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BIOASSAY-GUIDED FRACTIONATION OF ANTINOCICEPTIVE EFFECT OF LECANIODISCUS CUPANIOIDES USING MICE

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ABSTRACT

Objective: *Lecaniodiscus cupanioides* belong to the family Sapindaceae, used traditionally for the treatment of tooth ache, fever and swelling of the abdomen, boils and wounds most especially in the western part of Nigeria. In this present study, we evaluated the effects of the methanol extract and fractions obtained from Lecaniodiscus cupanioides using acetic acid-induced writhing, hot plate test and formalin-induced paw lick and the possible mechanism of action of the most potent fraction. **Materials and Methods**: Methanol extract and the partitioned chromatography fractions (n-HF, DCMF, EAF, and AF) of the plant obtained according to the standard methods were screened for anti-nociceptive activity. Male Swiss albino mice (20-22 g) were used (n=5 in each group). The experimental models used to assess the anti-nociceptive activity were Acetic acid-induced writhing, formalin, and hot-plate tests. Morphine (5 mg/kg, i.p.) and acetylsalicylate (ASA, 150 mg/kg, i.p.) were used as standard drugs in hot plate, formalin and acetic acid-induced writhing test respectively. **Results:** The methanol extract (ME) and fractions (EAF, and AF) significantly reduced abdominal constrictions in acetic acid, hot plate, and licking behavior of both acute and chronic phases of formalin test. While, fraction DCMF abolished the pain due to acetic acid and second phase of formalin. ME and AF significantly elevated the reaction time to pain in a dose related-manner in hot-plate test compare with control vehicle while fraction n-HF was not significant. The anti-nociceptive effect exhibited by AF in the formalin-induced paw licks was reversed by the systemic administration of naloxone in inflammatory phase, while both phases were blocked by atropine. **Conclusion:** Lecaniodiscus cupanioides methanol extract and fractions (EAF, AF and DCMF), showed anti-nociceptive effect. The effect produced is both peripherally and centrally mediated and the study revealed that the phyto-constituents that are polar in nature may responsible for the central effect of the plant. Aqueous fraction probably exhibited its action via opioid or muscarinic cholinergic pathways.

KEYWORDS: Lecaniodiscus cupanioides; Writhing test; Formalin test; Hot plate test; Anti-nociceptive.

INTRODUCTION

Medicinal plant has been used traditionally to treat pain in Africa without noticing its side effect. Therefore, plant-derived drugs may be introduced with little or no complication and within the reach of common people. Pain is a discomforting feeling in a certain part or all over the body. The significant of medical science is to maintain good human health and to alleviate pain. Knowing the concept of pain, therefore, is one of the necessary materials in realizing this objective. Pain is the major symptom that makes patient visit hospital because it is considered as an indicator of disease, all around the world and in all cultures.[1][2]

Various classes of pain-relieving drug have been reported. These are narcotics and non-narcotics, the examples of narcotic are morphine, codeine while, non-narcotics are non-steroidal anti-inflammatory. Most of these drugs have serious side effect, such as abdominal discomfort, therefore they are contraindicated in ulcer patient.[3] Also, morphine a narcotic analgesic was abused; patients develop tolerance to it which necessitates dose escalation regardless of disease progression limiting its effectiveness and usage.[4] In tropical countries, people have limited access to these modern medicines and even when available, the poverty level of many patients makes it impossible for them to procure those drugs.[5] Therefore, many people are shifting their attention towards the use of herbal medicine. Medicinal plants have been reported to have many secondary metabolites with different biological activity[6][7], which justifies the research on pharmacological properties of plants species and their potential use in drug development. Nowadays herbal drugs are employed for the treatment of pain rather than synthetic drugs. Many studies have reported that extracts of medicinal plants possess anti-inflammatory and analgesic activities.[8] However, in most instances, the

claims of therapeutic used of medicinal plants have not been fully supported by scientific evidence.

