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Article

Essential Oil from the Stem Bark of *Casuarina equisetifolia* Exerts Anti-inflammatory and Anti-nociceptive Activities in Rats&

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Herein the chemical constituents and the anti-pain properties of the essential oil from the stem bark of *Casuarina equisetifolia* L. (Casuarinaceae) grown in Nigeria were evaluated. The essential oil was obtained by hydrodistillation method in an all glass Clevenger-type apparatus, and characterized by gas chromatography (GC-FID) and gas chromatography-mass spectrometry (GC-MS). The hot plate method was used to determine the anti-nociceptive property whereas the anti-inflammatory activity was evaluated by carrageenan-induced and formalin experimental models. The pale-yellow essential oil was obtained in yield of 0.21% (v/w), calculated on a dry weight basis. The main constituents of the essential oil were methyl salicylate (30.4%), a-zingiberene (15.5%), (*E*)-anethole (9.5%), b-bisabolene (8.6%), b- sesquiphellandrene (6.9%), and ar-curcumene (6.2%). In the anti-nociceptive study, the rate of inhibition increases as the doses of essential oil increases with optimum activity at the 30th and 60th min for all tested doses. The essential oil displayed anti-nociceptive activity independently of reaction time at the highest tested dose (200 mg/kg). The essential oil of *C. equisetifolia* moderately reduced pain responses in early and late phases of the formalin test. The oil inhibited the paw licking in the neurogenic phase (60-63%) compared to the late phase of the formalin test. The carrageenan-induced oedema model revealed the suppression of inflammatory mediators within the 1st - 3rd h. Thus, *C. equisetifolia* essential oil displayed both anti-nociceptive and anti-inflammatory activities independent of the dose tested. The anti-inflammatory and anti-nociceptive activities of *C. equisetifolia* essential oil are herein reported for the first time.

Keywords: Methyl salicylate. Zingiberene. Formalin activity. Carrageenan.

INTRODUCTION

Casuarina equisetifolia J.R. & G. Forst. (Casuarinaceae) is a deciduous tree that occurs in open coastal habitats including sand beaches, rocky coasts, and sand dunes. The trees can grow up to over 100 ft (30.5 m) in height. The species is native to Australia and Southeast Asia and was introduced into other parts of the world. The foliage leaf is reddish-brown to gray bark while the branchlets resemble pine needles and are very thin, 4-8 in. (10-20 cm) long and gray-green coloured. The male flowers occur in terminal spikes, while the female flowers are in small axillary clusters. The tiny fruits contain one seed each. The fruits are contained in woody, cone-like structures that are 0.75 in (2 cm) long (Park *et al*., 2019). The antioxidant property of the extracts of *C. equisetifolia* was attributed to quercetin, lupeol and gallic acid present in the plant (Aher *et al*., 2009). A polyherbal gel containing *C. equisetifolia* was reported to displayed anti-acne activity (Yousra *et al.,* 2014). The root extracts of *C. equisetifolia* exhibited i*n vitro* anti-inflammatory, antioxidant and antibacterial activities (Vtk, Gowrie, 2018). Extract of *C. equisetifolia* ameliorates

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gentamicin-induced nephrotoxicity and oxidate damage by scavenging oxygen radicals (El-Tantawy, Mohamed, Abd Al Haleem, 2013). Reports indicated that various extracts of *C. eqisetifolia* have shown anti-ulcer, anti-diabetic, antihyperlipidemic, antispasmodic, anti-diarrhoeal, cytotoxicity, and hepatoprotective effects (Al-Snafi, 2015).

