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Anti-inflammatory and analgesic properties of *Cannabis sativa* L. leaf ethanol extract in animal model

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ABSTRACT

These studies evaluate the anti-nociceptive and anti-inflammatory properties of Cannabis sativa leaf ethanol extract and to determine its activity at graded doses using animal models. The extract was obtained, rinsed, air dried, macerated and prepared into aqueous extract. Swiss albino mice and Wistar rats were sundered into four groups (n=4). The reference drugs (indomethacin, diphenhydramine, dexamethasone aspirin, and pentazocine), the negative control, and the test groups were administered 15 and 30 mg/kg of the extracts. The results for the analgesic study, elicited a significant decrease in the number of writhes triggered by acetic acid compared with the control ($p< 0.05$). The hot plate-induced test has a significant increase in the percentage inhibition of pain at 30 and 60 minutes when compared with untreated control ($p< 0.05$). Graded doses of the extract (15 and 30 mg/kg) exhibited a significant reduction in formalin-induced pain when compared to the control ($p<0.05$). The anti-dematogenic effects of the extract at dose 15 and 30 mg/kg had a significant reduction in paw-edema carrageenan-induction at 1, 4, 5 hours and dextran-induced inflammation compared with the control. More so, graded doses of the extract inhibited xylene ear edema with a significant reduction compared to the control ($p< 0.05$). This study revealed the effect of Cannabis sativa effective as a potent anti-inflammatory and analgesic agent, hence required further investigation of the possible mechanism and compound implicated for the action.

***Keywords*:** Anti-inflammatory, analgesic, *Cannabis sativa*, wistar rats.

INTRODUCTION

Cannabis sativa L. (*Cannabis*) is also referred to as marijuana and usually grown worldwide. It belongs to family *Cannabaceae*. Over time, several varieties of *Cannabis* have been discovered, from selection and breeding programmes. Although, the *Cannabis* derived from these method opens room for further controversies in botanical classification. The genus was separated into three species namely: *C. sativa* L., *C. indica* Lam. and *C. Ruderalis*. The plant is an annual plant, which has its male as well as its female reproductive system in different plants. From when it was discovered, its use has been hinged on both recreational use and for the relief of pain. Apart from being a stimulant as well as sedative, the plant has active ingredients found in the resin of the plant. Over 538 chemicals of several classes have been reported to come from *Cannabis* (ElSohly and Slade, 2005). Amongst the several classes, the most relevant of them includes non-cannabinoids, cannabinoids, steroids, simple esters, nitrogenous compounds, terpenoids, phenols, glycoproteins, fatty acids, simple acids, simple aldehydes, simple ketones, hydrocarbons, vitamin; usually vitamin K, sugars and related compounds, lactones, amino acids and proteins, simple alcohols, enzymes, certain elements, flavonoids, pigments. The most important medicinal uses of *Cannabis sativa* are for analgesic purposes and also to ease certain nervous disorders. It is also used in the management of gout, insomnia, glaucoma, epilepsy, rheumatism, neuralgia etc. Tetrahydrocannabinol (THC) can be used to treat certain ailments such as; glaucoma, nausea cachexia, pains, glaucoma and for making child birth easier (Cohen and Andrysiak, 1982; Martin *et al*., 1993).

Despite the numerous importance of *C. sativa*, it is however banned and illegalized worldwide except in countries like Uruguay. It remains a controversy as to whether it should be legalized or not. However, most of the unfavourable claims against the use of marijuana were disproved by some research findings (Cherek *et al.*, 1993; Brick, 1990; Carter, 1980; Carter and Doughty, 1976). It is therefore very necessary and important to explore the good aspects and important uses of *C. sativa*. In this regard, the aim of this research was to access its analgesic and anti-inflammatory properties.

