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Antinociceptive and anti-arthritic properties of hydroethanolic leaf extract of *Clausena anisata* (Willd.) Hook. f. ex Benth (Rutaceae) in Rodents: possible mechanism of actions

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Summary: The leaves of Clausena anisata (Willd.) Hook. f. ex Benth (Rutaceae) is used in Traditional African medicine for the treatment of various ailments including arthritis. The present study sought to investigate the antinociceptive and anti-arthritic properties of hydroethanolic leaf extract of Clausena anisata (HeCA). HeCA (100, 200 or 400 mg/kg, p.o.) was administered 1 h before intraplantar injection of formalin 1%v/v in saline to evaluate antinociceptive effect. Moreover, its possible mechanism of antinociceptive action was investigated through pretreatment of mice with antagonists of receptors implicated in nociception. Anti¬inflammatory effect of the extract was investigated using the carrageenan-induced paw oedema and complete Freund's adjuvant (CFA)-induced arthritis models in rats. HeCA (400 mg/kg) treatment significantly reduced the duration of paw licking/biting during both in the early (42.12%) and late (75.79%) phases of formalin-induced nociception. However, the antinociceptive effect elicited by HeCA was reverse by pretreatment of mice with naloxone, prazosin, yohimbine, ketanserin, L-arginine, and parachlorophenylalanine (PCPA). HeCA produced dose-dependent and time course decrease in carrageenan-induced paw oedema. Pre- and post-treatment of rats with HeCA ameliorated CFA-induced arthritis evidenced in the significant decrease in arthritic index comparatively similar to the effect of celecoxib. CFA- induced oxidative and nitrosative stress were attenuated by subchronic treatment with HeCA. Findings from this study shows that C. anisata possesses antinociceptive activity through possible interaction with opioidergic, noradrenergic, L-arginine-nitric oxide and serotonergic pathways as well as anti-arthritic property which could be attributed to its ability to prevent the release of inflammatory mediators and oxidative stress.

Keywords*:* Complete Freund's adjuvant; L-arginine-nitric oxide; nociception; antioxidant; rheumatoid arthritis; serotonergic.

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INTRODUCTION

Clausena anisata (Wild) Hook .F. ex Benth (Rutaceae) is a tropical shrub or tree up to 10 meters high growing in and on evergreen forests. It is commonly known as "mbiet ekpene'' (Ibibios, Niger Delta region) and "Agbasa'' (Yoruba, Southwest) of Nigeria. The plant is traditionally used as effective remedies for worm infections, respiratory ailments, hypertension, malaria, fever, rheumatism, and other inflammatory conditions, headaches, pains, toothaches, convulsions and others (Hutchings et al., 1996). A mixture of Clausena anisata, Afraegle paniculata and Azadirachtha indica is taken against gut disturbance and a concoction of the latter called "Agbo" (Yoruba, Southwest, Nigeria) is used as an antimalarial medicine. The Ibibios use the plant to treat measles (Ajibesin et al., 2005), malaria, pains and inflammations. In the Temeke district (Tanzania) traditional healers employ C. anisata against epilepsy and as an anticonvulsant. All members of the genus contain different bioactive compounds from different chemical classes (Arbab et al., 2012). The plant has been reported to contain coumarins, limonoids, carbazole alkaloids, monoterpenoids furanocoumarin lactones and essential oils (Usman et al., 2010). Reports of antimicrobial (Gundidza et al., 1994), antibacterial (Senthikumar and Venkatesalu, 2009), antidiabetic (Ojewole, 2002), anticonvulsant (Makanju, 1983), and antineoplastic (Ito et al., 2009) have been published. Thus, this study was carried out to investigate the antinociceptive and anti-arthritic activities of the hydroethanolic leaf extract of Clausena anisata in rodents

MATERIALS AND METHODS

Plant material

The fresh leaves of Clausena anisata were collected from Abatadu Village, Ikire, Osun state. Botanical Identification and authentication was done by Mr. T.K Odewo a forestry expert of the herbarium section, Department of Botany, University of Lagos, Akoka, Lagos State, Nigeria. A voucher specimen number

LUH 5703 was deposited in the herbarium for reference.

Preparation of the extract: The leaves of Clausena anisata were washed and air dried. Dried leaves were then grinded and weighed. Eight hundred and twenty grams of the powdered leaves were soaked with 2 L of 70% ethanol in distilled water for 72 h after which the preparation was decanted using the Whatman No.1 filter paper (size 9 cm). The filtrate was concentrated on Rotavapor and the concentrate was oven dried at 40°C. The percentage yield was 2.29% of dark-brownish extract.

Preliminary phytochemical analysis: The preliminary qualitative phytochemical analysis was done using the method of Harbone (1973). Quantitative estimations of total phenolic, tannins, alkaloid, saponins and cardiac glycosides contents were carried out as described by El-Olemy et al. (1994) and Senguttuvan et al. (2014).

Experiment animals: Male Sprague-Dawley rats (90-110 g) and albino mice (15-19 g) were obtained from the laboratory Animal Centre, College of Medicine, University of Lagos. The animals were maintained under standard environmental conditions (12 h/12 h light/dark cycle) and had free access to standard rodent pellet diet (Livestock Feed Plc, Lagos, Nigeria) and water. The animals were acclimatized in the laboratory conditions for a week before the commencement of the study. The experimental procedures adopted in this study were in strict compliance with the ethical standards of the Research Grant and Animal Experimentation Committee of the College of Medicine, University of Lagos, Nigeria and in accordance with the United States National Institutes of Health Guidelines for Care and Use of Laboratory Animals in Biomedical Research (1985).