The phytochemical compounds of *C. eqisetifolia* include casuarine-6-α-D-glucoside (Wormald *et al.,* 1996), coumaroyl triterpenes and *d*-gallocatechin (casuarin) (Takahashi *et al.,* 1999). The chemical composition of essential oil hydrodistilled from the leaves of *C. equisetifolia* comprised mainly of pentadecanal (32.0%) and 1,8-cineole (13.1%), while caryophyllene oxide (11.7%), *trans*-linalool oxide (11.5%), 1,8-cineole (9.7%), α-terpineol (8.8%) and α-pinene (8.5%) were the significant compounds of the fruit oil (Ogunwande *et al.,* 2011). In another analysis, large amounts of a-phellandrene (40.6%), p-cymene (15.7%), 1,8-cineole (14.1%) and terpinolene (8.4%) were identified in the leaf oil of *C. equisetifolia* (Essien *et al.,* 2016). Adeosun et al. (2017) reported the main constituents of essential oil isolated from the stem bark of *C. equisetifolia* to comprise mainly of fatty acids namely *n*-hexadecanoic acid (18.67%), *cis*-13-octadecanoic acid (17.83%), tridecane (11.84%) and undecane (10.45%). Previous study has shown that essential oil isolated from the stem bark of *C. equisetifolia* displayed significant mosquitocidal potential against *Anopheles gambiae* complex and *Aedes aegypti* (Adeosun *et al.,* 2017). Also, essential oil from the leaf of *C. equisetifolia* exhibited significant antimicrobial activity with minimum inhibitory concentration values of 39-625 µg/mL, on pathogens employed in the assays (Essien *et al.,* 2016). Although the essential oils of *C. equisetifolia* were reported to have exhibited antimicrobial (Essien *et al.,* 2016) and mosquitocidal (Adeosun *et al.,* 2017), however, no report could be seen on the potential anti-inflammatory and anti-nociceptive activities.

Herein further data on our ongoing studies to identify potential pain relief agents from natural sources (Ogunwande *et al.,* 2019a,b; Avoseh *et al.,* 2020 a,b,c; Lawal *et al*., 2020), the anti-inflammatory and anti-nociceptive efficacies of essential oil from the stem bark of *C. equisetifolia* grown in Nigeria is reported, for the first time.

MATERIAL AND METHODS

Collection and handling of C. equisetifolia stem bark

The stem barks of *C. equisetifolia* were collected from trees growing in front of Okunuga Hall, Faculty of Law, Lagos State University, Ojo, in March 2018. The stem bark of *C. equisetifolia* was identified by Curators at the Herbarium Headquarters, Forestry Research Institute of Nigeria (FRIN), Ibadan, where voucher a specimen, FHI 111880, was deposited. Before the hydrodistillation process, the plant samples were air-dried under laboratory shade for two weeks at ambient temperature to reduce the moisture contents. Also, sediments and other unwanted materials were separated from the samples by handpicking and washing. Afterwards, samples were air-dried and pulverized to coarse powder in a locally made grinder.

Hydrodistillation of the essential oil

In this process, 300 g of air-dried and pulverized stem bark of *C. equisetifolia* was used. The samples were carefully introduced into a clean 5 L flask. Distilled water was added until it covered the sample completely. Hydrodistillation was carried out with a Clevenger-type distillation unit designed according to the specification as described previously (Ogunwande *et al.,* 2019a,b; Avoseh *et al.,* 2020 a,b,c). The distillation time was 4 h and conducted at normal pressure. The volatile oils were collected separately into clean weighed sample bottles. The oils were kept under refrigeration (4^{o}C) until the moment of analysis.

Chemical analysis of the essential oil

The essential oil was comprehensively analyzed for the constituents with the aid of gas chromatography-flame ionization detector (GC-FID) and gas chromatography-mass spectrometry (GC/MS). Gas chromatography (GC-FID) analysis was accomplished with an HP-5890 Series II instrument equipped with an HP-Wax and HP-5 capillary columns (both 30 m x 0.25 mm, 0.25 µm film thickness), working with the following temperature program: 60ºC for 10 min, rising at 5ºC/ min to 220ºC.

The injector and detector temperatures were maintained at 250ºC; carrier gas was nitrogen (2 mL/min); detector dual, FID; split ratio 1:30. The volume injected was 0.5 µL. The relative proportions of the oil constituents were percentages obtained by FID peak area normalization.

Gas chromatography-mass spectrometry (GC-EIMS) analysis was performed with a Varian CP-3800 gas-chromatograph equipped with an HP-5 capillary column (30 m x 0.25 mm; film thickness 0.25 µm) and a Varian Saturn 2000 ion trap mass detector. Analytical conditions: injector and transfer line temperature 220ºC and 240ºC, respectively; oven temperature programmed from 60ºC-240ºC at 3ºC /min.; carrier gas helium at a flow rate of 1mL/min.; injection volume 0.2 µL (10% *n*-hexane solution); split ratio 1:30. Mass spectra were recorded at 70 eV. The acquisition mass range was m/z 30-300 at a scan rate of 1 scan/sec.

