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Analgesic, Anti-Inflammatory and Anti-Oxidant Activities of *Clausena anisata* leaf and Stem Extracts and Quantitative and Qualitative Phytochemical Analysis of their Contents

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ABSTRACT

Clausena anisata is a medicinal plant use to treat diseases including inflammatory, pain and oxidative stress conditions. Nonetheless, these activities have not been evaluated for its stem. This study aimed to evaluate and compared analgesic, anti-inflammatory and anti-oxidant activities of ethanol extracts of leaf (CLE) and stem (CSE) of C. anisata. Anti-inflammatory and anti-oxidant activities were assessed by carrageenan induced paw edema and DPPH free radical scavenging assays respectively. Hot plate and writhing assays were used to test the analgesic activity. Total phenolics and flavonoids were quantified using UV-Vis spectrophotometry methods. CLE and CSE produced ($p<0.05$) dose-depended anti-inflammatory activity (76.35-85.83%) comparable to that of indomethacin (78.26%) and ($p< 0.05$) analgesic activity in the writhing assay. The extracts also produce ($p<0.0001$) overall analgesic activity in the hot plate assay comparable to tramadol, although, their activities were short lived. EC_{50} values obtained in the anti-oxidant activity showed that ascorbic acid (standard) was 86 and 74 times potent than CLE and CSE respectively. Total flavonoids and phenolics obtained per 100 mg of CLE or CSE were 2.46 ± 0.08 and 3.93 ± 0.122 mg QE (Quercetin Equivalent) respectively and 6.69 ± 0.54 and 7.45 ± 0.64 mg GAE (Gallic Acid Equivalent) respectively. Flavonoids and phenolic contents of CSE were higher than CLE. This may partly explain where CSE was more active than CLE in all the assays. These results show that CLE and CSE possessed significant anti-inflammatory and analgesic activities and weak anti-oxidant activity.

Keywords: Pain, Inflammation, Alkaloid, Coumarin, Triterpene.

INTRODUCTION

Pain is a protective mechanism that helps a living organism to get rid of obnoxious stimuli in other to avoid harm. However, alterations in the pain pathway produce hypersensitivity, so that pain metamorphosize from short-term warning signal to chronic devastating condition [1]. Likewise, inflammation is a key element of the immune system that is activated by any stimulus that poses actual or apparent hazard to tissue homeostasis [2]. Acute inflammatory reaction is self-limiting and leads to tissue repair and return of tissue homeostasis. Conversely, chronic inflammation is implicated in diseases such as diabetes, fatty liver, obesity, rheumatoid arthritis and atherosclerosis [3-4].

Moreover, free radicals are valuable substances in living systems that aid in gene expression, receptor activation, and signal transduction [5]. Nonetheless, excess free radicals produce oxidative stress which is implicated in cardiovascular disease, cancers, diabetes and inflammation [6,7]. Anti-oxidants molecules derived from plants are known to obstruct the generation of free radicals and relieve diseases caused by oxidative stress [6,8]. Moreover, some investigators have shown that plant-based products with high quantity of flavonoids and phenolics also have high antioxidant and anti-inflammatory activities [6,9,10].

Clausena anisata (Wild.) Hook f. ex. Benth (Rutaceae) is a small perennial tree that grows up to 10 m high. Various parts of the plant are use as remedy for several diseases such as epilepsy, malaria, gut disturbances, convulsion, toothache, oral candidiasis, fungal infections, and high blood pressure in traditional medicine [11-13]. Anti-bacterial, anti-hypertensive, anti-pyretic, anti-inflammatory, larvicidal, anti-oxidant, wound healing and *in vitro* anti-diabetic activities were evaluated for leaf extracts of the plant [14-19]. Furthermore, extracts of the root of *C. anisata* were reported to demonstrate analgesic, anti-inflammatory, anti-pyretic and hypoglycemic activities [20-22]. Finally, antimicrobial and cytotoxic activities of the fruit, leaf, stem bark, twigs and root of *C. anisata* were also evaluated [23]. However, anti-inflammatory, analgesic and anti-oxidant activities have not been performed on *C. anisata* stem. Moreover, total flavonoid and phenolic content of the leaf and stem of the plant have also not been determined. We therefore sought to evaluate and compare these activities of *C. anisata* stem and leaf extracts in other to provide scientific justification for some of their uses in traditional medicine.