Drugs and chemicals: Complete Freund's adjuvant (CFA), ethanol, prazosin, formalin, carrageenan, ketanserin, yohimbine, L-arginine, reserpine, naloxone, glibenclamide (Sigma Aldrich, Louis, MO, USA), celecoxib (Pfizer manufacturing Deutschland GmbH, Illertissen, German), morphine (Martindale Pharma, Essex, United Kingdom).

Acute toxicity test: Female albino mice were fasted for 12 h before the study but had access to water. Five groups of mice (n = 5) received; normal saline 10 ml/kg, C. anisata (250, 500, 1000 and 5000 mg/kg,p.o.). They were observed for toxic symptoms and behavioral changes (sedation, hyperactivity, diarrheal, writhing, piloerection, restlessness etc.) for 2 h post-administration and 14 days for signs of delayed mortality (Ishola et al., 2014a).

PHARMACOLOGICAL STUDIES

Antinociceptive activity

Formalin induced nociception test: Mice (17-19 g) were fasted for 12 h before the study and randomly assigned into five groups of six mice each. The treatment include; Group I: normal saline (10 ml/kg, p.o), Group II: morphine (3 mg/kg, p.o), Group III-V: C. anisata (100, 200, and 400 mg/kg, p.o., respectively). One hour after oral administration, 20pl of formalin (1%v/v in saline) was injected subcutaneously into the right hand paw of each mouse. The time (in seconds) spent in licking or biting the injected paw, indicative of pain, was recorded for each animal. The responses of the mice were observed for the first 5 min and 15-30 min post-formalin injection. The values were recorded and percentage inhibition calculated (Ishola et al., 2011).

Elucidation of possible mechanism of antinociception in mice: To investigate the roles of opioid system in the antinociceptive effect of HeCA, mice (n =6) were pretreated with naloxone (5 mg/kg, s.c, non-selective opioid receptor antagonist) (Rajendran et al., 2000) or vehicle (10 ml/kg), 15 min after pretreatment, HeCA (400 mg/kg, p.o.) or vehicle (10 ml/kg, p.o.), 1 h later, formalin test was carried out. The effect of serotonin synthesis on HeCA-induced antinociception was also investigated. Mice were pretreated with pCPA (100 mg/kg, i.p., tryptophan hydroxylase inhibitor) (Ishola et al., 2014b) or vehicle, for 4 consecutive days, 1 h after last treatment, HECA (400 mg/kg) was administered. Similarly, the effect of monoamine neurotransmitter depletion was also assayed. Mice were pretreated with reserpine (2 mg/kg, i.p.; vesicular monoamine transporter inhibitor) (Ishola et al., 2014b), 24 h prior to oral administration of HeCA (400 mg/kg). One hour later, the formalin test was carried out.

The specific role of serotonin receptor subtypes was also investigated, mice were pretreated with ketanserin (5 mg/kg, i.p.; non-selective 5HT2A/C receptor antagonist) (Alchaider, 1991) or vehicle, 15 min later, HECA (400 mg/kg) was given, 1 h post treatment, the formalin test was carried out.

The contribution of L-arginine-nitric oxide pathway in HECA-induced antinociception was assayed. Mice were pretreated with L-arginine (750 mg/kg, i.p., nitric oxide precursor) (Morgan et al., 1992) or vehicle, and 15 min later, they received HECA (400 mg/kg, p.o.) or vehicle (10 ml/kg, p.o.), 1 h later, formalin test was carried out. The possible participation of adrenergic system in the antinociceptive effect of HeCA was also evaluated, mice were pretreated with prazosin (1 mg/kg, i.p.; a1-adrenoceptor antagonist) or yohimbine (1 mg/kg, i.p.; a2-adrenoceptor antagonist) (Kaur et al., 2005; Ishola et al., 2014b) or vehicle (10 ml/kg,

p.o.), 15 min later, HECA (400 mg/kg, p.o.) or vehicle (10 ml/kg, p.o.) was administered. One hour after treatment, effect in formalin test was done.

The effect of ATP-sensitive potassium channels pathway on HeCA-induced antinociception was also examined. Mice were pretreated with glibenclamide (10 mg/kg, i.p; ATP-sensitive potassium channels blocker) (Alves et al., 2004), 15 min later, HECA (400 mg/kg, p.o.) was administered and 1 h later, formalin test was carried out.

Anti-inflammatory activity

Acute inflammation (Carrageenan-induced paw oedema model): Male Sprague-Dawley rats were fasted for 12 h before the study and randomly assigned into five groups of five rats each. The animals were treated 1 h before intraplantar injection of 100 pl of carrageenan (1%w/v in normal saline) into the right hind paw as follows: Group I: Normal saline (10 ml/kg, p.o), Group II: celecoxib (3 mg/kg, p.o), Group III-V: C. anisata (100, 200, and 400 mg/kg, p.o., respectively). The circumference of the injected paw was measured before and at 1, 2, 3, 4, 5 and 6 h post-carrageenan injection using the cotton thread method of Bamgbose and Noamesi, (1980).