Lecaniodiscus cupanioides Planch. ExBth (Sapindaceae) is one of the medicinal plant used traditionally for the treatment of wounds, boils, burns and bruises, tooth ache, fever and abdominal swelling caused by liver abscess.[9][10] Yemitan and Adeyemi, (2005)[11] reported the anticonvulsant property of Aqueous root extract of the plant. The plant has also been shown to have analgesic and hepatotoxic properties.[12][13] The aim of this work was to investigate the fractions from Lecaniodiscus cupanioides and the possible mechanism of action of the most active fraction.

MATERIALS AND METHODS

Plant material and Extraction

Lecaniodiscus cupanioides was collected in autumn year 2018, from a village in Ola-Oluwa Local Government, Nigeria. The plant was Air-dried and then powdered using an electrical mill (mesh number 100). Powdered plant was macerated for 24 hours using absolute methanol. Methanol extract was filtered and evaporated using rotary evaporator under reduced pressure at 40 ºC to yield powdered extract (85 g). The methanol extract was successively partitioned using solvent of different polarity into n-hexane, dichloromethane, ethylacetate, and water. The fractions were concentrated using rotary evaporator to give n-hexane (12.5 g), dichloromethane (18.6 g) ethylacetate fraction (22.20 g, 21.69%) and water or aqueous fraction (35.52 g, 40.13%). 20ml volume of each of the extract or fraction was prepared by dissolving 2g or 2000 mg of the extract in 20ml 0.9% saline to give a 100 mg/ml solution for some of the tests. Equal amount of the ethylacetate fraction was dissolved in DMSO (3%) in 0.9% saline. The partitioning of the methanol extract into n-hexane, dichloromethane and ethylacetate was to locate the fraction with the active principle, particularly to see if the polar medium (aqueous) or the non-polar medium (n-hexane) or semi polar medium (ethylacetate) will have the active pharmacological principle.

Experimental animals

Male Swiss albino mice weighing 20-22 g were obtained from animal house of College of Health Sciences, Ladoke Akintola University, Ogbomoso. They were housed under standard conditions with 12-hour light and 12-hour dark cycles. Five mice were placed in each cage and were given food and water ad-libitum. The animals were allowed to acclimatize prior experimentation. Animal procedures were performed according to guidelines for the care of experimental animals of College of Health Sciences.

Acetic acid-induced writhing test

The writhing test is a chemical method used to induce pain of peripheral origin by injection of irritant principles like phenylquinone and acetic acid in mice. Reduction in the frequency of writhing behaviour of mice was taken as an index of analgesic activity. This method was performed as previously described.[14][15] The mice were randomly grouped into five with five animals per group. Group 1, received the vehicle (normal saline, 10 ml/kg), group 2 received aspirin (150 mg/kg) as standard while groups (3-5) received the extract in the doses of 40, 80, and 160 mg/kg body weight. Thirty minutes later nociception was induced by intraperitoneal administration of 0.6 % aqueous solution of acetic acid and then placed in an observation chamber as described by Koster et al. (1959).[14] Nociception which is the presence of abdominal writhing evidenced as arching of the back, the extension of hind limbs and contraction of the abdominal musculature was measured manually as the number of abdominal constriction observed within a 20-minute period. The same procedure was carried out for partitioned fractions (n-HF, EAF, DCMF, and AF) of the plant (Lecaniodiscus cupanioides).