MATERIALS AND METHODS

Collection and extraction of the plant materials

C. anisata was collected in March 2012, from the arboretum of Centre for Plant Medicine Research (Centre) at Ayikumah in the Greater Accra Region of Ghana and authenticated by Mr. Ofori Lartey, a botanist at the Centre. Samples (CPM 0112 and CPM 0212) of the leaf and stem respectively were deposited in the Centre's herbarium. The leaf and the stem were separated from the plant respectively and chopped into pieces. The plant materials were separately air dried for 34 days and pulverized with hammer mill. The leaf (0.5 kg) was macerated with ethanol (16 l × 2) for 4 days and filtered and combined. The extract was dried at 45^{o}C with rotary evaporator to obtain a dark green semi solid labeled CLE (49.58 g; yield-9.92 $\%^{w}/_{w}$). The stem (0.5 kg) was also taken through a similar process to obtain brown solid labeled CSE (3.817 g; yield-0.76 $\%^{w}/_{w}$).

Animals

The three species of animals (Swiss albino rats, ICR mice and C3HP mice) used in this study were obtained from the Animal House Unit of the Pharmacology Department of the Centre. The animals were housed in aluminum cages under standard environmental conditions of 25 ± 2°C, 40-60% humidity with 12 h light/12 h dark cycle with free access to sterile water and feed. Mice used in the acetic acid-induced assays were fasted overnight just before the experiment. The animals were handled and cared for according to the guidelines of the Committee for the Update of the Guide for the Care and use of Laboratory Animals, 2011[24].

Drugs and chemicals

Carrageenan and Acetic acid were procured from Sigma Chemical Co. (St. Louis, USA). Indomethacin and diclofenac sodium salt powders were purchased from Cayman Chemical Company (Ann Arbor, USA) and Sigma-Aldrich (St. Louis, USA) respectively. Tramadol hydrochloride was purchase from Bristol Laboratories Limited. DPPH was purchased from Tokyo Chemical Industries Limited (Tokyo, Japan). $AlCl_3$ and $NaNO_3$ were supplied by Park Scientific Limited (Northampton, U.K.). Gallic acid and Folin - Ciocalteu reagent were also supplied by Nice Chemicals Limited, (Kerala, India) and Research – Lab Fine Chem Industries, (Mumbai India) respectively. NaOH was also obtained from Daejung Chemicals and Metals Co. Ltd (Gyeonggi-do Korea). Finally, NaCl was purchased from Timstar Laboratory Supply Ltd (Cheshire, U.K.). Propylene glycol was used as an emulsifier and analytical grade ethanol was used for extraction.

Phytochemical screening of the extracts

About 100 ml each of the extract were added to 50 ml of distilled water in ceramic evaporating dishes and evaporated on water bath to remove the ethanol. The aqueous portions of the extracts obtained were screened for the presence or absence of thirteen (13) classes of phytochemical constituents respectively [25].

Determination of total phenolic content

Phenolic content of CLE and CSE were determined as previously reported [26] with slight modifications. Briefly, absorbance of triplicate concentrations of 100-0.195 µg/ml of Gallic acid (standard compound) at 765 nm λ_{max} was measured and used to obtain a standard calibration curve. The extracts, 800 µl of 5-0.3125 mg/ml of CLE or CSE was added to 4 ml of Folin-Ciocalteu reagent (diluted 1:10 with de-ionized water). The mixture was neutralized with 3.2 ml of sodium carbonate solution (7.5% w/v) after 8 min and incubated in the dark at room temperature for 2 h. Absorbance was measured at the stated wavelength using double beam UV/VIS spectrophotometer (Biobase BK – D590). The total phenolic contents of CLE or CSE was determined from the linear equation of a standard curve. The content of total phenolic compounds was expressed as 100 mg/mg gallic acid equivalent (GAE) ± SEM.