Chronic inflammation (Complete Freund's adjuvant-induced arthritis model): Male Sprague-Dawley rats (90-110 g) were fasted for 12 h before the study and randomly assigned into seven groups of seven rats each. Arthritis was induced by intradermal injection of 100 pl of Complete Freund's adjuvant (CFA) into the right hind paw (Ahmad et al., 2006). The adjuvant contained Mycobacterium tuberculosis (10 mg) in 10 ml of paraffin oil. Treatments were given orally, 1 h before injection of CFA as follows: Group I: Control untreated group (Normal saline 10 ml/kg), Group II: Normal saline (10 ml/kg) + CFA, Group III: Celecoxib (3 mg/kg) + CFA, Group IV- VI: HeCA (100, 200 and 400 mg/kg p.o., respectively) + CFA, Group VII: HeCA (400 mg/kg; treatment commenced 4 days post CFA injection). The treatments were given for 28 and 24 days, respectively, for pre- and post-treatment groups, respectively. Arthritis was assessed by means of physical and biochemical measurements (Anburajan et al., 2012). The paw diameter and body weight were measured on day 0, i.e. before the injection of CFA and thereafter every 4th day for 28 days using cotton thread method of Bamgbose and Noamesi, (1980). Arthritic index was calculated using the method described by Kokkola et al. (2003) using ordinal scales as follows: 0 = unaffected; 1 = 1 type of joint affected; 2 = 2 types of joints affected; 3 = 3 types of joints affected; 4 = 3 types of joints affected and maximal erythema and swelling (maximum obtainable score per rat was set at 8 (4 points x 2 hind paws). On day 28 post-arthritic induction, the animals were anaesthetized with chloral hydrate (400 mg/kg, i.p.). Blood samples were collected for heamatological analysis through ocular puncture. The animals were euthanized by cervical decapitation.

Haematological analysis

The fully automated clinical haematological analyzer (Pentra-XL 80, Horiba ABX, USA) was used to evaluate the haematological parameters including total white blood cell count (leucocyte count), red blood cell count (RBC), haemoglobin (Hb), platelet count (PLT), neutrophil, mean cell haemoglobin concentration (MCHC), mean red cell volume (MCV) and mean cell haemoglobin, haematocrit (PCV).

Measurement of in vivo antioxidants and MDA levels

The determination of serum catalase (CAT), superoxide dismutase (SOD), reduced glutathione (GSH), nitrite, and malondialdehyde (MDA) were carried out using the protocol of Awodele et al. (2013).

Statistical Analysis

The results obtained were expressed as mean ± SEM and statistical level of significance between treatment groups was analyzed by one- or two-way Analysis of Variance (ANOVA) followed by Tukey's or Bonferroni post hoc multiple comparison tests, respectively (whichever is applicable), using Graphpad prism 6 (Graphpad software Inc., CA, USA).

RESULTS

Acute toxicity test

The extract up to 5000 mg/kg, p.o. did not induce mortality up to the 14th day of observation but the following toxicity behaviours were observed; writhing, hyperactivity, tachypnea and stooling.

Phytochemical Analysis of HeCA

The preliminary phytochemical screening of the hydroethanolic leaf extract revealed the presence of alkaloids, flavonoids, tannins, saponins, steroids and cardiac glycosides. Also, the quantitative analysis of the extract indicates; total phenolic 162.50±0.31 mg GAE/g, tannins 95.26±0.14 mg CE, alkaloids 47.31±0.27 mg/100g, saponins 16.39±0.43 mg DE/ 100g, and cardiac glycosides contents 57.48±0.13 mg DE/100 g. (GAE, gallic acid equivalent; CE, catechins equivalent, DE, dried extract).

Effect of HeCA on formalin-induced nociception in mice:

Intraplantar injection of formalin produced flinching or biting behaviour indicative of pain; the observed

Table 1: Effect of *Clausena anisata* leaf extract on duration of paw licking in formalin test

Treatment (mg/kg)	0-5 min	Inhibition (%)	15-30 min	Inhibition (%)
Vehicle 10ml/kg	54.39±4.00		106.20±16.65	
Morphine 3	15.63±2.24[c]	71.26	19.55±6.45[c]	81.59
HeCA 100	37.20±1.12[a]	31.61	115.00±13.85	-8.30
HeCA 200	33.39±5.25[a]	38.61	54.08±15.12[c]	49.08
HeCA 400	31.48±3.17[a]	42.12	25.71±1.01[c]	75.79

Values are expressed as Mean ± S.E.M. (n=6) [a]p<0.05, [c]p<0.001 versus vehicle 10 ml/kg treated group. Statistical level of significance using one way ANOVA followed by Tukey *post hoc* multiple comparison tests.

duration of paw licking in vehicle treated control are; 54.39±4.00 s and 106.20±16.65 s in the early and late phases, respectively (Table 1). However, the formalin-induced nociception in early and late phases in mice were significantly reduced by HeCA (100-400 mg/kg) treatment, to 31.48±3.17s (42.12% inhibition) and 25.71±1.01 s (75.79% inhibition) in the early and late phases, respectively, at 400 mg/kg (peak effect). In addition, morphine (3 mg/kg) treatment, significantly reduced the duration of nociception by 71.26% and 81.59% in the early and late phases, respectively (Table 1).