Formalin-induced paw lick

The formalin-induced paw lick is a popular chemical model of nociception. It involves the injection of a dilute solution of formalin unto the surface of the rodent's hind paw, following which stereotypical behaviours such as flinching, licking and biting of the affected hind paw[16] were scored. The behaviour lasts for approximately one hour, classified into an acute phase (time period immediately following injection) which measures the direct activation of nociceptors and the late phase (15 or 20 minutes after injection) which measures the inflammatory response.[17] Animals were randomly divided into groups (n=5) to be intraperitoneally pretreated with normal saline (vehicle) at 10 ml/kg, standard analgesic morphine sulphate (5 mg/kg), methanol extract (40, 80 and 160 mg/kg) for thirty minutes before exposure to nociceptive stimuli. Each mouse was injected on right hind paw with formalin (1%, 2 ul) as described by Hunskaar and Hole (1987).[18] Nociception was assessed as latency of animal to lick its paw at 0-5 minutes (early phase) and 20-30 minutes (late phase). Anti-nociceptive activity was expressed as the reduction in the duration of paw lick. The same procedure was carried out for partitioned fractions (n-HF, EAF, DCMF, and AF) of the plant (Lecaniodiscus cupanioides).

Hotplate

The hot plate test described by Eddy and Leimbach (1953)[19] is another test for measuring response to pain in laboratory animals. It was used to measure the effectiveness of analgesics by observing the reaction to pain induced by heat. In the hot plate test behaviours such as jumping and hind-licking were observed in response to the presence of a noxious thermal stimulus. Licking is a rapid response to painful thermal stimuli and a direct indicator of the threshold of nociception; jumping is a more detailed response, measures the latency to jump as well as the emotional component of escaping.[20] Mice were randomly divided into groups (n=5) and was pretreated intraperitoneally with methanol

extract (40, 80 and 160 mg/kg). Control group received either normal saline as the vehicle (10 ml/kg i.p) or standard analgesic, morphine sulphate (5 mg/kg). Thirty minutes later the pain episode was induced by thermal stimulus as previously described by Hunskaar et al., (1986)[21], each mouse was placed on a hot plate maintained at 55±0.5ºC. Nociception was assessed when the animal began to lick its hind paw or attempt to jump off the hot plate. The time taken to lick the hind paw was taken as reaction time, Anti-nociceptive activity was expressed as the increase reaction time. The same procedure was carried out for partitioned fractions (n-HF, EAF, DCMF, and AF) of the plant (Lecaniodiscus cupanioides).

Assessment of mechanism of action of the most potent fraction (Aqueous fraction)

Aqueous fraction is the most potent of the portioned fractions, hence, it was chosen for this study. To study the possible mechanisms by which Aqueous fraction exerts its anti-nociceptive activity, formalin-induced test was employed based on its biphasic action.

Involvement of the opioid system

Aqueous fraction (160 mg/kg, i.p), morphine (5 mg/kg, i.p.), or vehicle (10 ml/kg, i.p) was administered thirty minutes after intraperitoneal administration of naloxone (2 mg/kg) a nonselective opioid receptor antagonist before formalin injection, respectively.

Involvement of the adenosinergic system

Theophylline a nonselective adenosine receptor antagonist at a dose of 10 mg/kg was administered to a group of mice. Thirty minutes after which Aqueous fraction (160 mg/kg, i.p), morphine (5 mg/kg, i.p.), or vehicle (10 ml/kg, i.p) was administered and 30 mins after morphine administration.

Involvement of muscarinic receptors

The same procedure was repeated as above, in this case, atropine, a nonselective muscarinic receptor antagonist at a dose of 0.5 mg/kg was given intraperitoneally.

Statistical analysis.

Data were analyzed using One-way analysis of variance (ANOVA) followed by post-hoc tests (Student Newman Keul's) which was used to determine the source of a significant effect. Results were expressed as Mean ± SEM., while $p<0.05$ was taken as accepted level of significant difference from control or vehicle.

RESULTS

Acetic acid-induced writhing test

In this test, the methanol extract of Lecaniodiscus cupanioides leaves significantly ($p<0.05$) reduced the abdominal constrictions of mice in a dose-related manner. Similarly, acetylsalicylate (150 mg/kg, i.p.) profoundly inhibited the acetic acid-induced writhes (Figure 1).