Determination of total flavonoid content

Flavonoid content in CLE or CSE was measured using the aluminum chloride UV spectrophotometry assay as described previously [27] with slight modifications. Quercetin solution (1 ml) of concentrations ranging from 3.9 $\times 10^{-3}$-1 mg/ml was added to 4 ml of distilled water in 10 different test tubes. $NaNO_3$ (5% w/v; 0.3 ml) was added and incubated for 5 min. $AlCl_3$ (10% w/v; 0.3 ml) was added to the mixture, followed by addition of 2 ml of 1 M NaOH at the 6th min. The volume was made up to 10 ml with distilled water and mixed thoroughly. CLE or CSE (0.3125-5 mg/ml) was dissolved in methanol and treated separately just like the standard. The absorbance was determined at 510 nm using UV/VIS spectrophotometer (Biobase BK – D590) and in triplicates. Calibration curves were plotted and their linearity determined in order to estimate the flavonoid contents. The flavonoid content was expressed as mg of quercetin equivalence (QE) per 100 mg ± SEM of CLE or CSE respectively.

DPPH free radical scavenging assay – anti-oxidant activity

The DPPH free radical scavenging assay was used to evaluate the antioxidant activity of CSE and CLE as described by Oyaizu [28] with slight modifications. Briefly, 2-fold serial dilutions of CSE or CLE was prepared to obtain 7 different concentrations (2.5-0.039 mg/ml). The standard, ascorbic acid was also prepared in 3-fold dilutions (1-1.5 × 10^{-4} mg/ml). DPPH solution (20 µg/ml) 3 ml was added to 1 ml of each sample and incubated in the dark for 30 min at room temperature. The absorbance was read at 517 nm against a blank using UV/VIS spectrophotometer (Biobase BK – D590) in triplicates. The negative was prepared by adding 3 ml of DPPH solution to 1 ml of methanol and treated under the same conditions as the samples. Percentage inhibition was calculated as:

$$\text{Antioxidant activity} = (A\text{-}B)/A \times 100\%$$

Where A is optical density of blank and B is the optical density of sample.

The effective concentration (EC_{50}) of each sample was estimated from a regression curve.

Anti-inflammatory activity assay

Anti-inflammatory activity of the extracts was evaluated using carrageenan induced paw oedema model in rats [29]. Male Sprague-Dawley rats (254-294 g) were divided into 8 Groups (n=5). Group 1-3 were given CLE 50-200 mg/kg p.o. Group 4-6 were also given CSE 50-200 mg/kg p.o. Group 7-8 were given 10 mg/kg p.o. of indomethacin as standard drug or 1 ml each of 1% propylene glycol aqueous solution as vehicle control respectively. Inflammation was induced by the sub-plantar injection of 0.1 ml carrageenan in normal saline (1% w/v) in the right hind paw of each animal. Baseline paw volume (V_o) of the right hind limbs were measured prior to induction of inflammation and at an hourly interval (V_t)

until the 5th h by volume displacement using plethysmometer after extract or drug was given. (V_t-V_o) was taken as oedema (Olivera de Lima et al.,). Total oedema induced during the 5-hour period was measured as net Area Under the time course Curves (AUC). Anti-inflammatory activity was calculated using the formula:

Antiinflammatory activity = ((AuC Control-AUC Treated)/ AUC Control) × 100%

Analgesic activity assays

Acetic acid induced writhing assay

Analgesic activity of the extracts was also evaluated in the acetic acid induced-writhing model as previously described [30]. In short, 40 male Swiss albino mice (30-36 g) were divided into 8 Groups (n=5). Group 1-3 were given reconstituted CLE 50-200 mg/kg p.o. in 1% propylene glycol aqueous solution. Moreover, Group 4-6 were also given CSE 50-200 mg/kg p.o. Group 7 and 8 received diclofenac sodium, 10 mg/kg p.o. as standard drug and 0.2 ml of 1% propylene glycol aqueous solution as vehicle control respectively. Each mouse was then injected with 1 ml/100 g (i.p.) of 1 % v/v aqueous acetic acid 45 min after treatment and quarantined in transparent Perspex cages. Writhing movements and stomach contortions made by each mouse was counted for 20 min after induction. Analgesic activity (AE) was calculated using the formula:

$$AE = ((MRc-MRt)/MRc) \times 100\%$$

Where: MRc=average writhing count of the vehicle treated control group; MRt=average writhing count of treated group.

Hot plate assay

The mouse hot plate test used to evaluate the analgesic activity was performed as previously described [31]. Briefly, 40 male C3HP mice (23-33 g) were divided into 8 Groups (n=4). Each mouse was placed on an electric hot plate (UGO Basile hot/cold plate 35 100) maintained at 55 ± 0.5°C. Latency time (To), recorded as analgesic response was measured as the time taken for a mouse to lick, lift, shake or stamp any of the hind limbs or jump. Baseline latencies (T_o) were calculated as means of two pre-treatment determination of each mouse. Mean baseline latency of mice used for this test ranged from 5.4-10.6 s. Plant extracts were reconstituted with 1% propylene glycol aqueous solution and administered as follows: Group 1-3 were given CLE 5-200 mg/kg p.o. Group 4-6 also received CSE 5-200 mg/kg p.o., whereas Group 7 was given tramadol 5 mg/kg p.o. (as standard drug) and Group 8 served as vehicle control respectively. Latency of each mouse was then measured at 0.25, 0.5 and 1 h. Thereafter, the latency was measured at hourly (T_t) intervals for 4 h after each treatment per mouse. Analgesic activity was calculated as the sum of percentage maximal probable effect (% MPE) over the experimental period. % MPE was calculated as [32]:

$$\%MPE=((To-T_t)/To) \times 100$$

Statistical analysis

Graphpad Prism 5 was used for all statistical analysis. Data obtained for the time course curves were analyzed using Two Way ANOVA followed by Bonferroni's multiple comparison test to determine statistically significant. Whereas, One-way ordinary ANOVA was employed to analyse data in the writhing assay followed by Dunnett's multiple comparison test for statistically significant. Results were considered to be statistically significant when $p < 0.05$ by comparing the test group with the control group.

RESULTS AND DISCUSSIONS

Phytochemical screening

The results from the phytochemical screening of the extracts are shown below in Table 1.

Table 1: Phytochemical constituents present or absent in ethanol extract of *C. anisata* leaf or stem

Class of constituent	Leaf (CLE)	Stem (CSE)
Free reducing sugars	+	+
Alkaloids	+	+
Coumarins	+	+
Triterpenes	-	+
Saponins	-	-
Flavonoids	-	-
Anthraquinones	-	-
Phytosterols	-	-
Phenolic compounds	-	-
Polyuronoids	-	-
Tannins	-	-

+ = detected; - not detected

Results of the phytochemical screening, shows the presence of alkaloid, coumarins, and free reducing sugars CLE whereas CSE also contain the same constituents as CLE in addition to triterpenes (Table 1). Some coumarins, alkaloids and triterpenes were reported to possess anti-inflammatory, analgesic and anti-oxidant effects [20,33-35]. Hence, the presence of these phytochemical constituents in CLE and CSE contributed to the anti-inflammatory, analgesic and anti-oxidant activities of CLE and CSE.

Total phenolic content

Figure 1A-C below, shows the results obtained from the determination of the total phenolic content of the extracts. From Figure 1, the total phenolic content of 100 mg of CLE or CSE was estimated to be 6.69 ± 0.54 and 7.45 ± 0.64 mg GAE respectively. The results show that the phenolic content of CSE is slightly higher than that of CLE.