Mechanism of antinociceptive action of HeCA:

As shown in table 2, pretreatment of mice with naloxone (non-selective opioid receptor antagonist) prevented the anti-nociceptive effect elicited by HeCA in formalin test. Post hoc analysis revealed that naloxone pretreatment significantly (P<0.0001) reversed the effect of the extract in early and late phases. However, monoamine vesicular storage depletor, reserpine, failed to block the anti-nociceptive effect elicited by HeCA in murine formalin-induced nociceptive model (Table 2).

Four consecutive days pretreatment of mice with pCPA (serotonin synthesis inhibitor) blocked the effect of the extract. Pretreatment of mice with selective 5HT2A/2C receptor antagonist, ketanserin completely reversed the antinociceptive effect elicited by HeCA (P<0.001). pretreatment of mice with L-arginine (precursor of nitric oxide synthesis) abolished the antinociceptive effect of the extract in both phases of formalin test. In another series of experiment, the pretreatment of mice with prazosin (an a1 adrenoceptor antagonist) (P<0.001) or yohimbine (a2 adrenoceptor antagonist) (P<0.0001) prevents the antinociceptive effect elicited by HeCA (Table 2). In contrast, pretreatment of mice with glibenclamide (ATP-sensitive K+ channels blocker) failed to prevent the HeCA-induced antinociceptive effect in mice.

Effect of HeCA on carrageenan-induced paw oedema in rats: The intraplantar injection of carrageenan induced a time course increase in paw circumference (indicative of oedema), that peaked 3 h post injection (0.70±0.05 cm). However, pretreatment of rats with celecoxib (3mg/kg), produced time course and significant reduction of early and late phases of carrageenan-induced oedema, with peak effect 84.14% inhibition of oedema at 6h. Although, low doses of HeCA (100 and 200 mg/kg) could not attenuate the early stage oedema (1-2 h) but they effectively inhibit late phase of oedema. Moreover, HeCA (400 mg/kg) produced time course and significant reduction of oedema in both phases with peak effect 98.57% inhibition of oedema at the 5th h. In addition, two way ANOVA revealed significant effect of HeCA and celecoxib treatment [F(5,150)=48.43,P<0.0001] (Table 3).

Effect of HeCA on CFA-induced arthritis in rats

As shown in the table 4, intraplantar injection of complete Freund's adjuvant (CFA) into the right hind paw of rats produced time course and significant (p<0.001) increase in paw diameter which peaked on day 4 and thereafter gradually reduced by day 28 but still significant increase when compared to normal untreated rats. However, two way ANOVA revealed significant effect of subchronic treatment with HeCA (100-400 mg/kg) [F(6,245)=10.83,P<0.001], time course and significant decrease in paw oedema induced by CFA were observed. Moreover, the peak arthritogenic effect of CFA on day 8 was significantly reduced by HeCA 400 mg/kg (35.71%)

but the standard reference drug, celecoxib failed to produce a significant reduction. Moreover, the anti-arthritic effect of celecoxib was evident by day 12 of treatment (27.08% inhibition; P<0.05), with peak anti-arthritic effect on day 28 (55.07% inhibition; p<0.001).

In addition, post treatment with HeCA (400 mg/kg, p.o.) from day 4 post-induction, produced time course and significant (p<0.01, p<0.001) decrease in paw oedema-induced by CFA from day 12 to day 28 (44.93% inhibition on day 28), but in pretreatment group, peak anti-arthritic effect recorded in HeCA 200 mg/kg, treatment (57.97% inhibition, p<0.001) (Table 4). The results presented in Table 5 depict changes in body weight of the rats from week 1 to week 3 of the study. Intraplantar injection of CFA induced reduction of the body weight of vehicle- treated, control when compared to untreated control group. However, the decrease in body weight observed in vehicle-treated arthritic rats were

Table 2: Possible mechanism of antinociceptive action of HeCA in mice

Treatment	Dose (mg\kg)	0-5 min	Inhibition (%)	15-30 min	Inhibition (%)
Vehicle	10ml/kg	117.60±8.66		106.70±7.66	
HeCA	400	31.48±3.17***	73.18	25.71±1.01***	75.90
NAL + Veh	5	110.32±12.56		99.76±18.67	
NAL + HeCA	5 + 400	83.48±11.77[d]	29.01	95.18±12.79[d]	10.80
Reserp + Veh	2	17.60±8.66		106.70±7.66	
Reserp+ HeCA	2 + 400	58.55±4.01	50.21	15.40±3.04	85.57
PCPA + Veh	100*4d	96.17±16.52		98.80±16.54	
PCPA+ HeCA	100*4d +400	72.27±4.29[c]	38.54	111.30±7.20[d]	-4.31
KET + Veh	5	109.60±12.18		98.06±19.27	
KET + HeCA	5 + 400	97.28±12.85[d]	17.27	120.20±10.34[d]	-12.65
L-ARG + Veh	750	122.33±18.77		106.70±16.32	
L-ARG + HeCA	750 + 400	100.10±8.78[d]	14.88	69.63±6.72[d]	34.74
Prazosin + Veh	1	119.21±11.08		97.15±7.66	
Prazosin +	1 + 400	89.74±4.10[d]	23.69	92.91±7.19[d]	12.92
YOH + Veh	1	102.60±10.78		91.06±16.76	
YOH + HeCA	1 + 400	89.62±13.29[d]	23.79	48.39±2.91	54.65
GLIB + Veh	10	112.23±12.87		89.45±12.09	
GLIB+ HeCA	10 + 400	52.71±5.28	55.18	4.42±1.55	95.86

Values are expressed as mean ±S.E.M. (n=6), ***p<0.001 versus vehicle-treated control; cp<0.001, dp<0.0001 versus HeCA 400 mg/kg treated. (Veh-vehicle, NALnaloxone, KET-ketamine, GLIB-glibenclamide; reserp-reserpine; YOH-yohimbine PCPA, para-chlorophenylalanine for 4 consecutive days).