The identification of the constituents of *C. equisetifolia* was based on a comparison of the retention times with those of authentic samples, comparing their linear indices relative to a series of *n*-alkanes (C_6-C_{36}). Further identifications were also made possible by the use of a homemade library of mass spectra built up from pure substances and components of known oils, and MS literature data as described in our previous studies (Ogunwande *et al.,*2019a,b; Avoseh *et al.,* 2020 a,b,c). Moreover, the molecular weights of all the identified substances were confirmed by GC-CIMS, using MeOH as CI ionizing gas.

Biological tests

Drug and Chemicals

Carrageenan drug of analytical grade was obtained from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). Ibuprofen injection (May and Baker), Diclofenac Injection (Dizpharm, Nigeria Ltd.) and Piroxicam tablets were purchased from Lagos State University Pharmacy.

Animal study

Wistar rats (150-200 g) of both sexes were accommodated in the Biochemistry Department animal facility of Lagos state University, Ojo-Lagos. The rats were kept in a metal steel cage, where they had an unrestricted supply of water and standard pellet food. They were acclimatized for two weeks (14 days), before the commencement of the experiment (Rao, Eswaraiah, 2018). All experimental procedures were conducted as approved by the Research Ethical Clearance Committee (RECC) of the University (Approval no: 012/2019/LASU/BCH).

The rats were assigned at random to 5 different groups consisting of 6 rats per group:

Group 1- Control group (Saline solution);
Group 2- Piroxicam / Diclofenac/ Ibuprofen treated group 100 mg/kg (Standard Group);
Group 3- 100 mg/kg of *C. equisetifolia* essential oil;
Group 4- 200 mg/kg of *C. equisetifolia* essential oil; and
Group 5- 400 mg/kg of *C. equisetifolia* essential oil

The rationale for selecting the studied doses was that rats of similar weight were grouped to obtained average weight. The weight recorded was similar across the groups of animals. The dose was therefore determined from the weight of animals in the assigned group. Essential oil of *C. equisetifolia* was dissolved in saline vehicle and administered to the animals in the order of 100, 200 and 400 mg/kg.

Toxicity study

The essential oil was tested for acute toxicity study using Wistar rats. Twenty-five Wistar rats (both sexes, 150- 200 g each) divided into 5 rats in each group were used for the toxicity study. Wistar rats were administered 500, 1000, 1500 and 2000 mg/kg of *C. equisetifolia* per oral route. One group received normal saline that served as a negative control. Animals were observed for 14 days for deaths, weight loss and eating behavioural changes.

Hot Plate test for anti-nociceptive study

The experiment was carried out according to the method described previously (Ogunwande *et al.,* 2019a,b; Avoseh *et al.,* 2020 a,b,c). Thirty (30) mature Wistar rats of both sexes were randomly divided into 5 groups

of equal rats. The rats were fasted for 12 h with the provision of clean water *ad libitum*. Each rat was placed upon the heated metal plate (Hot plate) maintained at the temperature of about 50-55 ^{0}C within the restraining glass cylinder (Treede, 2018).

Group 1 rats received 10 mL/kg of saline solution and served as control;
Group 2 rats received Piroxicam (100 mg/kg (Piroxicam) (standard control);
Groups 3, 4 and 5 received 100, 200 and 400 mg/kg of *C. equisetifolia* respectively per 0 s (p.o.).

Rats' response to the heat varies and such changes include kicking of the hind foot, jumping about, licking of the foot, raising the foot, and holding the foot tightly to its body or shaking of the foot. The reaction time was recorded 30, 60, 90 and 120 min after the administration of the treatments. The maximum reaction time was fixed at 30 s to prevent any injury to the tissues of the paws. If the reading exceeds 30 s, the activity is considered as maximum analgesia.

