principle is based on its ability to reduce Cumene Hydroperoxide while oxidizing reduced glutathione (GSH) to oxidised glutathione (GSSG). The generated GSSG is reduced to GSH with consumption of NADPH by glutathione reductase (GR). The decrease in NADPH (read at 340 nm) is proportional to GPx activity. Twenty-five (25) μ L of the 40 mM NADPH solution was diluted into 975 μ L distilled water to generate 1mM NADPH standard. Then, 0, 20, 40, 60, 80, and 100 μ L of the 1 mM NADPH standard were added in duplicate into the microplate to generate 0, 20, 40, 60, 80, and 100 nmol/well standard. These were brought to 100 μ L with assay buffer, and absorbance was read at 340 nm to plot the NADPH standard curve. Then, 40 μ L of reaction mixture (consisting of 33 μ L assay buffer, 3 μ L of 40 mM NADPH solution, 2 μ L of GR solution, and 2 μ L of GSH solution) were pipetted into wells containing 50 μ L each of serum and assay buffer (reagent control, RC). It was mixed well, and incubated for 15 minutes to deplete all GSSG in the sample. Ten (10) μ L of Cumene Hydroperoxide solution were then added to start GPx reaction and mixed well. Absorbance was read with microplate reader (Spectramax Plus; Molecular Devices,

Sunnyvale, CA, USA) at 340 nm at T1=0 min and T2=5 min to read A1 and A2.

Change in absorbance $\Delta A_{340nm} =$

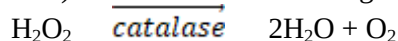
(Serum A1-Serum A2) – (RC A1-RC A2)

This ΔA_{340nm} was then applied to the NADPH standard curve to get the NADPH amount of the serum (B).

GPx activity = B / (T1-T2) x 50 μ L

2.2.5.4 Catalase activity

The continuous catalase activity was determined through spectrophotometric reading (modification of the method used by Claiborne, 1985). The continuous spectrophotometric rate reduction determination (A_{480nm} , light path = 1cm) is based on the following reaction



Absorbance was read at 480nm at interval of 30 seconds for the duration of 3 minutes.

2.2.5.5 Reduced Glutathione concentration

Reduced glutathione (GSH) was measured according to the method of Ellman (1959). Equal quantity of the serum was mixed with 10% trichloroacetic acid and centrifuged to separate proteins. To 0.01ml of this supernatant, 2ml of phosphate buffer (pH 8.4), 0.5 ml of 5, 5-dithio, bis (2-nitrobenzoic acid) and 0.4 ml double-distilled water was added. Mixture was vortexed and the absorbance was taken at 412 nm within 15 min. The concentration of reduced glutathione was expressed as mU/mL.

2.2.5.6 Total antioxidant capacity

Based on the manufacturer's instruction, total anti-oxidant capacity (TAC) measurement in the serum was done with a spectrophotometric microplate reader (Spectramax plus, Molecular Devices, Sunnyvale, CA, USA) using OxiSelect TAC assay kit that uses the single electron transfer mechanism (Cell Biolabs, Inc. San Diego, CA. cat no: STA-360). It is based on the reduction of copper (II) to copper (I) by anti-oxidants such as uric acid. Upon reduction, the

copper (I) ion further reacts with a coupling chromogenic reagent that produces a colour with a maximum absorbance at 490 nm. The net absorbance values of anti-oxidants were compared with a known uric acid standard curve, and are proportional to the sample's total reductive capacity. After adding 20 μ L of serum or uric acid standard to microplate wells, 180 μ L of reaction buffer was added to each well and mixed thoroughly. The initial absorbance was read at 490 nm. The reaction was initiated by adding 50 μ L of the copper ion reagent into each well and then incubated for 5 minutes in an orbital shaker. Then, 50 μ L of stop solution was added to each well to terminate the reaction. The absorbance was read again at 490 nm. Uric acid standard curve was generated by plotting its net absorbance against the uric acid concentration. Then, the uric acid equivalent (UAE) of serum was extrapolated from the standard curve.