Table 3: Effect of HeCA on carrageenan-induced paw oedema in rats

Treatment (mg/kg)	Time course of paw edema (cm)					
	1h	2h	3h	4h	5h	6h
Vehicle	0.21±0.05	0.64±0.05	0.70±0.05	0.70±0.07	0.70±0.05	0.70±0.05
Celecoxib 3	0.13±0.0[a] (38.10)	0.37±0.04[a] (42.19)	0.37±0.05[a] (47.14)	0.29±0.0[c] (58.57)	0.11±0.0[c] (84.29)	0.09±0.03[c] (87.14)
HeCA 100	0.17±0.04 (19.05)	0.63±0.05 (1.56)	0.60±0.09 (1.43)	0.47±0.0[a] (32.86)	0.33±0.0[b] (52.86)	0.23±0.06[b] (67.14)
HeCA 200	0.19±0.04 (9.52)	0.49±0.04[a] (23.44)	0.41±0.01[a] (41.43)	0.29±0.0[b] (58.57)	0.14±0.0[c] (80.00)	0.03±0.02[c] (95.71)
HeCA 400	0.11±0.0[b] (47.62)	0.53±0.03(17.19)	0.39±0.03[a] (44.29)	0.23±0.0[c] (67.14)	0.10±0.0[c] (98.57)	0.01±0.01[c] (98.57)

Values are expressed as mean ±S.E.M. (n=6) ap<0.05, bp<0.01, cp<0.001, versus vehicle 10 ml/kg control treated group; Values in parenthesis represent % inhibition of oedema.

Table 4: time course **e***ffect of HeCA on CFA-induced arthritis in rats*

Treatment (mg/kg)	Change in paw circumference (cm)						
	Day4	Day8	Day12	Day16	Day20	Day24	Day28
Vehicle 10 ml/kg	0.02±0.02	0.05±0.02	0.10±0.02	0.13±0.02	0.15±0.02	0.15±0.02	0.15±0.02
Vehicle 10 ml/kg + CFA	1.12±0.04	0.98±0.07	0.96±0.10	0.94±0.07	0.89±0.04	0.77±0.05	0.69±0.07
Celecoxib 3 + CFA	0.80±0.06 (28.57)	0.73±0.03 (25.51)	0.70±0.04[a] (27.08)	0.70±0.05[a] (25.53)	0.63±0.05[a] (29.21)	0.38±0.06[b] (50.65)	0.31±0.07[c] (55.07)
HeCA 100 + CFA	0.91±0.03 (18.75)	0.77±0.04 (21.43)	0.71±0.04[a] (26.04)	0.66±0.04[a] (29.79)	0.60±0.05[a] (32.58)	0.56±0.06[a] (27.27)	0.33±0.05[b] (52.17)
HeCA 200 + CFA	0.90±0.06 (19.64)	0.97±0.06 (1.02)	0.84±0.08 (12.50)	0.69±0.06[a] (29.79)	0.60±0.04[a] (32.58)	0.53±0.05[a] (31.17)	0.29±0.06[c] (57.97)
HeCA 400 + CFA	0.94±0.03 (16.07)	0.63±0.12[a] (35.71)	0.69±0.08[a] (28.13)	0.67±0.07[a] (28.72)	0.51±0.09[b] (42.70)	0.41±0.09[b] (46.75)	0.37±0.09[b] (46.38)
HeCA 400 PT +CFA	- -	0.90±0.04 (8.16)	0.74±0.06[a] (22.92)	0.73±0.05[a] (22.34)	0.56±0.09[a] (37.08)	0.56±0.07[a] (27.27)	0.38±0.08[a] (44.93)

Values are expressed as mean ±S.E.M. (n=6), ***p<0.001 versus normal untreated rats; ap<0.05, bp<0.01, cp<0.001 versus vehicle 10 ml\kg + CFA treated group. CFA complete Freund's adjuvant; PT, post treatment from day 4; Values in parenthesis indicate % inhibition of oedema.

Table 5: Effect of HeCA on body weight of CFA-induced arthritic rats

Treatment (mg/kg)	**Change in body weight (g)**		
	Week 1	**Week 2**	**Week 3**
Vehicle 10 ml/kg	8.88±2.06	15.88±3.81	21.38±4.41
Vehicle 10 ml/kg + CFA	7.00±2.16	15.50±4.76	18.88±6.24
Celecoxib 3 + CFA	13.13±1.17	17.50±1.97	26.75±2.53[a]
HeCA 100 + CFA	10.71±2.61	14.57±2.10	23.29±3.32
HeCA 200 + CFA	11.38±2.42	15.88±2.90	28.38±12.47[b]
HeCA 400 + CFA	13.25±2.24	20.75±2.52	22.75±3.14
HeCA 400 PT + CFA	14.83±1.01	20.67±0.80	25.33±1.02

Values are expressed as mean ±S.E.M. (n=6); ap<0.05, bp<0.01 in comparison with vehicle treated CFA-induced arthritis control group. CFA-complete Freund's adjuvant; PT, post treatment from day 4.