Formalin test

This test was based on the method described earlier (Treede, 2018), with slight modification. Briefly, formalin solution (1% in distilled water; 0.5 mL/paw) was injected into the hind paw plantar surface (i.pl. injection) of the grouped rats. The time (s) spent in intense licking or biting the affected paw was rated during two-time intervals: 0–5 min (first phase or neurogenic pain) and 15–30 min (second phase or inflammatory pain). Oral treatment (p.o.) with sodium diclofenac (10 mg/kg; positive control), vehicle (water, 10 mL/kg), *C. equisetifolia* essential oil (100, 200, 400 mg/kg), were given 60 min prior to formalin injection. Percentage inhibitions are evaluated using;

$$\text{Percentage inhibition} = [(1 - T/C)] \times 100$$

Where T is the number of times treated rats licked/bite the injected paw; C is the number of times control mice licked/bit the treated paw.

Carrageenan-induced paw edema in rats (Anti-inflammatory Analysis)

Animals in each afore-mentioned groups were induced subcutaneously with 0.1 mL of 1% freshly prepared Carrageenan in the right hind paw of rats treated by oral administration of vehicle (water, 10 mL/kg; control), Ibuprofen (10 mg/kg; positive control) and *C. equisetifolia* essential oil doses (100, 200 and 400 mg/kg) following a previous procedure (Ogunwande *et al.,* 2019a,b; Avoseh *et al.,* 2020a,b,c) with slight modification. Paw volume of the injected rats was measured hourly on a plethysmometer (Ugo Basile, Italy mod. 7140) commencing 1 h before (basal values) and up to 4 h following Carrageenan injection. Edema was calculated as the difference (L) between injected and control paw. The area under the curve (AUC) time versus Δ paw volume was calculated for each animal and the edema was expressed as the mean±S.E.M. of AUC (Ferreira *et al*., 2006).

Statistical analysis

Unless otherwise specified, data represent the Mean ± S.E.M. of the evaluated parameter and were analyzed for statistical significance by one-way analysis of variance (ANOVA) followed by Dunnett's multiple for the Formalin induced assay while two-way ANOVA and post-hoc Bonferrotti test were used for the Carrageenan and the hot plate test using GraphPad Prism (version 7.02, San Diego CA, USA, www.graphPad.com). The minimum levels of significance, $p < 0.05$, p 0.01 and $p < 0.001$, were considered as the statistically significant difference of the means.

RESULTS AND DISCUSSION

Chemical constituents of the essential oil

The yield of the essential oil was 0.12% (v/w), calculated on a dry weight basis. Table I indicates the compounds identified from the GC/MS spectra along with their retention indices and percent compositions. Twenty-two compounds accounting for 92.4% of the volatile contents were identified from the oil sample. Oxygenated

monoterpenes (40.8%) and sesquiterpene hydrocarbons (48.8%) represent the main class of compounds identified in the oil. The monoterpene hydrocarbons and oxygenated sesquiterpene classes of compounds were not identified in the oil sample. The main constituents of the oil were methyl salicylate (30.4%), a-zingiberene (15.5%), (*E*)-anethole (9.4%), b-bisabolene (8.6%), b-sesquiphellandrene (6.9%) and *ar*-curcumene (6.2%).

TABLE I - Compounds identified in the stem bark essential oil of *C. equisetifolia*

Sr. No	Compounds[a]	RI[b]	RI[c]	Percent[d]
1	1,8-Cineole	1034	1932	0.4
2	Linalool	1101	1100	0.3
3	Nonanal	1102	1101	2.8
4	*trans*-Pinocarveol	1139	1140	0.2
5	Borneol	1165	1167	0.9
6	Methyl salicylate	1192	1190	30.4
7	(*E*)-Anethole	1283	1282	9.4
8	Bornyl acetate	1287	1287	0.4
9	a-Isocomene	1388	1387	3.3
10	b-Elemene	1392	1393	0.6
11	b-Elemene	1420	1419	1.9
12	a-Humulene	1456	1452	0.7
13	Sesquisabinene	1460	1458	0.4
14	b-Santalene	1463	1464	1.1
15	b-Acoradiene	1471	1470	1.0
16	g-Himachalene	1475	1473	1.7
17	b-Chamigrene	1476	1476	0.2
18	ar-Curcumene	1483	1482	6.2
19	a-Zingiberene	1496	1496	15.5
20	b-Bisabolene	1509	1507	8.6
21	b-sesquiphellandrene	1534	1532	6.9
22	(*E*)- g-Bisabolene	1535	1533	0.7
	Total			**92.4**
	Oxygenated monoterpenes (Sr. No. 1,2, 4-8)			**40.8**
	Sesquiterpene hydrocarbons (Sr. No. 9-22)			**48.8**
	Non-terpenes (Sr. No. 3)			**2.8**