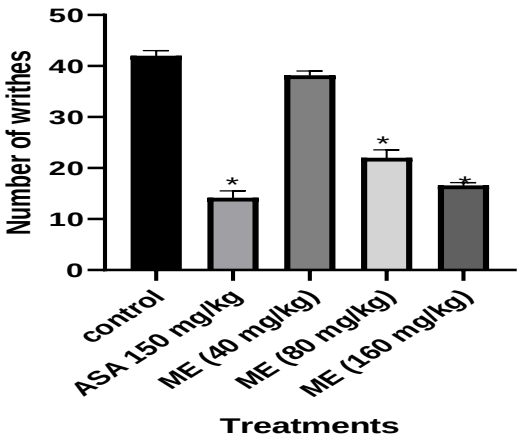

Figure 1: Effect of methanol extract of Lecaniodiscus cupanioides on acetic acid-induced writhing test in mice. Each column represents the mean±SEM (n=5 per group). *P<0.05 compared to treated groups. ANOVA followed by Newman-Keuls Multiple Comparison test

Hot plate test

The anti-nociceptive effect of methanol extract and morphine in hot plate test are given in Figure 2. ME at 40, 80 and 160 mg/kg doses produced a significant ($p<0.05$) increased in the reaction time to the thermal stimulus in a dose-related manner. The effect produced by the extract at 160 mg/kg is compared to be similar to the effect of standard drug (morphine).

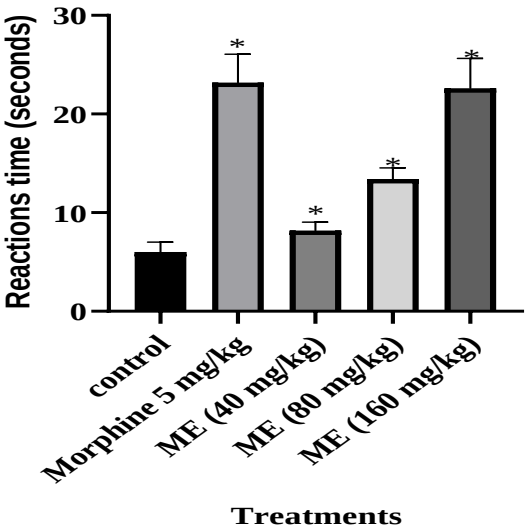

Figure 2: Effect of methanol extract of Lecaniodiscus cupanioides on hot plate test in mice. Each column represents the mean±SEM (n=5 per group). *P<0.05 compared to treated groups. ANOVA followed by Newman-Keuls Multiple Comparison test.

Formalin-induced paw licks

Figure 3 shows the effect of pretreatment of methanol extract and morphine on formalin-induced pain in mice. The intraperitoneal administration of methanol extract

(40, 80, and 160 mg/kg) thirty minutes before the injection of formalin inhibited both and inflammatory phases of formalin-induced licking though inhibition did not reach statistical significance with the dose of 40 mg/kg. Morphine (5 mg/kg, i.p.), the positive standard analgesic control, produced a marked inhibition of both the neurogenic and inflammatory pain phases. Although, the effect produced was not statistically significant at a dose of 40 mg/kg dose.