Table 6: Effect of HeCA on arthritic index in rats

Treatment	Day	Day 4	Day 8	Day 12	Day 16	Day 20	Day 24	Day 28
Vehicle+CFA	4.00±0.	6.67±0.4	7.30±1.27	7.50±2.47	7.00±2.42	6.67±1.84	6.45±1.61	6.00±1.44
Celecoxib 3+CFA	4.00±0.	5.86±0.5	5.71±0.52[a]	4.86±0.40[b]	4.71±0.47[c]	4.00±0.38[a]	3.00±0.38[c]	3.00±0.38
HeCA 100+CFA	4.00±0.	7.00±0.4	7.00±1.13	5.50±0.62[b]	4.50±0.50[c]	3.67±0.56	3.17±0.31[c]	3.17±0.31
HeCA 200 + CFA	4.00±0.	6.50±0.3	6.33±0.33	4.83±0.40[b]	3.67±0.49[c]	3.33±0.33	2.83±0.16	2.83±0.17
HeCA 400 + CFA	4.00±0.	6.50±0.3	6.33±0.33	4.83±0.40[b]	3.67±0.49[c]	3.33±0.33[c]	2.83±0.16	2.83±0.17
HeCA 400 PT +	-	-	4.67±0.49[b]	4.17±0.65[c]	3.50±0.43[c]	3.50±0.43[c]	3.50±0.43[c]	3.50±0.43

Values are expressed as mean ±S.E.M. (n=6); ap<0.05; bp<0.01, cp<0.001 versus vehicle treated CFA-induced arthritic group. CFA-complete Freund's adjuvant; PT, post treatment from day 4.

Table 7: Effect of HeCA on haematological parameters.

Group	WBC	RBC	Plat	Neut	Hb	PCV	MCV	MCH	MCHC
1	9.40±1.90	7.42±0.20	681.00±36.86	3.23±0.18	13.60±0.32	41.30±1.01	56.20±1.42	18.37±0.15	33.23±0.09
2	6.67±1.51	7.33±0.32	739.30±53.99	3.62±0.25	13.63±0.24	42.87±1.18	55.60±2.94	19.27±0.12	33.70±0.26
3	12.53±4.52	7.49±0.12	614.30±28.05	4.02±0.26	14.47±0.15	45.07±0.69	57.47±0.35	19.30±0.23	31.33±0.54
4	9.47±3.03	7.74±0.15	718.70±14.31	4.45±0.39	14.53±0.23	45.53±0.90	76.60±4.22	18.97±0.24	31.97±0.58
5	7.77±3.43	7.73±0.14	784.00±41.48	4.04±0.46	14.1±0.23	45.67±0.32	57.53±1.09	19.17±0.12	31.17±0.24
6	13.63±3.36	7.68±0.11	716.00±57.30	4.51±0.23	14.2±0.26	45.87±1.41	58.73±2.28	19.10±0.20	31.47±0.33
7	14.5±4.03[a]	7.55±0.24	666.00±30.92	4.70±0.19	14.57±0.18	46.20±0.32	58.00±0.87	19.07±0.41	30.97±0.41

Values are expressed as mean ±S.E.M. (n=6) aP<0.05 versus vehicle 10 ml\kg + CFA or vehicle only treated. WBC (103/µL), white blood cells; RBC ((103/µL), red blood cells; Plat (104/µL), platelet count; Neut (103/µL) neutrophil count, Hb (g/dl), Haemoglobin; PCV, Packed Cell Volume; MCV (fl), Mean Corpuscular Volume; MCH (pg), Mean Corpuscular Haemoglobin; MCHC (g/dl), Mean Corpuscular Haemoglobin Concentration. 1= Vehicle 10 ml/kg, 2= Vehicle 10 ml/kg + CFA, 3= Celecoxib 3 + CFA,4= HeCA 100 + CFA, 5=HeCA 200 + CFA, 6=HeCA 400 + CFA, 7=HeCA 400 PT+ CFA

Table 8: Effect of HeCA treatment on CFA-induced oxidative and nitrosative stress in rats

Treatment (mg/kg)	GSH(U/mg)	SOD(U/mg)	CAT(U/mg)	MDA(U/mg)	Nitrite
Vehicle 10 ml/kg	1.08±0.10	3.73±0.01	31.52±1.37	0.09±0.01	8.13±1.50
Vehicle 10 ml/kg + CFA	0.63±0.21**	3.87±0.18	31.57±1.64	0.20±0.01***	12.10±0.12*
Celecoxib 3 + CFA	0.95±0.07	3.96±0.12[a]	36.96±0.25[a]	0.10±0.04[b]	10.10±0.70[a]
HeCA 100 + CFA	0.79±0.12	3.49±0.08	31.19±0.58	0.11±0.01[b]	9.75±1.47[b]
HeCA 200 + CFA	1.57±0.36[a]	4.80±0.84[b]	39.89±6.92[a]	0.24±0.10	10.90±0.40[a]
HeCA 400 + CFA	1.59±0.05	2.87±0.19	25.29±2.19	0.13±0.03[a]	8.90±0.12[b]
HeCA 400 PT + CFA	0.99±0.07	2.87±0.19	25.29±2.19	0.13±0.03[a]	8.60±0.12[b]