MATERIALS AND METHODS

Plant material and extraction

Cannabis sativa fresh leaves were collected from Ovia North East Local Government Area of Benin City, Edo state, Nigeria, in November, 2018. The plant sample was identified and Authenticated by Dr. Timothy Odaro of the Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Benin, Benin City and a Voucher number was issued (UBW-V528). The leaves were dried using a laboratory oven at a temperature of 40^{o}C, for a period of 8 days till the leaves turned crispy. The dried leaves were then grounded to powder form using an electric mill (Tigmax Petrol Gx160-5.5hp). Five hundred (500) grams of the powdered leaves was cold macerated in 2.5 l of ethanol (Owolabi *et al.,* 2008) for 72hours and was shaken regularly. Afterwards, the solution was decanted and the extract filtered (0.45 micron pore size) from the solution and evaporated to dryness at 40^{o}C. The dehydrated extract was then stored in a clean, dried bottle and kept at 4.4^{o}C for further use.

Experimental animals

The study was performed with both sexes of albino mice (20-28 g) and Wistar rats of 16 weeks old (180-210 g). Wistar rats and albino mice were received from the Animal house, Faculty of Pharmacy, University of Benin, Nigeria. They were fed using standard rodent feed procured from Top Feed Palletized Finisher and had clean drinking water *ad libitum*. Before the start of each study, the animals were fasted during the night. The animals were uncovered to a day lighting and handled according to the standard experimental procedures accepted by Institutional Ethical Committee, Faculty of Life Sciences, University of Benin, Edo state, Nigeria with an ethical number of LS20187.

Anti-inflammatory activity

Carrageenan-induced paw edema

For this study, four groups of four Wistar rats of 16 weeks old (180-210 g) each were used. The extract at 15 and 30 mg/kg doses were administered orally to the test groups. Ten (10) mg/kg of indomethacin was orally administered to the positive control while distilled water (3 mL/kg) was administered to the negative control group. After an hour, 0.1 mL, 1 % w/v carrageenan suspension in normal saline injected into the subplantar tissue of the right hind paw of the animal. The thickness of the paw was measured using Vernier caliper at 0 hour and after every one hour interval for 5 hours (Thambi *et al.*, 2006).

Dextran-induced paw edema

Wistar rats weighing between 135-180 g were randomly divided into four groups of four animals each for this study. The extract (15 and 30 mg/kg) were administered to the test group orally, while distilled water (3 ml/kg) was administered orally to the negative control group. The reference group were given 60 mg/kg of diphenhydramine. The animals were treated one hour before they were injected with 1.5 % w/v dextran in normal saline of 0.1 mL into right hind paw subplantar tissue (Glauce *et al.*, 1998). Vernier calipers were used to measure the thickness of the paw at (0, 1 to 5 hours).

Xylene-induced ear edema

For this study four groups consisting of randomly selected four Swiss albino mice were used. The extract (15 and 30mg/kg) were administered orally to the test groups. The method described by Akindele *et al.* (2007) was adopted. Distilled water (3 ml/kg) was given to the untreated control group while the reference group was administered dexamethasone (1 mg/kg). After thirty (30) minutes, ear-edema was induced in the mice by the application of a drop of xylene to the interior surface of the right ear. About 15 minutes after, they were sacrificed in a mild chloroform, by maintaining a good animal ethics for ethical purpose, and the left and right ears were isolated, sized and weight taken and recorded.

Analgesic activity

Mouse writhing assay

Four groups consisting of randomly selected four male Swiss albino mice were used for this study. The test groups were administered the extract (15 and 30 mg/kg) orally, while distilled water (10 ml/kg) was administered to the group induced without treatment (negative control). The reference drug used was aspirin (100 mg/kg).Thirty (30) minutes after administration of reference drug and extract, acetic acid in normal saline (0.6% v/v) was given to the mice intraperitoneally with an injection and writhes were counted for 30 minutes at five minutes interval.