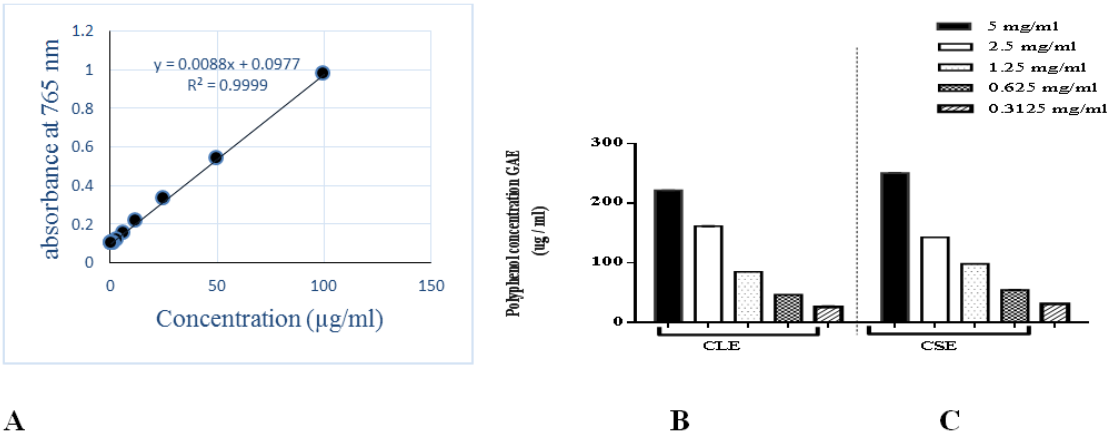

Figure 1: Calibration curve for total phenolic content determination of CLE and CSE using gallic acid (A) and total phenolic contents obtained from the calibration curve for CLE (B) and CSE (C) respectively

Total flavonoid content

Figure 2D-F shows the results of the flavonoid contents of CLE and CSE. The total flavonoid content per 100 mg of CLE or CSE was estimated to be 2.46 ± 0.08 and 3.93 ± 0.122 mg QE respectively. The results also show that CSE has higher flavonoid content than CLE.

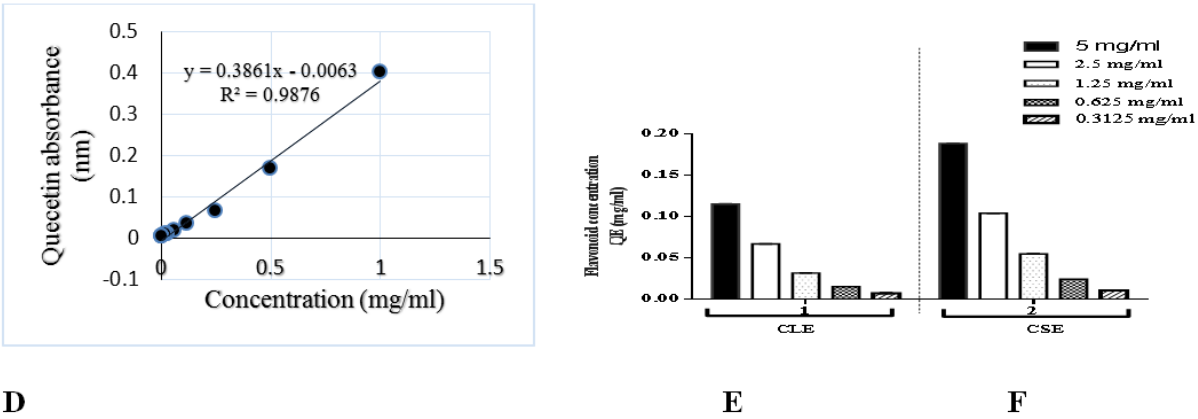

Figure 2: Calibration curve for total flavonoids content determination of CLE and CSE using quercetin (D) and flavonoids contents of CLE (E) and CSE (F) respectively

DPPH free-radical scavenging activity

Figure 3 shows the results of the free radical scavenging action of ascorbic acid, CLE and CSE. The R^2 and EC_{50} values calculated are tabulated below in Table 2.