Values are expressed as mean ±S.E.M. (n=6), *p<0.05; **p<0.01; ***p<0.001 versus vehicle 10 ml/kg only treated group; ap<0.05, bp<0.01 versus vehicle 10 ml/kg + CFA treated group. Statistical level of significance analyzed using one way ANOVA followed by Tukey *post hoc* multiple comparison test; CFA-complete Freund's adjuvant; PT, post treatment from day 4. GSH, reduced glutathione; SOD, superoxide dismutase; CAT, catalase; MDA, malondialdehyde.

significantly reversed by HeCA (200 and 400 mg/kg) and celecoxib treatments [$F(2,120) = 32.31, P<0.001$], compared with the control group (vehicle 10 mg/kg + CFA) by the 3 week.

Effect of HeCA on Arthritic Index

Intraplantar injection of CFA produced a time course and significant increase in arthritic index that peaked on day 12 (7.50±2.47), as shown in table 6. However, the increase in arthritic index induced by CFA was significantly reduced by pretreatment of rats with HeCA (100-400 mg/kg) and post-treatment with HeCA (400 mg/kg). Similar observations were recorded in celecoxib pretreated rats (Table 6).

Effect of HeCA on haematological parameters in arthritic rats.

In this study, one way ANOVA revealed no significant change in haematological parameters except, a significant increase in level of WBC in HeCA 400 mg/kg (pre and post treatment) following 28 days subchronic administration of the extract when compared to control (vehicle 10 ml/kg + CFA) as shown in table 7. But no significant change was recorded in celecoxib treatment group.

Intraplantar injection of CFA significantly increased the level of lipid peroxidation and nitrite generation with concomitant decrease in the level of glutathione, catalase and superoxide dismutase. However, subchronic oral administration of HeCA (100 - 400 mg/kg) significantly attenuated the increase in the level of lipid peroxidation and nitrite generation with concomitant increase in the level of glutathione, catalase and superoxide dismutase following deficit induced by CFA injection (Table 8). Similarly, celecoxib pretreatment also reversed the increase in MDA and nitrite levels with increase in the activities of catalase and superoxide dismutase.

DISCUSSION

The results of this study demonstrate antinociceptive and anti-arthritic properties of the hydroethanolic leaf extract of Clausena anisata. Clausena anisata inhibited both early and late phases of formalin induced nociception test. This test which represents a model of persistent pain can also be used to determine the ability of new compounds to affect peripheral or central nociceptive pathways due to its biphasic nociceptive characteristics, known as the early and late phase, resulting from formalin administration (Malmberg et al, 1992). The early phase classified as the neurogenic pain, is an acute response observed immediately after the administration of formalin which lasts for 0-5 mins. The late phase, classified as an inflammatory pain is a tonic response resulting from inflammatory processes generated by inflammatory mediators such as histamine, serotonin, PGE and bradykinin (Verma et al., 2005). Centrally acting drugs (e.g. opioids) inhibit both phases, while peripherally acting drugs (e.g. NSAIDS) inhibit only the late phase. The results also suggest that Clausena anisata possesses central antinociceptive effect.

Moreover, the ability of the extract to attenuate the late phase of formalin test suggests not only anti-nociceptive effect but also anti-inflammatory activity (Zakaria et al., 2007).

To elucidate possible mechanism of antinociceptive effect of the extract, various pathways implicated in pain signaling mechanisms were evaluated. Most of the drugs used in managing chronic pain targets opioidergic pathway and results obtained from this study suggest central antinociceptive effect of the extract (Zakaria et al., 2007). Hence, possible participation of opioid receptors in the antinociceptive effect of the extract was investigated through subcutaneous injection of naloxone (non-selective opioid receptors antagonist). Findings from the study showed that the pretreatment of mice with naloxone reversed the antinociceptive effect elicited by the extract in formalin-induced nociceptive test, suggesting possible interaction of the extract with opioid receptors (Knights et al., 2004). Serotonin is known to play complex modulatory roles in pain signaling mechanisms, serotonergic descending pathway from rostral ventromedial medulla to the dorsal horn is crucial to spinal nociceptive processing (Millan, 2002; Suzuki et al., 2004). Conversely, pretreatment of mice with reserpine (vesicular monoamine depleter) failed to reverse the antinociceptive effect of the extract. However, 4 days pretreatment of mice with parachlorophenylalanine (serotonin synthesis inhibitor) prevented the antinociceptive effect of the extract. Moreover, pretreatment of mice with ketanserin (5-HT2A/C receptor antagonist; Alchaider, 1991; Knights et al., 2004) prevented the antinociceptive effect elicited by the extract, suggesting participation of serotonergic systems in the antinociceptive action of C. anisata.