2.2.5.7 Malondialdehyde level

The assay method of Hunter *et al.*, (1963), modified by Gutteridge and Wilkins (1982) was adopted. Malondialdehyde (MDA), a product of lipid peroxidation, was estimated by taking 1ml of serum combined with 2ml of TCA-TBA-HCl reagent and mixed thoroughly. Stock solution of TCA-TBA-HCl composed of 15g of TCA, 0.375g of TBA and 0.25N of HCl were prepared. The solution was heated for 15min in a boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at 1000 rpm for 10 min. The absorbance of the supernatant was measured at 532 nm against a blank that contains all the reagents.

2.2.6 Data analysis

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

3.0 Results

Ethanol extract of *C. sativa* (EECS) significantly ($p < 0.05$) decreased the activities of catalase, superoxide dismutase (SOD), glutathione peroxidase, glutathione reductase and total antioxidant capacity (TAC) Figures 1 and 2. However, it also significantly ($p < 0.05$) increased the activities of lactate dehydrogenase and malondialdehyde (MDA) Figure 3 and 4. It was also evident that the co-administration of EECS and melatonin significantly ($p < 0.05$) increased the activities of catalase, SOD, glutathione peroxidase, glutathione reductase and TAC (Figures 5, 6 and 7). Co-administration of EECS and melatonin significantly ($p < 0.05$) decreased the activities of lactate dehydrogenase and MDA.

4.0 Discussion

Oxidative stress occurs when the number of ROS are more than that of anti-oxidants that scavenge them, thus, resulting in pathological effects (Ruder *et al.*, 2009). Stimulation of FSH secretion causes an increase in estrogen, thereby triggering the generation of catalase in the dominant follicle, and thus, preventing apoptosis caused by follicular ROS (Behrman *et al.*, 2002).

Previous studies have established the pro-oxidant role of melatonin (Zhang and Zhang, 2014). One of melatonin's most important features is its ability to act as a free radical scavenger, thus protecting against oxidative stress. Several research works have been done on the importance of melatonin as an antioxidant for the treatment of diseases which are caused by excessive apoptosis, as a result of oxidative stress (Pandey *et al.*, 2003). This study showed a significant increase in lactate dehydrogenase level in the cannabis-treated rats which has played a significant role in the generation of NADPH, a fuel for ROS generation (Said *et al.*, 2005) compared with other groups. Melatonin ameliorated this effect possibly by acting directly and/or indirectly as a hormone preventing attack by free radicals (Reiter *et al.*, 2005). The significant decrease in TAC in cannabis-treated rats compared with other groups showed that oxidative stress was

involved in their gonadotoxic effects. This is consistent with the increase in lactate dehydrogenase activity which was previously observed in the cannabis-treated rats and this could have led to accumulation of ROS. This effect was ameliorated when combined with melatonin, possibly by acting as an antioxidant (Reiter *et al.*, 2005). The study recorded a significant decrease in catalase, SOD, glutathione peroxidase and glutathione reductase levels in the cannabis-treated rats compared with other groups which were ameliorated by melatonin perhaps by potentiating their antioxidant capacities and thus detoxifying most of the ROS produced (Rodriguez *et al.*, 2004).

Malondialdehyde is a product of lipid peroxidation of polyunsaturated fatty acids which are degraded by ROS (Davey *et al.*, 2005). The study also revealed a significant increase in MDA level in the cannabis-treated rats compared with other groups which was ameliorated by melatonin possibly by acting directly and/or indirectly as a hormone preventing attack by free radicals (Reiter *et al.*, 2005).

Conclusion

This study concluded that the gonadotoxic effects of CS could be partially mediated by oxidative stress. However, these effects may be ameliorated by melatonin. Since the consumption of *C. sativa* is increasing globally because of its health benefits which have led to legalization in some parts of the world. Thus, the use of melatonin as supplement, could be recommended for *C. sativa* consumers to prevent its deleterious effects.