Formalin test

Male Wistar rats were randomly separated into four groups of four animals each for this experiment. The extract (15 and 30 mg/kg) were administered orally to the test group while the reference group was administered aspirin (100 mg/kg, subcutaneously). Distilled water was administered orally to the negative control group. At 30 minutes after they were administered, 1% of 20µl formalin was subcutaneously administered into the right paw of the rats. As a sign of response to pain, the time spent in biting and licking responses of the paw injected was recorded in seconds. The responses were measured for five (5) minutes subsequent to formalin injection (ûrst phase) and 15 to 30 minutes after injected with formalin (second phase) (Shibata *et al*., 1989)

Hot plate test

The method of the hot plate test described by Eddy and Leimback (1953) was used to evaluate the latencies of pain response. Swiss mice were randomly divided into four groups (n=5). The albino mice were independently positioned on a hot plate kept at constant temperature of 55 ^{o}C (Okpo *et al.,* 2001) the interval of time from placement and shaking or licking of the paw or jumping was recorded as an index of response latency. The pre drug latency period for each animal was determined and recorded. The negative control group was treated orally with distilled water at 10 mL/kg. The test groups of mice were administered with 15 and 30 mg/kg of the extract. Pentazocine (15 mg/kg) was given intraperitoneally and used as a standard. The animals were placed on the hot plate at 15, 30, 45, 60 minute, 15 minutes after treatment and the time recorded for either paw shaking or jumping was recorded.

Statistical analysis

Data from this study were analysed as Mean ±SEM. Means of the various groups were compared by ANOVA, using 2009 version of Graph Pad Prism computer software package. P-values <0.05 (95 % confidence interval) were regarded significant.

RESULTS AND DISCUSSION

Table 1 shows the result of carrageenan induced paw edema. The ethanol leaf extract of *Cannabis sativa* at 15 and 30 mg/kg, significantly inhibited paw edema in comparison to the negative control group. The 15 mg/kg extract dose was not significantly different from the indomethacin treated group. The 30 mg/kg extract at the first hour showed higher level of inhibiting paw edema as compared to the indomethacin and the 15 mg/kg extract. However, as compared to both groups, the 30 mg/kg had a non-significant effect in the fourth hour but showed paw edema inhibition at fifth hour.

The study investigated the antinociceptive and anti-inflammatory potential of *Cannabis sativa* leaf ethanol extract to determine its activity at different doses. Our study has shown that ethanol leaf extract of *Cannabis sativa* had anti-inflammatory activities. Inflammation is one of the processes utilized by the body to fix damaged tissue as well as eliminate injurious stimuli. Continuous inflammation would result in gradual tissue injury (Shin *et al*., 2008). Evidence has shown that inflammatory processes results in tissue damage, which eventually leads to several diseases like inflammatory bowel disorder and rheumatoid arthritis (Monaco *et al*., 2004; Akaogi *et al*., 2006). Therefore, research and the development of anti-inflammatory drugs have remained a necessary scientific pursuit (Gautam and Jachack, 2009).

This study employed carrageenan-induced paw edema, an already established protocol for the study of acute inflammation. As an agent for testing anti-inflammatory drugs, carrageenan has been proven to be a non-comparable choice due to its non-antigenic nature and elimination of subsequent systemic effect (Chakraborty *et al.*, 2006). Edema resulting from carrageenan occurs in two phases

Table 1: Effect of *Cannabis sativa* leaf ethanol extract on carrageenan-induced paw edema

Groups	Doses (mg/kg)	Pre-drug	0hr	1hr	2hr	3hr	4hr	5hr
Control (Dw)	0.5 ml/kg	-	0.60±0.03	0.68±0.02	0.70±0.03	0.63±0.01	0.67±0.02	0.65±0.01
Indomethacin	10	0.37±0.03	0.59±0.05	0.55±0.03*	0.72±0.01	0.53±0.03	0.55±0.02*	0.53±0.01**
C. sativa	15	0.33±0.01	0.52±0.05	0.57±0.03*	0.63±0.01	0.60±0.03	0.57±0.03*	0.54±0.03**
C. sativa	30	0.34±0.01	0.57±0.03	0.54±0.03**	0.70±0.04	0.62±0.04	0.59±0.03	0.56±0.02*

Values are means ± SEM; n=4; *= $p<0.05$. Dw – Distilled water

Table 2: Effect of *Cannabis sativa* leaf ethanol extract on xylene-induced ear edema in rats

Groups	Doses (mg/kg)	Left ear (g)	Right ear (g)	Differences in both ear (g)
Control (Dw)	0.5ml/kg	0.010±0.00	0.019±0.00	0.009±0.00
Aspirin	100	0.065±0.01	0.098±0.02	0.033±0.02***
C. sativa	15	0.058±0.00	0.092±0.01	0.034±0.01***
C. sativa	30	0.064±0.01	0.117±0.0	0.058±0.01**

Values are means ± SEM; n=4; **$p<0.05$. Dw — Distilled water.