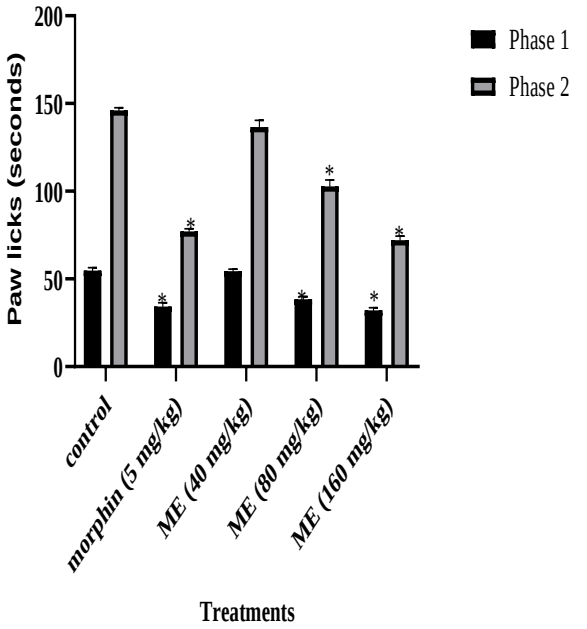

Figure 3: Effect of ME on formalin-induced paw lick. Each column represents the mean±SEM (n=5 per group). *P<0.05 compared to treated groups. ANOVA followed by Newman-Keuls Multiple Comparison test

Effect of partitioned fractions on acetic acid-induced Writhing test

The anti-nociceptive activities of the partitioned fractions (n-Hexane, dichloromethane, ethyl acetate and aqueous fractions) were initially evaluated using the acetic acid-induced writhing test. Administration of acetic acid by intraperitoneal injection to a negative control group of mice that had been orally pretreated with vehicle (normal saline) induced 45.5±21 contortions during the period of the test. On the other hand, the groups of animals pretreated with DCMF, EAF, and AF significantly increasing the percentages inhibitions in that order. The effect produced by EAF at 160 mg/kg is comparable with that of the standard drug (ASA, 150 mg/kg). Meanwhile, the most non polar solvent (n-HF) did not statistically inhibit abdominal contortion compared with the control. Group. Aqueous fraction appears to be the most active of all the fractions and can be kept for isolation and characterization in our next study (Figure 4).

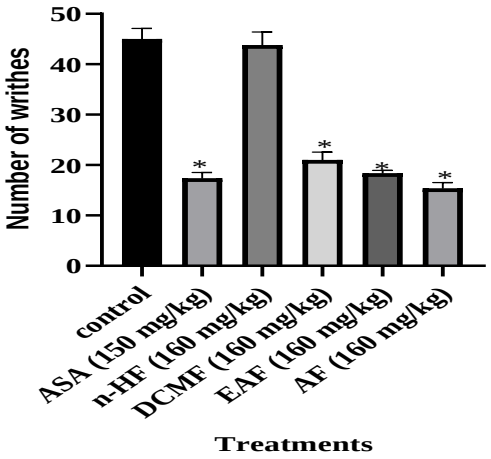

Figure 4: Effect of portioned fractions (n-HF, DCMF, EAF, and AF) on acetic acid-induced writhing test. Each column represents the mean±SEM (n=5 per group). *P<0.05 compared to treated groups. ANOVA followed by Newman-Keuls Multiple Comparison test

Hot plate test

Confirming the anti-nociceptive effect of partitioned fractions, and to investigate their mode of action, whether it is centrally or peripherally mediated. In the hot plate test, a significant ($p<0.05$) inhibition or increase in latency period was reported with morphine, EAF and AF fractions. Biological assays showed that the activity was concentrated in the EAF and AF fractions. While, DCMF and n-HF are not statistically effective. Aqueous fraction produces the greatest effect. These results suggest the anti-nociceptive effect of ethyl-acetate and aqueous fractions is in part mediated by its central analgesic action (Figure 5).

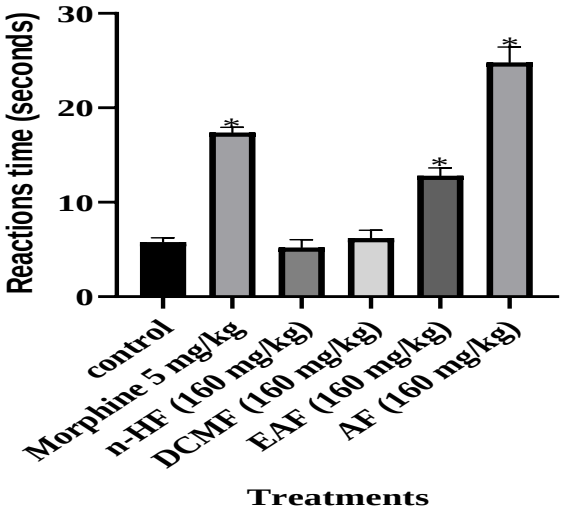

Figure 5: Effect of partitioned fractions (n-HF, DCMF, EAF, and AF). Each column represents the mean±SEM (n=5 per group). *P<0.05 compared to treated groups. ANOVA followed by Newman-Keuls Multiple Comparison test.