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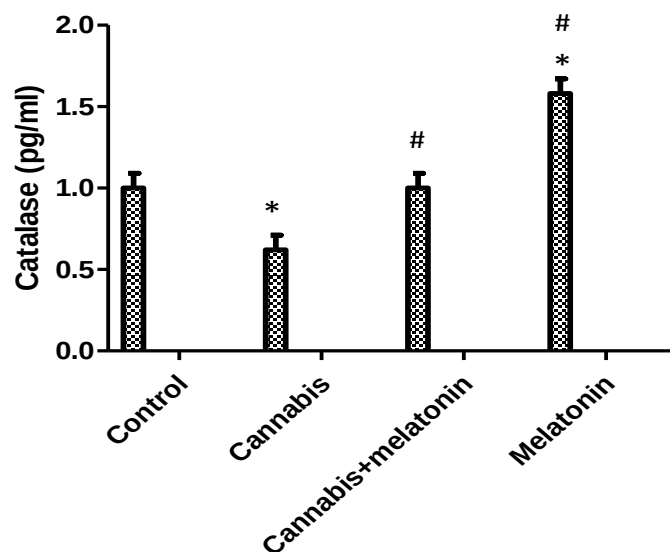


Figure 1: Catalase activity of rats that received EECS with or without melatonin, and melatonin alone.

Values are expressed as mean $\pm$ Standard Error of Mean; * p <0.05 vs Control; # p <0.05 vs cannabis; EECS: Ethanolic Extract of *Cannabis sativa*;

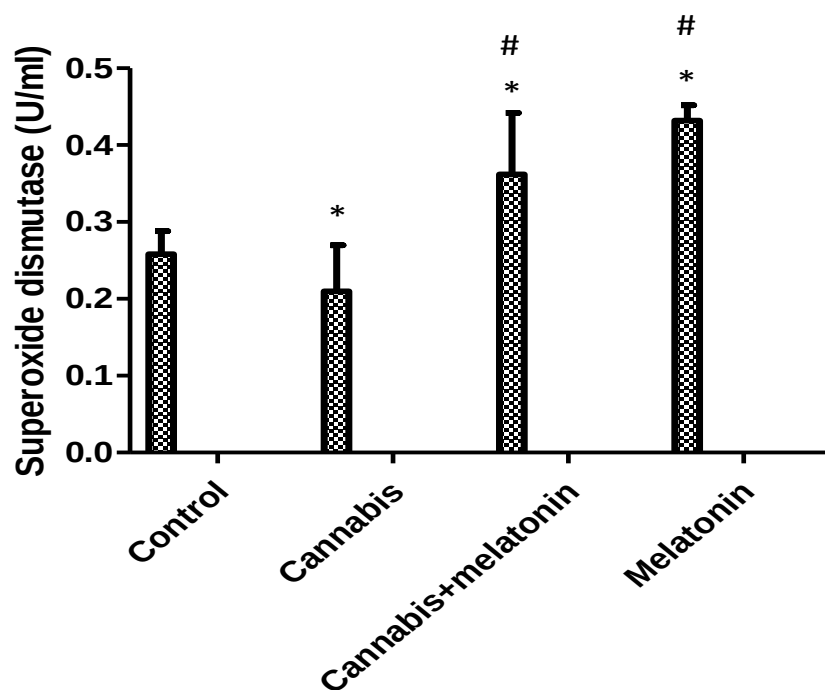


Figure 2: SOD activity of rats that received EECS with or without melatonin, and melatonin alone.

Values are expressed as mean $\pm$ Standard Error of Mean; * p <0.05 vs Control; # p <0.05 vs cannabis; SOD: Superoxide dismutase; EECS: Ethanolic Extract of *Cannabis sativa*;

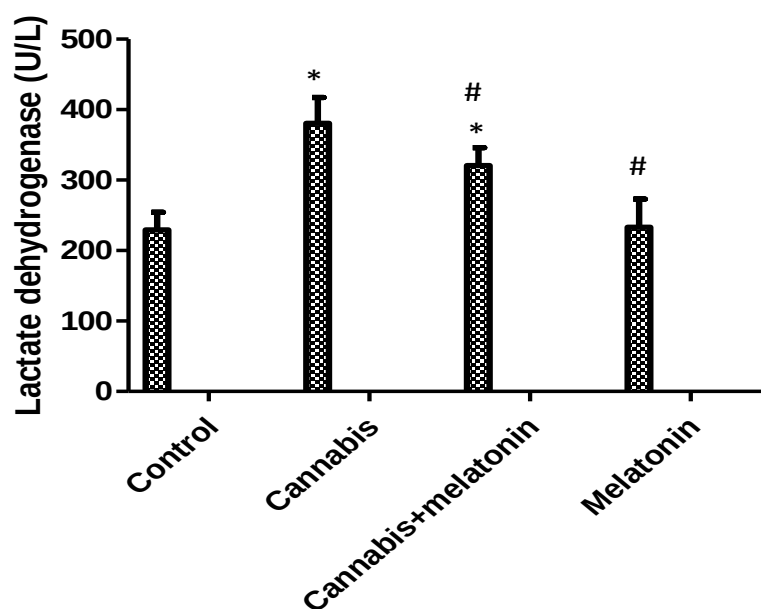


Figure 3: Lactate dehydrogenase activity of rats that received EECS with or without melatonin, and melatonin alone.