Table 3: Effect of *Cannabis sativa* leaf ethanol extract on acetic acid induced writhing with time dependent

Groups	Doses (mg/kg)	0-5 mins	5-10 mins	10-15 mins	15-20 mins	20-25 mins	25-30 mins
Control (Dw)	0.5 ml/kg	18.0±6.19	28.7±2.40	25.0±2.58	20.8±3.17	18.0±2.38	15.0±1.29
Aspirin	100	5.25±1.32*	18.0±1.00**	19.5±1.56	18.5±1.26	17.5±2.18	15.3±1.18
C. sativa	15	3.50±1.19*	15.7±0.33**	13.8±0.75**	11.0±1.35*	7.75±1.49*	5.75±1.11***
C. sativa	30	0.0±0.0**	15.3±1.86***	14.0±2.16**	9.50±1.94**	8.50±2.18*	6.50±1.04***

Values are means ± SEM; n=4; $p<0.05$. Dw — Distilled water

Table 4: Effect of *Cannabis sativa* leaf ethanol extract on hot plate-induced pain with time dependent

Groups	Doses (mg/kg)	0 sec	15 mins	30 mins	45 mins	60 mins
Control (Dw)	0.5 ml/kg	3.00±0.33	4.17±0.75	3.00±0.12	3.20±0.32	2.67±0.46
Pentazocine	100	2.35±0.13	7.75±2.00*	8.40±1.82**	10.92±0.89***	9.00±1.68***
C. sativa	15	2.83±0.24*	5.83±2.33	6.68±2.20*	6.83±2.18*	6.94±2.19**
C. sativa	30	2.70±0.43	6.12±2.57	6.70±2.38*	8.55±1.69**	8.64±1.75**

Values are means ± SEM; n=4; $p<0.05$.Dw — Distilled water.

(Vinegar *et al.*, 1987). The first phase takes place during one hour of carrageenan inflammation and it is as a result of cytoplasmic enzymes, serotonin and histamine discharge from the mast cells.

Arachidonic acid metabolites and platelet activating factor also have their distinct roles to play (Boughton-Smith *et al.*, 1993). Second phase of carrageenan-induced edema takes place after an hour and is mediated by release of proteolytic enzymes, prostaglandins, release of oxygen, free radicals arachidonate metabolites, migration of neutrophil and some neutrophil-acquired mediators (Boughton-Smith *et al.*, 1993; Bouriche *et al.*, 2003) and kinins are responsible for the continuity between the two phases (Vinegar *et al.*, 1987). Our findings have demonstrated that *C. sativa* ethanol leaf extract (15 and 30 mg/kg doses) had a significant ($p<0.05$, 0.01) inhibition of paw edema producing an inhibitory effect in the first and second phases of the inflammation (Table 1).

The antihistamine potential of the extract is demonstrated in the first phase which may be as a result of the extracts ability to reduce carrageenan-induced leakage of the microvasculature (Kuriyama *et al.*, 2000). Histamine can induce vascular permeability of endothelial cells thereby resulting

