

Antinociceptive Activities of Acetone leaf Extracts of *Carissa Spinorum* in Mice

Mworia JK*, Gitahi SM, Juma KK, Njagi JM, Mwangi BM, Aliyu U, Njoroge WA, Mwonjoria KJ, Mawia AM, Nyamai DW, Ngugi MP and Ngeranwa JJN

Department of Biochemistry and Biotechnology, Kenyatta University P. O Box 43844-00100, Nairobi, Kenya.

Abstract

Despite the progress that has occurred in recent years in the development of therapies for pain, there is still a need for effective and potent analgesics for pain. Pain is defined as unpleasant feeling essential for body's defense system. It acts as a warning signal against disturbances in the body. Conventional antinociceptives are expensive and have many side effects. Continued use of these drugs may lead to tolerance and resistance. Medicinal plants have been used to relieve pain and form a better alternative. Herbal antinociceptives are affordable and have arguably fewer side effects. *Carissa spinarum* (Linn) is used to treat rheumatoid pain, fever and inflammation related disorders. This plant is used locally by people in Embu County as analgesics. This study was designed to bioscreen the acetone leaves extracts *C. spinarum* (Linn) for anti-nociceptive potential. The plant parts were collected from Siakago-Mbeere north sub-county, Embu County, Kenya. The samples were prepared and extraction of the active compounds carried out using organic solvent acetone in the ratio 1:2. Swiss albino mice were divided into five groups of five mice each: Normal, negative, reference and experimental group. Pain was induced experimentally using formalin and acetic acid. The experimental groups were treated with 50 and 100 mg/kg dose quantities of plant extract prepared. The acetone leaves extracts of the plants were evaluated for antinociceptive properties in mice compared to the reference drug diclofenac sodium. Mice were injected intraperitoneally with doses of the herbs, diclofenac and the vehicle. Thirty minutes later the animals were injected with 0.01ml of 2.5% formalin in the sub planter region of the left hind paw and the other set with 0.4 ml of 5% acetic acid. The total time spent lifting; biting, licking the paw and writhing were counted and scored. The acetone leaves extracts tested at different dose levels lowered paw licking time in a dose dependant manner. Further, the phytochemical screening results showed that the acetone leaves extracts of *C. spinarum* (Linn) possess anti-nociceptive activities. The study has established that acetone leaves extracts of *C. spinarum* (Linn) are effective in management of pain.

Keywords: *Carissa spinarum* (Linn); Writhing; Nociception; Formalin; Acetic acid

Introduction

Pain is an unpleasant feeling that is essential to the body's defense system. It provides a rapid warning to the nervous system to initiate a motor response to minimize physical harm. Inadequate pain relief is a known problem worldwide. Surveys show that many patients still suffer from moderate to severe pain [1,2] despite an increased focus on pain and the development of new standards for pain management [3].

Conventional analgesics available over the counter include aspirin, paracetamol and ibuprofen. Others are diclofenac and morphine based pain killers such as codeine phosphate or tramadol, opioids and antidepressants. The management of pain is a daily challenge in modern medicine despite the currently available wide range of analgesics. Conventional analgesics are expensive, have arguably many side effects such as gastric disorders, kidney, liver and heart failure, prolonged bleeding after injury and diabetes and continued use may lead to addiction and drug resistance. Almost all pharmacological treatments may produce side effects [4]. Alternative medicines are thought to possess many safe and effective phytochemicals useful in treating various disorders including pain. Several medicinal plants have been tested for antinociceptive activities [5].

Materials and Methods

Collection and preparation of plant materials

Fresh leaves of *C. spinarum* (Linn) were collected from their natural habitats on the basis of ethnobotanical information with the help of local healers from Siakago division, Mbeere North Subcounty in Embu County, Kenya. The samples were cleaned to ensure they are free of dust and other contaminants. They were transported to the department

of Biochemistry and Biotechnology of Kenyatta University for studies. The samples were taxonomically identified and authenticated by an acknowledged taxonomist and a voucher specimen deposited at the Kenyatta University herbarium for future reference. The leaves were air dried at room temperature until completely dry. The dried leaves were then crushed by use of an electric mill to obtain fine powder which was stored at room temperature in air tight containers until used in extraction.