Values are expressed as mean $\pm$ S.E.M; * p <0.05 vs Control; # p <0.05 vs cannabis; EECS: Ethanolic Extract of *Cannabis sativa*

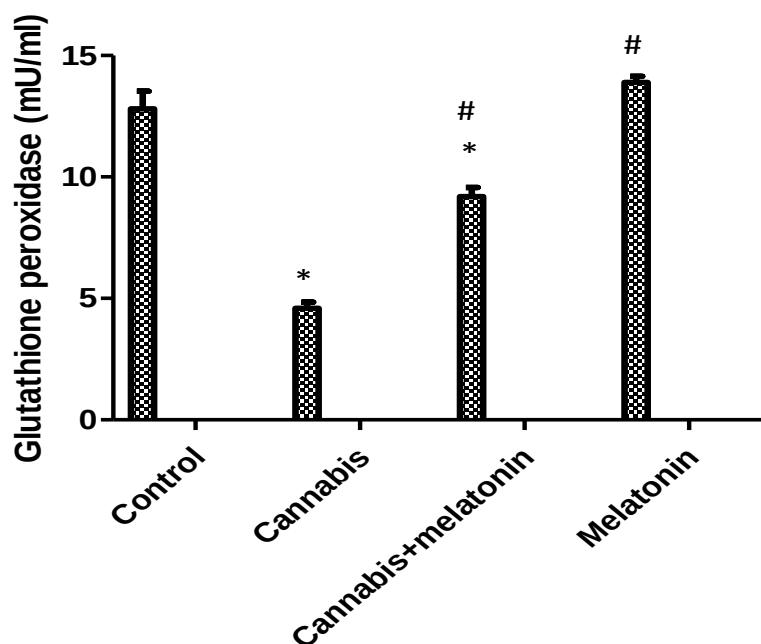


Figure 4: Glutathione peroxidase activity of rats that received EECS with or without melatonin, and melatonin alone.

Values are expressed as mean $\pm$ S.E.M; * p <0.05 vs Control; # p <0.05 vs cannabis; EECS: Ethanolic Extract of *Cannabis sativa*

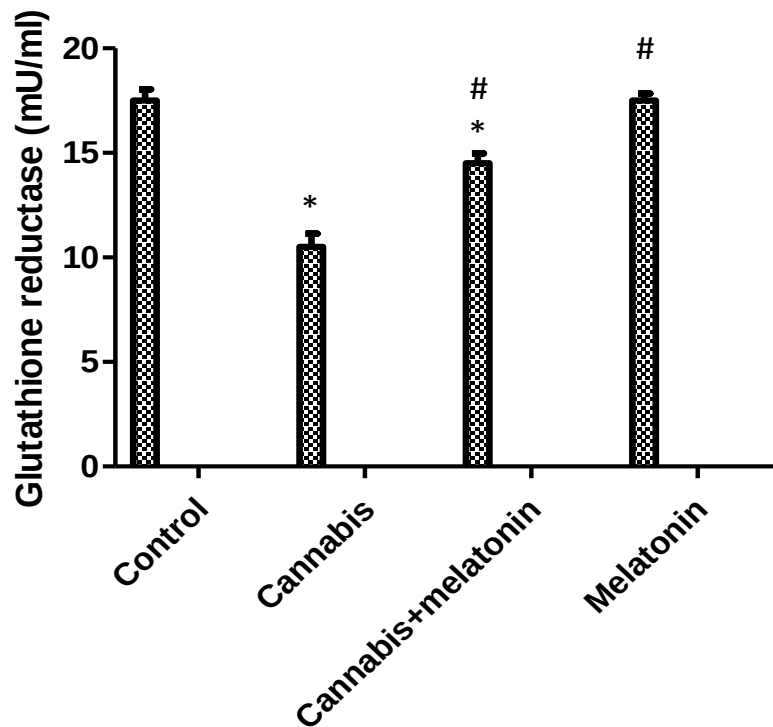


Figure 5: Glutathione reductase activity of rats that received EECS with or without melatonin, and melatonin alone.