Effect of partitioned fractions Formalin-induced paw licks

Figure 6 shows the effect of intraperitoneal administration of partitioned fractions before induction of paw licks by formalin. Biological assays showed that the activity was concentrated in the fractions EAF and AF, both in neurogenic and inflammatory phases. The effect in the fraction DCMF is significant only in the inflammatory phase and not in neurogenic as compared with indomethacin (not shown), while, the fraction n-HF is neither effective in neurogenic nor inflammatory.

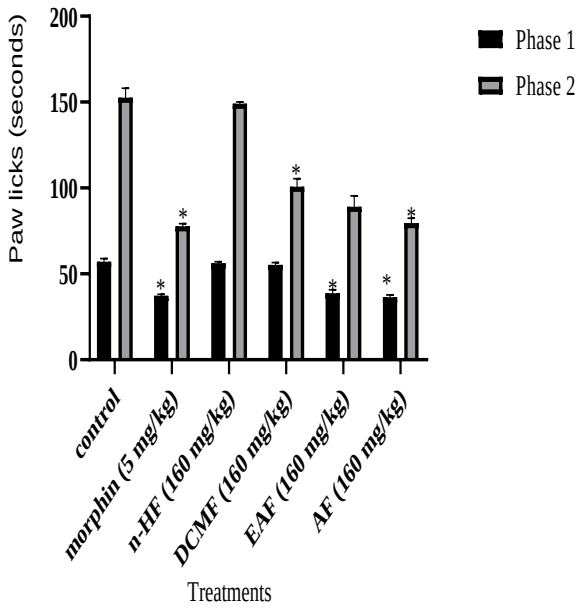

Figure 6: Effect of partitioned fractions (n-HF, DCMF, EAF, and AF). Each column represents the mean±SEM (n=5 per group). *P<0.05 compared to treated groups. ANOVA followed by Newman-Keuls Multiple Comparison test.

Assessment of mechanism of action of the most potent fractions (AF)

Figure 7 shows mice pretreated with naloxone significantly reversed (P<0.05) anti-nociceptive effect of AF in the inflammatory phase but not in neurogenic. Morphine anti-nociceptive effect was also nullified by Naloxone significantly (P<0.05) in both the neurogenic and inflammatory phases of formalin-induced pain.

Theophylline neither abolish the first phase nor the second phase of anti-nociception caused by AF, but significantly reversed anti-nociception caused by morphine in both phases of the formalin test (Figure 8).

Administration of Atropine significantly abolished the anti-nociception caused by the AF in both cases of formalin-induced pain. On the other hand, the effect of morphine was nullified in the second phase but not in the first phase (Figure 9).