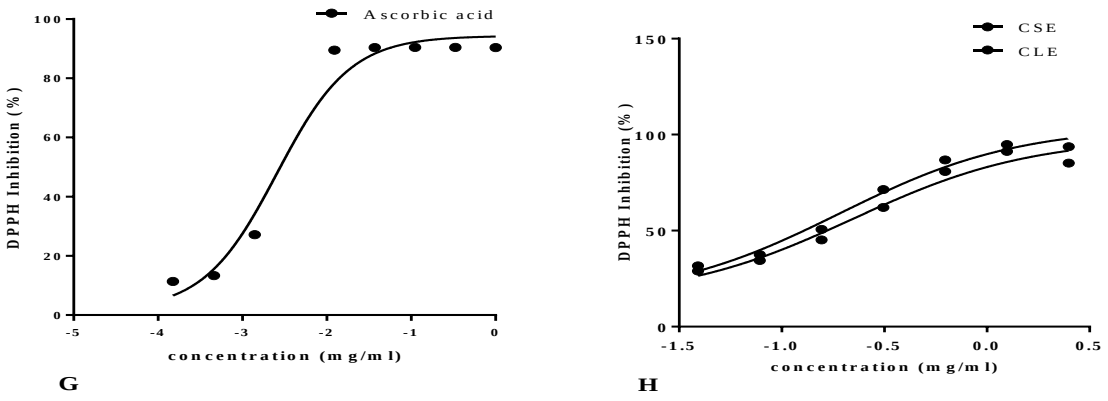

Figure 3: Regression curves of DPPH scavenging actions of ascorbic acid (G), CLE and CSE (H)

Table 2: R^2 values and EC_{50} of ascorbic acid, CSE and CLE

Sample	EC_{50} (mg/ml)	R^2
Ascorbic acid	0.0026 ± 0.0872	0.9768
CSE	0.1922 ± 0.0653	0.9842
CLE	0.2229 ± 0.0894	0.9701

The R^2 values obtained for the calibration curves in the regression analysis of the DPPH free-radical scavenging activity, total phenolics and total flavonoids contents determination of the extracts ranges from 0.9701 - 0.9999. These values were much closer to 1. This indicates that the ranges of the calibrations were very linear.

Anti-inflammatory activity

The results of the anti-inflammatory activity study on CLE and CSE are shown below in Figure 4. CLE and CSE significantly inhibited the formation of oedema in rats' paw comparable to the standard drug, indomethacin on the time course curves (Figures 4I and 4K). In addition, the overall anti-inflammatory response calculated as the net AUC were very significant for all doses of the extracts used in this study (Figures 4J and 4L).