Several lines of evidence have indicated the role of nitric oxide (NO) in the modulation of pain and analgesia (Cury et al., 2011). The reports of Moore et al. (1991) showed the involvement of brain NO system in the mechanisms underlying pain perception based on the fact that L-NG-nitro arginine methyl ester (L-NAME), a selective inhibitor of nitric oxide synthase, produces a potent, long lasting and centrally mediated antinociception in mice. Finding from this study showed that the pretreatment of mice with L-arginine prevented the antinociceptive effect elicited by C. anisata in formalin-induced paw licking model which suggest possible involvement of the L-arginine/nitric oxide/cyclic guanosine monophosphate pathway in the antinociceptive effect of the extract. Moreso, Morgan et al. (1992) have reported antinociceptive effect of L-NAME in formalin-induced paw licking and acetic

acid-induced abdominal constriction test. In addition, NO produced from L-arginine activates soluble guanylate cyclase which produces an increase in cyclic GMP (cGMP) levels (Meller and Gebhart, 1993).

In another series of experiment, the involvement of noradrenergic systems was also investigated, to have more robust understanding of the mechanisms of antinociceptive effect of the extract. Noradrenaline, through its action on ai- and a2-adrenoceptors, is involved in intrinsic control of pain. Peripheral noradrenaline that is mainly released by the sympathetic nervous system has little influence on healthy tissues, whereas in injured or inflamed tissues it has varying effects, including aggravation of pain in neuropathy. The peripheral pronociceptive effect has been associated with injury-induced expression of novel noradrenergic receptors, sprouting of sympathetic nerve fibers, and pronociceptive changes in the ion channel properties on primary afferent nociceptors, whereas an interaction with the immune system may contribute to peripheral antinociceptive effect of noradrenaline. At supraspinal levels, the effect of noradrenergic system on pain varied depending on many factors such as the type of the adrenoceptor, pathophysiological condition, and the brain area (Pertovaara, 2013). In the current study, the antinociceptive effect of the extract was blocked by prazosin (ai-adrenoceptor antagonist) and yohimbine (a2-adrenoceptor antagonist) pretreatment. The ability of the extract to increase noradrenergic activity or receptors may provide an effective way for suppressing pain, particularly in pathophysiological conditions. This synergistic pain inhibitory interaction of the extract with many neurotransmitter systems provide a possibility to maximize pain inhibition while minimizing side effects (Pertovaara, 2013). In another series of experiment, the involvement of ATP-sensitive K+ channels was investigated. In contrary, the K+ATp channels blocker, glibenclamide (Edwards and Weston, 1993) failed to inhibit the antinociceptive effect of the extract. Thus ruling out the involvement of K+ channels in its antinociceptive effect.

In carrageenan induced paw oedema model, the extract of Clausena anisata exerted pronounced effect at early stage of inflammation indicating possible inhibition of histamine, serotonin and kinnins that are involved in the early stage of carrageenan-induced oedema (Vane and Booting, 1987). The extract also reduced later stage of the oedema, an activity that may be due to its ability to inhibit prostaglandin which is known to mediate the second phase of carrageenan induced inflammation. Celecoxib a selective cyclooxygenase-2 inhibitor (COX-2- inducible upon inflammation) whose mechanism of action involves inhibition of prostaglandin inhibited significantly the paw swelling due to carrageenan injection indicating acute anti-inflammatory effect of the extract.

In chronic models of inflammation induced by CFA, rats developed arthritis-like symptoms on day 4 after intraplantar injection of CFA into the right hind paw. There was rapid development of a localized inflammatory response characterized by increased vascular leakage and subsequent swelling of the affected paw. However, oral administration of Clausena anisata extract reduced the inflammation associated with the arthritis as well as arthritic index. The anti-arthritic effect of Clausena anisata was similar to that of celecoxib, the findings corroborated the report of the in vitro assay by Amoo et al. (2013) that dried material of C. anisata effectively inhibited cyclooxygenase activity better than fresh material.

Intraplantar injection of CFA not only induced inflammation but also induced lipid peroxidation and nitrite generation with concomitant deficit in glutathione, catalase and superoxide dismutase activities. The extract showed potent antioxidant effect by attenuating the levels of malondialdehyde and nitrite as well as increase in glutathione, catalase and superoxide dismutase activities indicating antioxidant property of the extract. Interestingly, the deficit in catalase and superoxide dismutase as well as increase MDA and nitrite generations were reversed by subchronic treatment with celecoxib. Also, the decrease in body weight induced by CFA was reversed by the 3 week. Since a change in body weight is a useful index to assess the disease course and response to therapy of the drugs under investigation.

The results of phytochemical screening revealed the presence of alkaloids, flavonoids, tannins, saponins, reducing sugars, and cardiac glycosides which could be attributed to the observed antinociceptive and anti-inflammatory activities. Flavonoids particularly have been shown to possess anti-inflammatory activity through inhibition of the COX pathway (Liang et al., 1999). Studies of the constituents of plants of the genus Clausena have resulted in the isolation of some carbazole alkaloids (Ito et al., 2009). It has been reported that carbazole alkaloids possess various biological activities such as antitumor, antioxidative, antimutagenic, and anti¬inflammatory activities (Nakahara et al., 2002).

Conclusion

The results obtained from this study showed that Clausena anisata leaves extract possesses antinociceptive activity through interaction with opioidergic, noradrenergic, serotonergic and L-arginine-nitric oxide pathways, and anti-arthritic properties which could be attributed to its ability to prevent the release of inflammatory mediators as well as attenuation of oxidative and nitrosative stress. The results of this study support the ethnobotanical use of the plant in the treatment of painful and inflammatory conditions.

Conflict of interest

The authors declare that there are no conflicts of interest in respect of this study.

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