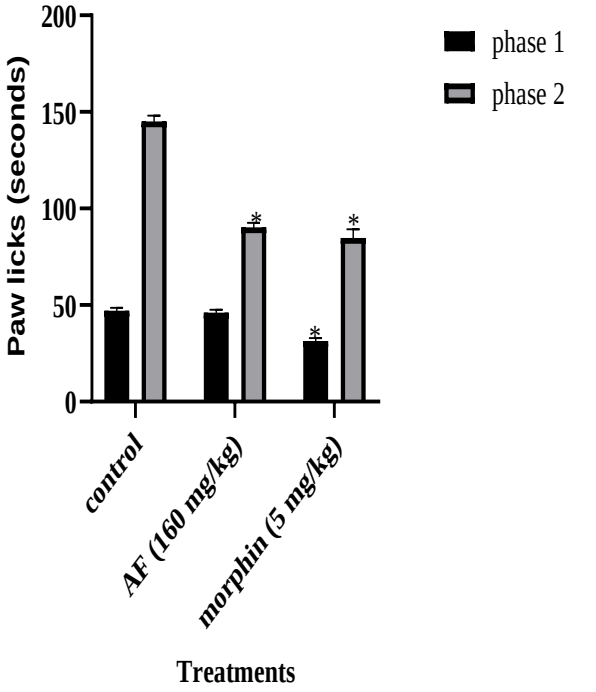

Figure 7: Blocking effect of naloxone on anti-nociception of fraction AF and morphine.
Each column represents the mean±SEM (n=5 per group). *P<0.05 compared to treated groups. ANOVA followed by Newman-Keuls Multiple Comparison test.

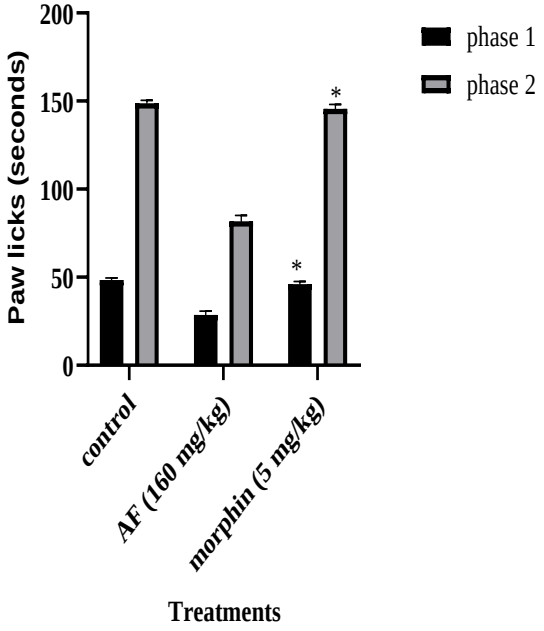

Figure 8: Blocking effect of theophylline on anti-nociception of fraction AF and morphine.
Each column represents the mean±SEM (n=5 per group). *P<0.05 compared to treated groups. ANOVA followed by Newman-Keuls Multiple Comparison test.

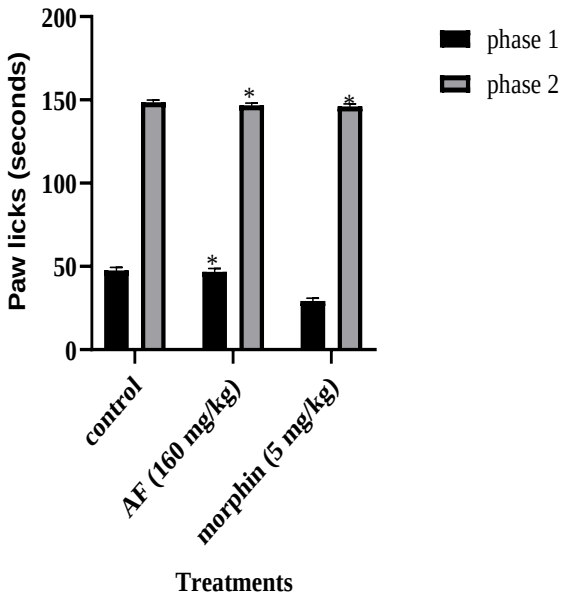

Figure 9: Blocking effect of atropine on anti-nociception of fraction AF and morphine.
Each column represents the mean±SEM (n=5 per group). *P<0.05 compared to treated groups. ANOVA followed by Newman-Keuls Multiple Comparison test.