Values are expressed as mean $\pm$ S.E.M; * $p < 0.05$ vs Control; # $p < 0.05$ vs cannabis; EECS: Ethanolic Extract of *Cannabis sativa*

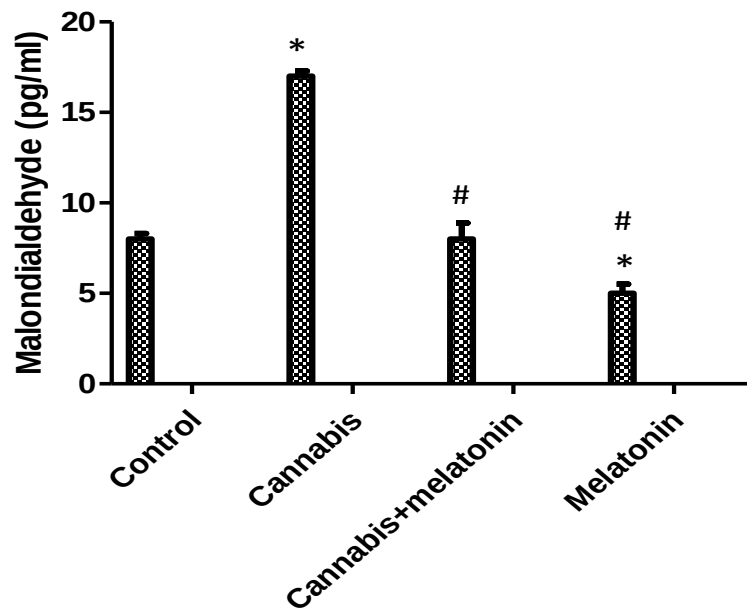


Figure 6: MDA activity of rats that received EECS with or without melatonin, and melatonin alone. Values are expressed as mean $\pm$ S.E.M; * $p < 0.05$ vs Control; # $p < 0.05$ vs cannabis; MDA: Malondialdehyde; EECS: Ethanolic Extract of *Cannabis sativa*

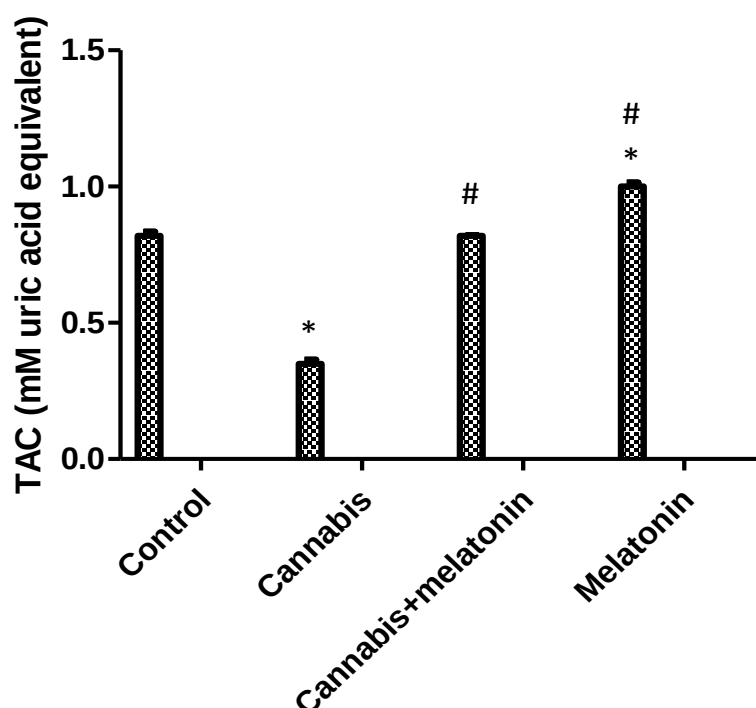


Figure 7: TAC activity of rats that received EECS with or without melatonin, and melatonin alone.

Values are expressed as mean $\pm$ S.E.M; * $p < 0.05$ vs Control; # $p < 0.05$ vs cannabis; EECS: Ethanolic Extract of *Cannabis sativa*

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