Extraction

200 gms of the plant powder was weighed and put into a conical flask and labeled. 750 ml of acetone were added and corked. The mixture was allowed to stand 12 hr. The extract was filtered into a container using Whatman No.1 filter papers. 250 ml of acetone was added to the remnant and allowed to stand for four hours. Second filtering was then done. The procedure was repeated until the solvent remained clear. The extract was then concentrated using rotary evaporator at 56°C. The extract was then placed in open beaker to allow any remaining acetone

***Corresponding author:** Mworia JK, Department of Biochemistry and Biotechnology, Kenyatta University, P.O. Box 43844-00100, Nairobi, Kenya, Tel: 254724420320; E-mail: kiambijoseph2013@gmail.com

Received August 06, 2015; **Accepted** October 21, 2015; **Published** October 26, 2015

Citation: Mworia JK, Gitahi SM, Juma KK, Njagi JM, Mwangi BM, et al. (2015) Antinociceptive Activities of Acetone leaf Extracts of *Carissa Spinorum* in Mice. Med Aromat Plants S1: 006. doi:[10.4172/2167-0412.S1-006](https://doi.org/10.4172/2167-0412.S1-006)

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to evaporate until a sticky solid was obtained. This was stored at room temperature until use in bioassays.

Experimental animals

Swiss albino mice were used in this study. The mice breeding colony was acquired and bred in the animal breeding and experimentation facility of the department of Biochemistry and Biotechnology, of Kenyatta University. The animals were kept in standard cages and maintained under the standard laboratory conditions at room temperature and with 12 hr dark and 12 hr light cycle. They were fed on rodent pellets diet and supplied with water *ad libitum*. The ethical guidelines and procedures for handling animals were followed in the study.

Experimental design

Formalin-induced antinociceptive assay: The formalin induced antinociceptive assay was carried out [6]. Swiss albino male and female mice weighing between 20-25 gms were divided into five groups of 5 animals each. Group I was intraperitoneally administered with 0.1 ml normal saline (negative control). Group II was administered with 0.01 ml of 2.5% formalin which was injected in the left hind paw (positive control). Group III was split into two groups of five animals each. Each group received treatment as follows; Group IIIa was intraperitoneally administered with 50 mg/kg body weight dose of each plant extract, group IIIb was intraperitoneally administered with 100 mg/kg body weight dose of each plant extract. Group IV was intraperitoneally administered with the standard drug, diclofenac (the reference drug) at a dose of 15 mg/kg body weight. Thirty after each dose administration, pain was induced by injecting 0.01 ml of 2.5% formalin in the left hind paw. The mice were placed in the prexi-glass box for observation. The time that the mice spent lifting, licking or biting the injected paw was recorded [7]. Two distinct periods of intensive licking, biting or lifting activity were identified and scored separately. The first period (early phase) was recorded between 0-5 minutes and the second period (late phase) was recorded between 15-30 minutes.

The percentage inhibition of the licking was calculated using the following formula

$$\frac{C - T}{C} \times 100$$

C

Where,

C = the vehicle control group value for the each phase,

T = the treated group value for the each phase.

Acetic-acid induced antinociceptive assay: This was carried out following the protocol used by Koster, Anderson and De Beer [8]. Swiss male and female mice weighing between 20-25 grams were divided into five groups of 5 animals each. The groups will receive treatment as follows: Group 1 was intraperitoneally administered with administered with 0.1 ml of the vehicle, (normal saline). Group II was intraperitoneally administered with 0.4 ml of 5% acetic acid solution. Group III was divided into two subgroups of five animals each. Each subgroup received treatment as follows; Group IIIa was intraperitoneally administered with 50 mg/kg body weight of each plant extract. Group IIIb was intraperitoneally administered with 100 mg/kg body weight of each plant extract. Group IV was intraperitoneally administered with standard drug, diclofenac sodium (the reference drug) at a dose of 15 mg/kg body weight. Thirty minutes after administration of the each

treatments pain was induced by injection of 20 ml/kg 5% acetic acid in the right side of the belly intraperitoneally. The mice under experiments were then placed in prexi-glass box for observation. The number of abdominal constrictions (writhes) was counted 5 to 15 minutes after acetic acid injection. The percentage inhibition of the writhing was calculated using the following formula described for section above.