[a]Order of elution on HP-5 column; [b]Retention indices on HP-5 column; [c]Literature retention indices (see Experimental); [d]Standard deviation were insignificant and excluded from the Table to avoid congestion; Sr. No, serial number

In contrary to a previous report, *n*-hexadecanoic acid, *cis*-13-octadecanoic acid, tridecane and undecane which were identified as the major compounds of the stem bark oil of *C. equisetifolia* (Adeosun *et al*., 2017), were not identified in the present investigated oil sample. In addition, pentadecanal, caryophyllene oxide, *trans*-linalool oxide, α-terpineol, α-pinene, a-phellandrene, p-cymene, and terpinolene, the characteristic compounds of previously reported leaf and fruit samples of *C. equisetifolia* oils (Ogunwande *et al*., 2011; Essien *at al*., 2016), were conspicuously absent in the presently investigated stem bark oil. Moreover, the content of 1,8-cineole in the present oil sample was much lower than previously reported for the leaf and fruit oils (Ogunwande *et al*., 2011; Essien *et al*., 2016). The occurrence of methyl salicylate and α-zingiberene as the major constituents in the present analysed oil sample could therefore represent a different chemical form of the essential oils of *C. equisetifolia*.

Toxicity assay on the essential oil

To ascertain the bio-safety of the essential oil doses, a toxicity assay was carried out. In this study, the results obtained at test doses of 500, 1000, 1500 and 2000 mg/kg body weight demonstrated a high safety margin with no sign of divergent effects on the reflex, health, weight, food and water intake habit and no mortality recorded for 14 days. Therefore, the highest dose of 400 mg/kg given to rats in this study was considered to be safe. This study is significant and agrees with previous studies where animals administered with 2000 mg/kg of methanolic extracts of *C. equisetifolia* showed normal behaviour without any alteration in passivity signs, motor activity and no mortality observed up to 14 days (Rao, Eswaraiah, 2018; Vaidya *et al*., 2020).

Effect of *C. equisetifolia* essential oil on hot plate model

This study evaluated the effect of the stem bark essential oil (CEOE) on the central anti-nociceptive effect using the hot plate model and the formalin-induced assay. Figure 1 shows the effects of *C. equisetifolia* essential oil on the hot plate model. The heat latency activity of the essential oil in the animal model was evaluated at 100, 200 and 400 mg/kg concentration. Figure 1 below shows the maximum pain threshold as a function of dose and time. The reaction of Wistar rats to heat was recorded from time zero after administration of the essential oil to the 90th min, to understudy the effect on heat resistance by the rats. In this result, 200 mg/kg extract of the essential oil significantly increased the thermal resistance of the rats throughout the reaction period at a significant value of p <0.001 (0th to 60th min) and p <0.01 for the 90th min. The lowest activity was recorded by the 100 and 400 mg/kg of essential oil, where inhibition was only significant at the 60th min and 30th min, respectively (p <0.01). Therefore, the anti-nociceptive property of the essential oil of *C. equisetifolia* was more pronounced in the 200 mg/kg dose.