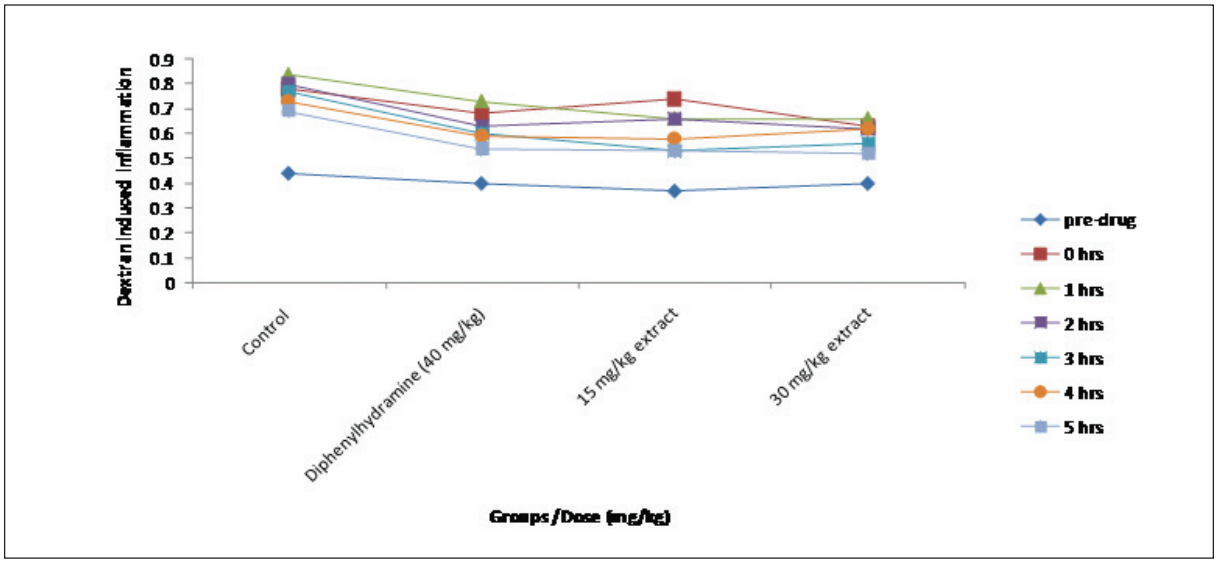

Fig. 1: Effect of *Cannabis sativa* leaf ethanol extract on dextran-induced inflammation in rats paw