Qualitative phytochemical screening: Freshly prepared acetone leaf extracts of *C. spinarum* were subjected to qualitative phytochemical screening to identify presence or absence of major secondary metabolites [9,10]. The classes of secondary metabolites, screened included alkaloids, saponins, tepenoids, sterols, flavonoids, cardiac glycosides and phenolics.

Data management and statistical analysis: The data on latency of pain response was obtained for all the animals in different groups, recorded and tabulated on a broad sheet using MS Excel. The results were expressed as mean \pm standard error of mean (SEM). Statistical significance of differences among group were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc analysis to separate the means and obtain specific significant differences. $P \leq 0.05$ was considered significant. Analysis was done using Minitab statistical software.

Results

Antinociceptive activities of acetone leaf extracts of *C. spinarum* (Linn) on formalin induced pain in mice

Formalin induced pain in two phases; early phase, which lasted between 1and 5 minutes and the late phase, which lasted between 15-30 minutes after formalin injection. Generally, the administration of acetone leaf extract of *C.spinarum* significantly reduced the formalin-induced pain in the early and late phase. This was indicated by reduction in paw licking time (Table 1).

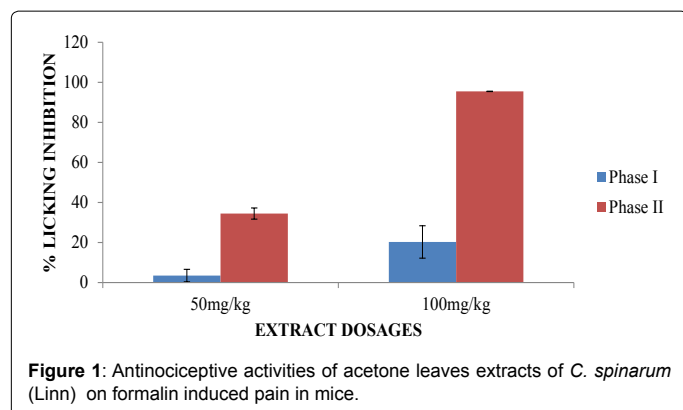
In the early phase, the treatment of mice with acetone leaf extracts of *C.spinarum* at the dose levels of 50 and 100 mg/kg body weight reduced paw licking by 3.47% and 20.27% respectively (Table 1). The antinociceptive effectiveness of the two extract dose levels was not significantly different from each other as well as the controls ($p>0.05$; Table 1).

In the late phase, the treatment of mice with acetone leaf extracts of *C.spinarum* at the dose levels of 50 and 100 mg/kg body weight reduced paw licking by 34.46% and 95.50% respectively (Table 1 and Figure 1). The antinociceptive effectiveness of the acetone leaf extracts of *C.spinarum* at the dose level of 100 mg/kg body weight was comparable

Animal Groups (N=5)	Treatment	Phase1	Phase2
Baseline	Formalin (2.5%)	300.40 \pm 20.8 ^a (-21.16)	287.20 \pm 3.84 ^c (-24.89)
Negative Control	DMSO (30%)	247.60 \pm 3.19 ^a (0.00)	271.60 \pm 2.98 ^a (0.00)
Positive Control	Diclofenac (15mg/kgbw)	209.60 \pm 3.53 ^a (15.34)	10.600 \pm 1.54 ^b (96.09)
Acetone Extract	50mg/kg bw	239.00 \pm 3.11 ^a (3.47)	178.00 \pm 2.77 ^{ac} (34.46)
	100mg/kg bw	197.40 \pm 8.08 ^a (20.27)	12.20 \pm 0.80 ^b (95.50)

Table 1: Antinociceptive activities of acetone leaf extracts of *C. spinarum* (Linn) on formalin induced pain in mice

- Values are expressed as Mean \pm SEM for five animals per group.
- Values with the same superscript are not significantly different by ANOVA followed by Tukey's post hoc test ($p>0.05$).
- The figures in blankets represent % inhibition.



to the reference drug (Diclofenac) which reduced the paw licking time by 96.09%. Although the antinociceptive effectiveness of the acetone leaves extracts of *C. spinarum* at the dose level of 50 mg/kg body weight was not significantly different from the baseline and negative controls ($p > 0.05$; Table 1), it showed a slight but significant antinociceptive effect compared to the positive control group and the group treated with the extract at the dose level of 100 mg/kg body weight ($p < 0.05$; Table 1).