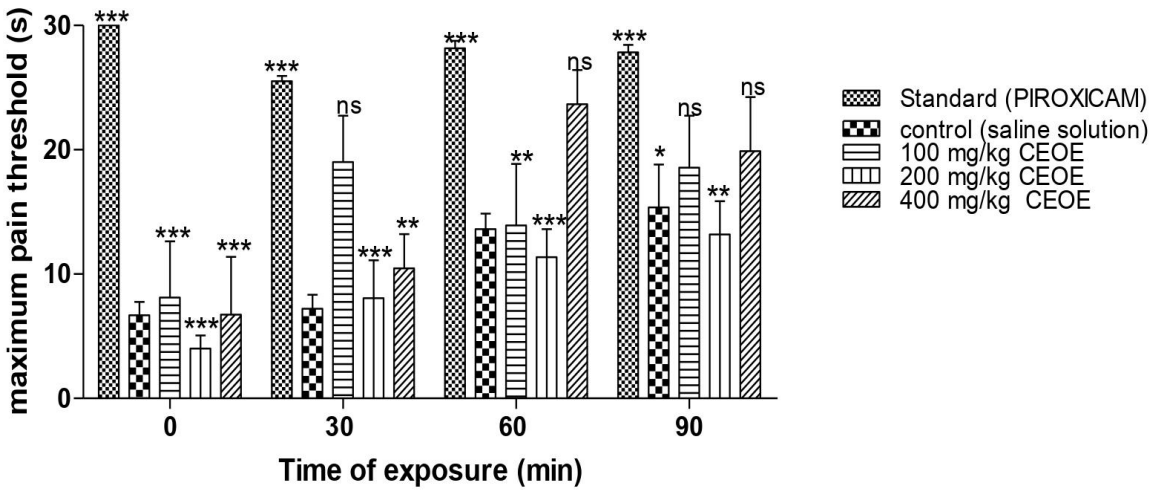

FIGURE 1 - Effect of *C. equisetifolia* on heat-induced nociception. Control, Standard and CEOE represent 1 mL saline solution, 100 mg/kg of piroxicam and 100, 200 and 400mg/kg of essential oil of *C. equisetifolia* respectively. ***$p < 0.01$ statistically compared to control.

The use of natural products as alternative drug therapy has been of high priority due to the natural products bio-safety, availability and little to non-toxicity. Several well-known drugs of natural products origin have been developed to control, prevent, diagnose, cure and treat diseases and ailments. Aspirin, morphine, and other similar drugs with pharmacological importance have been deployed for the management of different types of pain due to their basic structure and amelioration of certain pain mediators. Pain is a major cause of distress, both physical and psychological, and is also associated with an increased in-patient hospital stay, poor wound healing, and prolonged rehabilitation (Yam *et al*., 2018). The sensation of pain is associated with the activation of the receptors in the primary afferent fibers, which are hitherto silent but are activated in the presence of potential noxious stimulus which includes, thermal, mechanical, or injuries (Chen *et al*., 2016).

The nociception study was evaluated using the hot plate model. The model perfectly fits for the study of thermal changes caused at the Aδ- and C-fibers afferent nociceptors responding to noxious stimuli presented in animal bodies. In addition, the model predicts the mechanism of action of the essential oils as either a central nervous or peripheral nervous regulator (Yam *et al*., 2018). The inhibition rate observed in this study shows that the essential oil of *C. equisetifolia* at a dose of 200 mg/kg significantly regulated the thermal stimuli in the Wistar rats. A previous study revealed that *C. equisetifolia* increases cell survival by increasing proteasome activities and restoring mitochondrial membrane (Aher *et al*., 2010). Extracts from different parts of *C. equisetifolia* inhibited the proliferation of nociceptors by activating opiate receptors and nitric oxide release via the formalin model (Aher *et al*., 2010). These studies agreed with our findings as the essential oil displayed significant inhibitory effects during the nociceptive, neurogenic and anti-inflammatory phases.

Effect of *C. equisetifolia* essential oil on formalin-induced model

To analyze the effect of *C. equisetifolia* essential oil on both the peripheral and centrally mediated analgesia, the formalin-induced assay was adopted. The results as shown in Table II depicted the rate of inhibition at the analgesic (anti-nociceptive) and anti-inflammatory stages after the administrations of the essential oil. Elevated inhibitions were observed at the 100 and 200 mg/kg dose of the essential oil while the 400 mg/kg was relatively lower at the 1st phase. The rate of inhibition of the formalin by the standard was lower (46.81%) when compared to

the essential oil at doses of 100 mg/kg (60.28%) and 200 mg/kg (63.83%). At the anti-inflammatory phase, the inhibition rate varies as concentration changes. Essential oil of *C. equisetifolia* at a dose of 200 mg/kg showed a minimal inhibition of 33% more than other tested doses, similar to the neurogenic phase. The essential oil also exhibited similar inhibitory activities as the standard drug for the late (anti-inflammatory) phase.