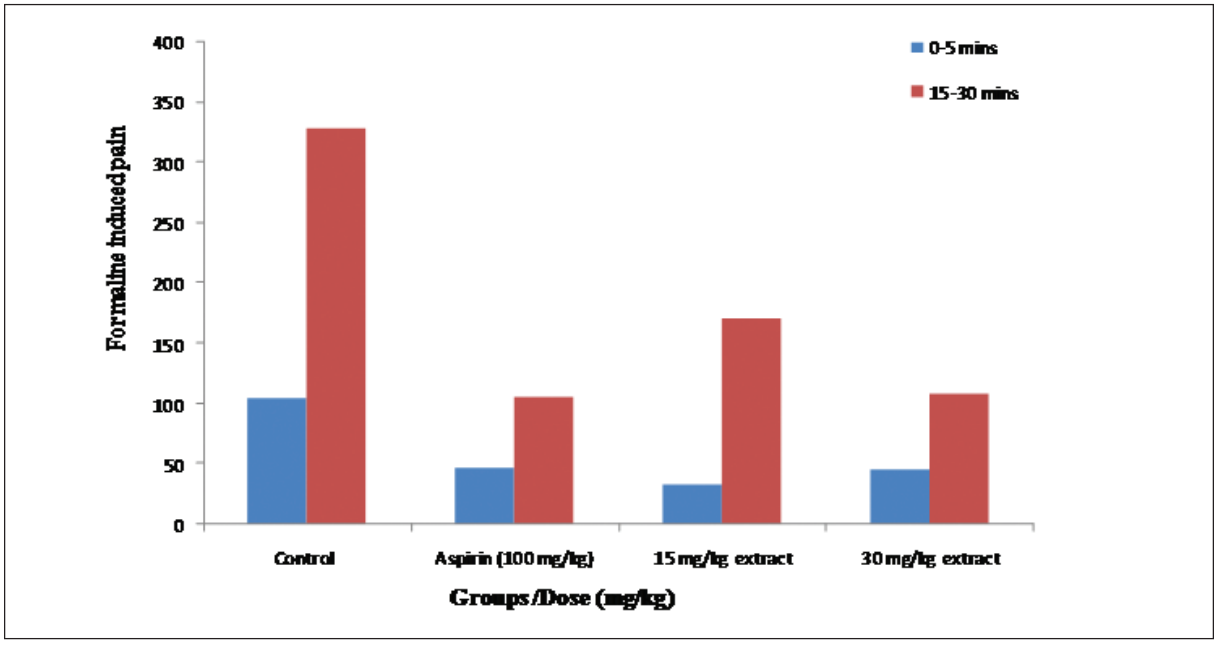

Fig. 2: Effect of Cannabis sativa leaf ethanol extract on formalin induced pain in rats

in outpouring of fluid and cells (Kuriyama *et al.*, 2000). Our study is in agreement with Musa *et al.* (2011). In this study carried out using *Cannabis sativa* seeds in petroleum oil extract in albino rats, the authors reported a decrease in edema. The edema ameliorating potential of *C. sativa* at second phase, suggesting a potential cyclooxygenase synthesis inhibited due to the fact that the carrageenan inflammatory model reveals prostaglandins actions (Di Rosa *et al.*, 1971; Ferreira *et al.*, 1974).

Figure 1 presents the results for dextran induced paw edema. The ethanol leaf extract of *Cannabis sativa* (15 and 30 mg/kg) treated groups hindered paw edema, in comparison with the negative control. *C. sativa* extract (15 mg/kg) had greater

paw edema inhibitory properties when compared to the positive control at the first, third, and fourth hour of the experiment, but had no inhibiting effect at zero hour in comparison to the positive control. At 30 mg/kg, the extract had a greater inhibitory effect of paw edema in comparison to the positive control at zero and one hour. More so, dextran-induced inflammation is mediated via serotonin released from mast cells (Lo *et al*., 1982).

These inflammatory mediators released bring about marked vascular changes such as vasodilatation, higher permeability and slow blood flow, finally leading to paw inflammation. Our study have demonstrated that ethanol leaf extract of *Cannabis sativa* inhibited significantly ($p<0.05$, 0.01) dextran-induced paw edema at both doses (15 and 30 mg/kg) (Figure 1). These observations were in agreement with the study reported by Wade *et al*. (2004) where they showed that the ethanol extract of *Leptadenia arborea* demonstrated an inhibitory effects in the edema size.

The ethanol leaf extract of *Cannabis sativa* (15 and 30 mg/kg) inhibited xylene-induced ear edema in comparison to the negative control (Table 2). At 15 mg/kg dose, *C. sativa* had inhibitory activity comparable to the positive control. Xylene induced ear edema is used to investigate the anti-inflammatory steroids to reduce the sensitivity of non-steroidal anti-inflammatory mediators (Zannir *et al.*, 1992). The indicators on acute inflammation after topical applications of xylene detected were; infiltration of inflammatory cells, severe vasodilation and edematous skin changes. This study showed that the xylene ear edema was inhibited by the extract at both doses significantly ($p<0.01$, 0.001), which suggests that *C. sativa* possess antiphlogistic effects. Our findings corroborates with the study conducted by Okpo *et al.* (2001) where they demonstrated the inhibitory potential of *Crinum glaucum* in xylene induced ear edema.

Acetic acid induced writhing (Table 3) shows that ethanol leaf extract of *Cannabis sativa* (15 and 30mg/kg) inhibited writhing in comparison to the negative control. The extract showed a higher inhibitory effects compared with the positive control drug.

In the formalin induced test (Figure 2), the extract (15 and 30 mg/kg) ameliorated formalin induced pain as compared to negative control group. This observed reduction was seen in both phases. However, at 15 mg/kg dose, a higher inhibitory effect in the first phase was observed when compared to (100 mg/kg) dose of aspirin. The 30 mg/kg dose had effect in both phases similar in comparison to that of aspirin. Furthermore, our study on the analgesic properties of *Cannabis sativa* showed the relationship between *Cannabis* and pain (Figure 2). Some random, controlled clinical studies have reported the effective quality of *Cannabis* as a pharmacotherapy for pain (Hill, 2015).