Antinociceptive effects of acetone leaf extracts of *C. spinarum* (Linn) on acetic acid induced pain in mice

Generally, the administration of acetone leaf extract of *C. spinarum* successfully reduced the acetic acid-induced pain (Table 2 and Figure 2). This was indicated by reduction in the number of writhing movement. The acetone leaf extracts of *C. spinarum* at the dose levels of 50 mg/kg and 100 mg/kg body weight reduced acetic acid induced pain in mice by 73.77% and 86.89% respectively (Table 3). However, the antinociceptive effectiveness of the two dose levels was not significantly different from each other but it was comparable to Diclofenac (reference drug), which reduced the number of writhing by 70.49 % ($p > 0.05$; Table 2).

Phytochemical composition of *C. spinarum*

Upon qualitative phytochemical screening, the acetone leaf extracts of *C. spinarum* was found to contain alkaloids, flavonoids, steroids, phenolics and tepenoids while saponins and cardiac glycoside were absent (Table 3).

Discussion

In this study, the acetone leaf extract of *C. spinarum* (Linn) showed a significant antinociceptive effect by reducing formalin paw-licking time in both phases and acetic acid induced writhing, with a more potent activity in the second phase. This suggests both central and peripheral antinociceptive effects [11]. This central antinociceptive effect could have been caused possibly by inhibition of the nociceptive effects of serotonin, adrenaline, noradrenaline, prostaglandins, bradykinin, acetylcholine and adenosine and peripherally by inhibiting the release of endogenous mediators such as PGE_2 , prostaglandins E_2 and PGE_{2a} in perionetal fluids as well as lipooxygenase which stimulates the nociceptive neurons [7].

The significant pain reduction by the plant extract in mice might be due to the presence of analgesic principles acting through the prostaglandin pathways. The mechanism of action of the plant

extract can be postulated to be similar to that of non-steroidal anti-inflammatory agents, such as ibuprofen, aspirin and diclofenac.

The NSAIDs block the production of prostaglandins by truncating the COX1 pathway [12]. This blockage reduces sensitization of the peripheral nervous tissue resulting in less nerve stimulation and ultimately less pain [12].

The results of this study are similar to other previous studies on evaluation of antinociceptive activities of medicinal plant extracts [13,14]. That the acetone leaf extract of *C. spinarum* (Linn) showed significant antinociceptive effects by reducing formalin paw-licking and acetic acid writhings time in both phases is consistent with Gitahi et al. [13] and Hesseinzadeh et al. [14] who worked on DCM methanolic leaf of *C. edulis* (Forssk) Vahl in laboratory animals and *M. officinalis* against pain respectively.

The dose ranges used in this study were within the dose ranges used by Gitahi et al. [13,15-18]. Giutahi et al. used dose ranges of 50, 100 and 150 mg/kg body weight while evaluating antinociceptive activity of DCM: methanolic leaf and root bark extracts of *C. edulis* (Forssk.) Vahl in laboratory animals [13].

Animal Groups (N=5)	Treatment	Number Of Writhings	% Inhibition
Baseline	Acetic acid 20ml/kgbw	19.60±3.70 ^a	-60.66
Negative Control	DMSO (30%)	12.20±1.50 ^a	0.00
Positive Control	Diclofenac(15mg/kgbw)	3.60±1.50 ^b	70.49
Acetone Extract	50 mg/kgbw	3.20±1.02 ^b	73.77
	100 mg/kgbw	1.60±0.40 ^b	86.89

Table 2: Antinociceptive properties of *C. spinarum*(Linn) in Acetic acid-induced pain in mice.

a. Values are expressed as Mean ± SEM

b. Values with the same superscript are not significantly different by ANOVA followed by Tukey's post hoc test ($p > 0.05$).