TABLE II - Formalin induced pain anti-nociceptive effect of oral treatment with *C. equisetifolia* essential oil [a]

		Neurogenic Phase (0-10 min)		**Inflammatory Phase (15- 30 min)**	
Treatment	Dose (mg/kg)	No. of Licks	Inhibition (%)	No. of Licks x	Inhibition (%)
Control	Saline	23.5 ±7.50	-	31.62 ±0.60	-
Standard (Piroxicam)[b]	100	12.5 ±1.74	46.81	16.50 ±2.83	54
C. equisetifolia	100	9.33 ±1.21	60.28	22.33 ±0.96	29
C. equisetifolia	200	8.50 ±0.80	63.83	21.33 ±0.47	33
C. equisetifolia	400	20.85 ±1.92	11.35	24.83 ±2.37	21

[a] Values are expressed as mean ± S.D. (n=6) statistically. [b]Rate of inhibition are expressed in comparison to control sample; - Low inhibition

The effect of the *C. equisetifolia* essential oil in the late phase of the formalin test suggests that the activity may be the result of peripheral action (Wang *et al*., 2011; da Silva *et al*., 2014). Formalin activates the primary afferent sensory neurons through a selected and direct action on the transient receptor potential cation channel, member A1 (TRPA1), which is highly expressed by a subset of the C-fiber nociceptors. Drugs that act centrally, such as opioids and narcotics, inhibit both phases of formalin-induced pain, while drugs such as aspirin, hydrocortisone and dexamethasone, which acts primarily peripherally, only inhibit the late phase (Lenardão *et al*., 2016). This model can be used to partially predict the mechanism of action of natural products in anti-nociception and anti-inflammation. Crude extract of *C. equisetifolia* was reported earlier to displayed mild anti-inflammatory activity (Vtk, Gowrie, 2018), although the constituents and mechanism eliciting such observation may differ from crude extracts to essential oils.

Carrageenan-induced model anti-inflammatory study

In this study, the rates of inflammatory inhibition were expressed as a change in volume of inflammation to doses of the essential oil. As shown in Figure 2, the essential oil of *C. equisetifolia* displayed very high inhibitory activities between the 1st to the 3rd h at a statistical significance value of $p < 0.001$ for all doses evaluated. However, inhibition rates decrease exponentially as the experiment approaches the 4th h post-administration. The standard drug (Ibuprofen), 100 and 400 mg/kg were all non-significant ($p > 0.05$) at this hour, however, the 200 mg/kg of the essential oil slightly displayed an inhibition rate of $p < 0.05$.