DISCUSSION

Acetic acid mouse writhing used routinely in the screening of compounds with potential peripheral analgesic activity.[22][23][24] The writhing response is regarded as a visceral inflammatory pain.[25][26] It is a chemical irritant known to cause tissue necrosis and later release of pro-inflammatory mediators such as bradykinin, histamine, prostaglandin, substance P and vasoactive polypeptide. These mediators induce pain either by the sensitization or activation of nociceptors which responsible for the encoding tissue injury.[27] In contrast the hot plate or tail immersion model of pain is usually used to detect potential centrally acting analgesics.[18] In our study, the extract (CME) and partitioned fractions (BF, ethylacetate (EAF), dichloromethane (DCMF), n-hexane (NHF) abolished the nociceptive effects of the chemically induced pain stimuli. The inhibitory effect observed with CME and the various fractions against the nociceptive action of an acetic acid in mice suggested the presence of phytochemicals with significant peripheral analgesic activity. This finding was further supported by the inhibitory property of the fractions in nociceptive (inflammatory pain) behaviour observed with the formalin test. The mechanism of analgesic effect of extract and fractions in acetic acid-induced writhing may be linked to the blockade of the effect or the release of endogenous substances that excites pain nerve endings similar to that of indomethacin and other NSAIDs.[28]

Formalin is another chemical used in this study which causes noxious stimuli to trigger pain. It is used to evaluate agents or drugs that possess central and/or peripheral analgesic activity.[29] Administration of this chemical was reported to induce neurogenic pain which is observed during the early phase (0-5 mins post injection) accompanied by the pain arising from inflammatory reaction to formalin injection observed during the late (20-40 minutes' post injection) phase.[18][30] The neurogenic pain is a mediation of central activity and was due to stimulation of nociceptive primary afferents nerve fibers and the release of pain mediators like kinin, histamine and serotonin. While, inflammatory pain is peripherally mediated and it is due to the peripheral release of chemical pain mediators which activate or sensitize nociceptors like prostaglandin.[31] Nonsteroidal anti-inflammatory drug was reported to have acted Peripherally and very effective against inflammatory pain produced by formalin.[29][32] While, centrally acting analgesics (like morphine) would mitigate formalin induced neurogenic and inflammatory pain. The inhibitory effect observed with CME and partitioned fractions against neurogenic and inflammatory pains may suggest peripheral and central acting analgesic activity similar to morphine.

Notable mediators of the nociceptive pathway such as naloxone, theophylline, and atropine were used to evaluate the mechanism through which AF exerts its anti-nociceptive effect. Because of the biphasic nature of the formalin test and its specificity, it was selected for this study.[4][33] Anti-nociceptive effect of aqueous fraction of formalin test in the second phase was nullified by naloxone suggesting a possible muscarinic receptor involvement in its actions.

Theophylline abolished anti-nociceptive effect of morphine but not the fraction AF. Adenosine has been reported to have blocked anti-nociceptive effect of morphine as previously described[34] and has been confirmed in this study. It acts at several P1 receptors (A_1, A_{2A}, A_{2B}, and A_3) all of which are coupled to G proteins.[35]

This study revealed that the anti-nociceptive effects of Aqueous fraction was completely abolished by the nonselective muscarinic receptor antagonist, atropine, implicates the muscarinic cholinergic system in the actions of the extract. The activation of muscarinic receptors induces anti-nociception in various pain paradigms including thermal, inflammatory, and neuropathic pain as it was previously described.[36][37][38] Therefore, this study also suggested that anti-nociceptive effects of Aqueous fraction may be due to the interaction with one of the muscarinic receptors.

CONCLUSION

In conclusion, Lecaniodiscus cupanioides possessed both peripherally and centrally mediated anti nociceptive activity and this effect resided in the main methanol extract as previously reported, the aqueous as well as the ethylacetate fractions of the extract but not in dichloromethane and n-hexane. Which means the active

pharmacologic principle located in the polar and semi polar medium. The anti-nociceptive effect involves an interaction with muscarinic cholinergic and opioid pathways.

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