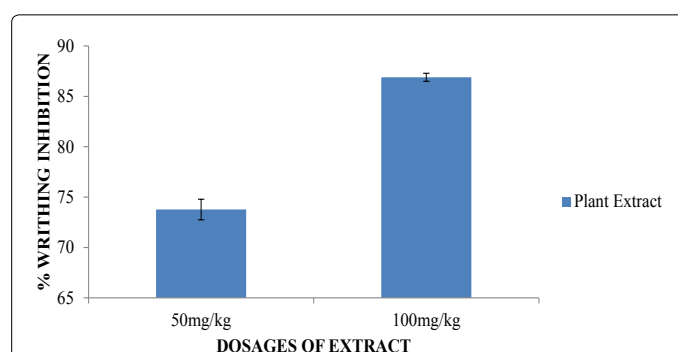


Figure 2: Antinociceptive properties of *C. spinarum*(Linn) in Acetic acid-induced pain.

Pytochemicals	<i>C. spinarum</i>
Alkaloids	+
Flavonoids	+
Steroids	+
Saponins	-
Cardiac glycosides	-
Phenolics	+
Terpenoids	+

Table 3: Phytochemical composition of acetone leaf extracts of *Carissa spinarum* (Linn). Present phytochemicals are denoted by (+) sign, absent phytochemicals are denoted by (-) sign.

The acetone leaf extract of *C. spinarum* (Linn) at the lower dose level of 50 mg/kg body weight was not as effective as the higher dose of 100 mg/kg body weight in both tests (Tables 1 and 2) This may mean that the effect is dose related or on the other hand there could have been fast metabolism, clearance and inactivation of the lower concentration of the active principle. Dose dependent antinociceptive effects were working with ethanolic extracts of *Ocimum kilim*, *scharicum baker ex gürke* and *ocimum kenyense ayob. ex a.j. paton* leaves [19] and DCM: methanolic leaf and root bark extracts of *C. edulis* (Forssk.)Vahl in laboratory animals [13].

In this study high concentration (100 mg/kg body weight) of the acetone leaf extracts of *C. spinarum* (Linn) reduced the writhes. This possibly may be explained by higher dissociation in higher concentration and faster filtration across the mucosal lining [20]. The antinociceptive effect of the acetone leaf extract of *C. spinarum* (Linn) can be attributed to one or more groups of the phytoconstituents detected in the extracts. The flavonoids too from *M. officinalis* have been associated with antinociceptive effects [21]. Flavonoids have been shown to cause anti-nociceptive effects by widely targeting prostaglandins which are involved in the pain perception through moderating opioidergic mechanism [22]. The anti-nociceptive effects of *M. officinalis* are attributed to the flavonoids present in the extracts. On the other hand, studies have shown that flavonoids widely target prostaglandins which are involved in the pain perception through moderating opioidergic mechanism [22]. Alkaloids present in *K. macrophylla* showed analgesic actions [23] while aqueous extracts of *Radix Aconiti Carmichaeli* exhibited antinociceptive effect probably due to the presence of high content of mesaconitine alkaloids [24]. Among other actions, naturally occurring terpenoids present antinociceptive properties by inhibiting platelet aggregation, and interfering at the intracellular level, with several steps of signal transduction mechanisms [25].

Conclusions

In conclusion, the present study has demonstrated antinociceptive potential of acetone leaves extracts of *C.spinarum* (Linn) in mice models. The significant reduction in formalin induced pain when treated with standard drugs as well as different doses of extracts reflects that *C.spinarum* (Linn) acetone leaves extracts at 100 mg/kg body weight were almost similar to the standard drug diclofenac sodium. For acetic acid induced pain, there was significant reduction of pain when treated with the standard drug diclofenac as well as *C. spinarum* (Linn) at doses 100 mg/kgbw and 50 mg/kgbw respectively. The acetone leaves extracts of *C.spinarum* contain different phytochemicals (secondary metabolites) which are responsible for antinociceptive activity (Table 3).The acetone leaves extracts of *C.spnarum* (Linn) demonstrated antinociceptive properties and acts possibly centrally and peripherally.

Acknowledgement

The authors gratefully acknowledge the technical support availed to them by Daniel Gitonga Mwaniki and James Adino.

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