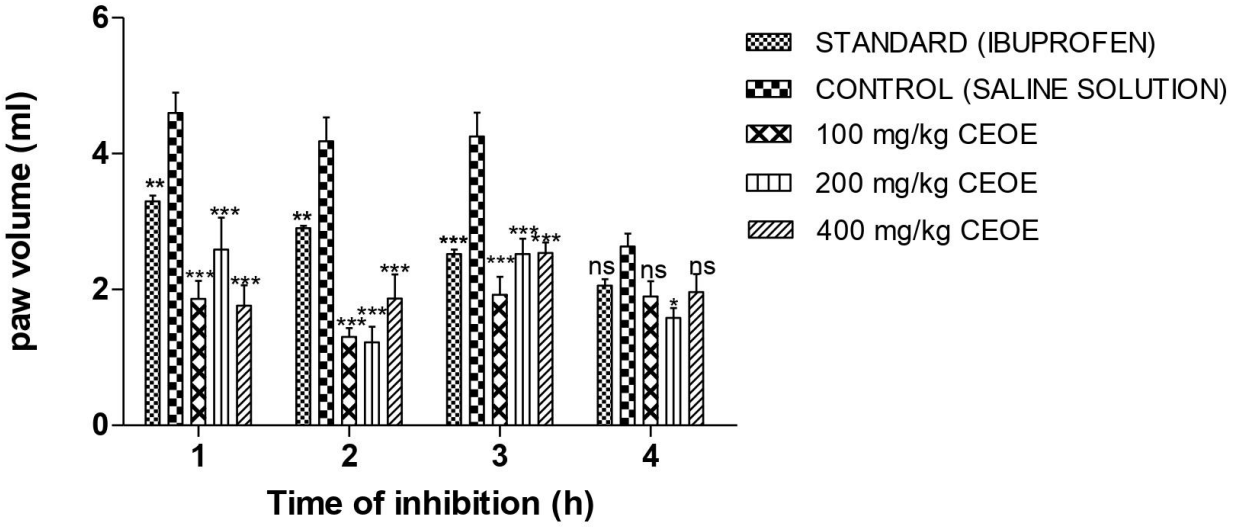

FIGURE 2 - Effect of *C. equisetifolia* on Carrageenan-induced inflammation. Control, Standard, and CEOE represent 1 mL saline solution, 100 mg/kg of Ibuprofen injection and 100, 200 and 400 mg/kg of essential oils of *C. equisetifolia* respectively. **p* <0.05, ***p* <0.01, *** *p* <0.001 statistically compared to control.

The Carrageenan-induced paw edema was adopted in the study of anti-inflammatory activity. This is a well-defined model for acute inflammation and has been applied in the study of the anti-edematous effect of extracts due to the production of different inflammatory mediators in the Wistar rats. The Carrageenan-induced model is a biphasic model leading to the release of inflammation mediators at different hours. In this specific model, the initial phase involves the release of mediators such as histamine, serotonin, and bradykinin lasting within the first 1st h, while the latter phase is characterized by infiltration of leukocytes and prostaglandins biosynthesis (Antônio, Souza Brito, 1998). The inhibitory activities of the essential oil (Figure 2) were significant within the 3rd h of reaction, suggesting an inhibition at both phases. This treatment steadily attenuated the paw edema induced by Carrageenan, as well as by numerous inflammatory mediators such as bradykinin, histamine, substance P and platelet-activating factor participating in the Carrageenan-induced inflammation (de Campos *et al*., 1994; Próspero *et al*., 2018). The anti-nociceptive and anti-inflammatory inhibitory capacities of *C. equisetifolia* observed in this study can be predicated on the terpenoids present in the essential oil (Sá *et al.,* 2013; Sarmento-Neto *et al.,* 2015). The major constituents identified in the essential oil of *C. equisetifolia* were known for their biological potentials. The action of methyl salicylate in ameliorating pain had been well documented. Methyl salicylate glycoside inhibited the production of inflammatory cytokines, blocks the activation of the NF-κB signalling pathway and non-selectively suppressed the activity of COX (cyclooxygenase) (Xin *et al*., 2014). Methyl salicylate was also reported to inhibit the release of lipopolysaccharide (LPS)-induced interleukin (IL)-6 and tumour necrosis factor (TNF)-α in a dose-dependent manner upon evaluation of the anti-inflammatory activity derivatives (Li *et al*., 2016). Essential oil with large contents of α-zingiberene, ar-curcumene and sesquiphellandrene produced a significant reduction in acute inflammation produced by Carrageenan, dextran and formalin-induced chronic inflammation, as well anti-nociceptive property with a significant reduction in acetic acid-induced writhing movements (Jeena, Liju, Kuttanract, 2013). Zingiberene exhibits anti-inflammatory property with a significant reduction in the level of inflammatory markers (TNF-α, IL-6, NF-κB, and IL-1β) in rats (Li *et al*., 2020). Anethole was reported to show moderate anti-inflammatory activity (Freire *et al*., 2005), and displayed anti-nociceptive effect in the writhing model induced by acetic acid, in the second phase of the formalin test (Ritter

et al., 2014). The inhibition of the pro-inflammatory mediators and nociceptive activities by the essential oil is suggestive of the synergistic action of the compounds present in the essential oil.

CONCLUSION

In summary, the study demonstrated that essential oil from the stem bark of *C. equisetifolia* acts as an anti-nociceptive and anti-inflammatory agent through suppression of several pain mediators at a dose-dependent pattern in Wistar rats. This therapeutic effect of *C. equisetifolia* essential oil may be related to the reduced production of nociceptor mediators, pro-inflammatory cytokines and inhibition of other pro-inflammatory signalling pathways. The essential oil displayed both central and peripheral pain mediated activities and can fit as either an opioid or non-opioid antagonist. The present findings provide an ethno-pharmaceutical use of the stem bark of *C. equisetifolia* for pain-related ailments. However, further study will be required to elucidate the mechanism of pain activity of the essential oil.

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