Several studies have reported the analgesic properties of tetrahydrocannabinol using animal models (Lim *et al*., 2003; Johanek *et al*., 2001), also experimental research carried out on investigating the properties of *Cannabis sativa* on human response to nociceptive has been on clinical pain samples or healthy adults. For instance, Wallace *et al*. (2007) validated the activities of smoked *C. sativa* at different doses (low, medium, or high doses vs. lethargic placebo) on intradermal capsaicin-induced pain reactions by means of a randomized, double-blind, verge trial in fifteen (15) healthy volunteers (mean age of 28.9; 58% male). They observed a significant reduction in pain at medium dose and increase pain at higher dose. The results showed a signiûcant reduction in pain at medium dose of *C. sativa* and a significant increase in pain at high dose.

The study also reported no significant difference was seen with the low dose of *C. sativa*. The conclusion derived was that medium dose of smoked Cannabis could be a good therapy for pain. Another experimental study by the same authors was carried out with 18 healthy female volunteers who tested the effect of orally administered *C. sativa* extract (vs. potent placebo) on sunburn and intradermal capsaicin pain reactions using a double-blind, cross over trial (Wallace *et al*., 2007).

They observed that the extract of *Cannabis* did not result in any analgesic effect. They concluded that the lack of understanding of the dose-dependent therapeutic and psychotropic effects may limit the use of *C. sativa* as an analgesic agent. A review of cannabinoids for medical usage among patients with chronic pain has shown excellent reduction in pain and numerical pain rating (Whiting *et al*., 2015). The authors therefore concluded that

moderate evidences are available to support the analgesic properties of cannabinoids (Whiting *et al*., 2015). Our findings revealed that ethanol leaf extract of *Cannabis sativa* possessed analgesic properties.

One of the widely used methods to evaluate anti-nociceptive activity is the writhing on animal models caused by acetic acid (Gene *et al.*, 1998). This method is highly sensitive even at lower dose when compared to the tail-flick test in detecting the anti-nociceptive potentials of bioactive agents (Collier *et al.*, 1968; Bentley *et al.*, 1981). Our study demonstrated that *C. sativa* ethanol leaf extract caused a significant ($p < 0.005$, 0.01, 0.001) reduction in acetic acid induced-writhes at all doses. *C. sativa* extract generally was more effective than 100mg/kg of aspirin dose, which was the control drug used and it was sustained throughout the 30 min period suggesting peripheral mediation for the extract's analgesic effect.

The extract (15 and 30mg/kg) inhibited hot plate induced pain in mice from the 30th minute all through to the 60th minute in comparison to the negative control (Table 4). Pentazocine (15mg/kg), a known centrally active drug had a better inhibitory effects than the extract treated groups.

The findings from our study showed that *C. sativa* ethanol extract (15 and 30 mg/kg) was able to cause a significant ($p<0.005$, 0.01) inhibition in the two phases of formalin-induced pain. However we observed a significant inhibition in the first phase in the group administered 15 mg/kg in comparison to the group administered aspirin (100 mg/kg), while the 30 mg/kg dose had effects in both phases similar to that of aspirin as shown in Figure 2. In addition, Formalin induced pain in rats is a model that is of great importance in explaining the mode of action of analgesia as well as pain (Tjolsen *et al*., 1992). Centrally mediated bioactive agents like the narcotics are able to inhibit both phases (Santos *et al*., 1994). This suggests that analgesic activity was centrally mediated.

Hot plate tests are usually employed to evaluate nociceptive effects mediated centrally. We observed in our study that *C. sativa* (15 and 30 mg/kg) was able to cause a significant extension of reaction latency to pain in the hot plate as shown in Table 4which suggests that the analgesic activity was centrally mediated. The abnormal calmness some minutes after administration of the extract observed in the animal models suggests psychotropic effects of the extract.

CONCLUSION

The study showed that *Cannabis sativa* had a dose-dependent anti-inflammatory and analgesic activities. Hence, this study elicited a scientific validation the ethno-medicinal uses of the plant.

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