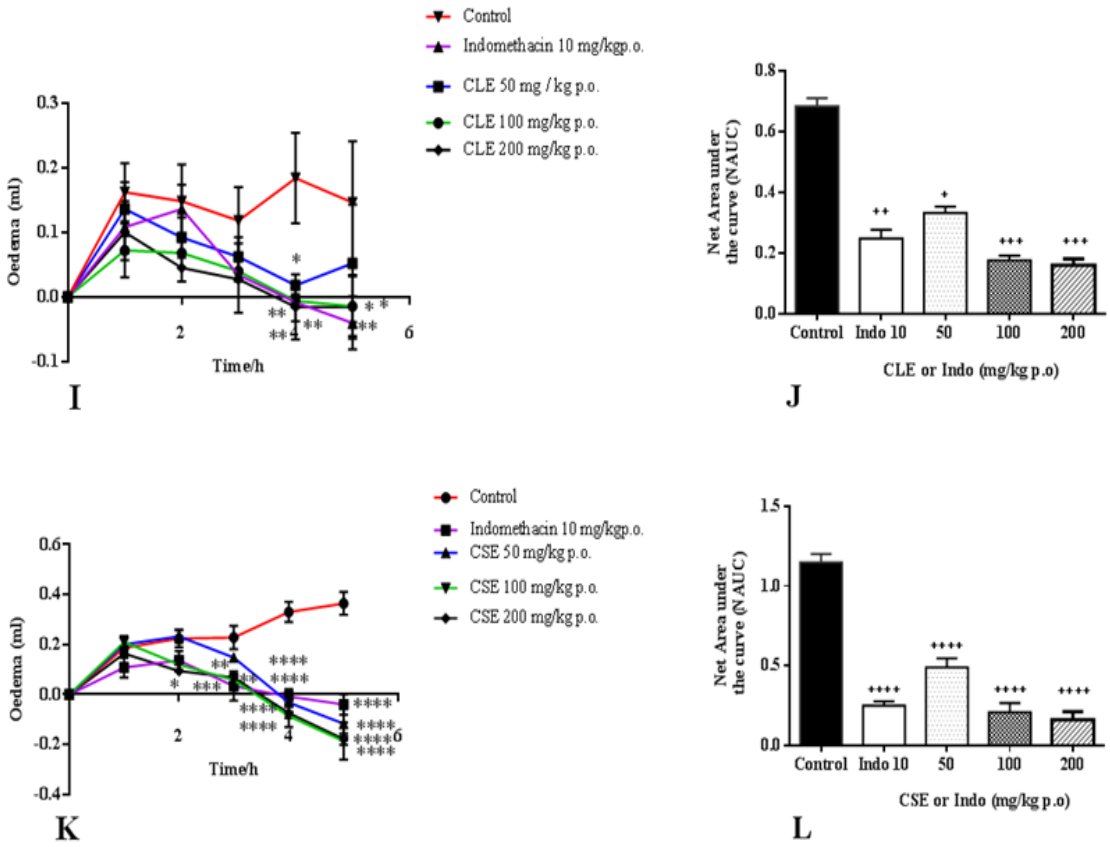

Figure 4: Inhibitory effect of CLE or CSE (50-200 mg/kg p.o.) and indomethacin (10 mg/kg p.o.) on carrageenan-induced paw oedema in rat's paw on Time Course Curve (TCC) (I and K). *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001 compared to vehicle treated control group (Two-way ANOVA followed by Bonferroni's post hoc test). J and L represents total inflammatory response calculated as the area under TCC (mean ± SEM (n = 5). $^{+}$p < 0.05, $^{++}$p < 0.01, $^{+++}$p < 0.001 and $^{++++}$p < 0.0001 compared to vehicle treated control group (One-way ANOVA followed by Dunnett's post hoc test)

The highest anti-inflammatory activity at 200 mg/kg p.o. of the extracts were 76.35 and 85.83% respectively for CLE and CSE. And that of indomethacin was 78.26%. These results indicate that the extracts possess very significant anti-inflammatory activity.

It has been demonstrated that injection of carrageenan into the paw of rats leads to increased production of prostaglandins (PGs) and inducible isoform of cyclooxygenase enzyme 2 (COX -2) [36]. Dudhgaonkar and co-workers also demonstrated that carrageenan – induced paw inflammation is characterized by released of prostaglandins and free radicals when the phlogistic agent is administered from 1^{st}-6^{th} h [37]. The antioxidant activity of the extracts was therefore evaluated in other to ascertain if the extracts' ability to mop up these free radicals also contributes to their anti-inflammatory activity. However, the EC_{50} values obtained showed that the extracts possessed weak anti-oxidant activity since CSE and CLE are 74 and 86 times less active than the standard drug ascorbic acid respectively. Meanwhile, CSE and CLE showed very significant ($p<0.01$) inhibition of the oedema formation from 3^{rd} -6^{th} h on the time course curve (Figure 4). Therefore, the inflammatory activity of the extracts may be due to their ability to potentiate the synthesis or action of PGs and COX-2 rather than their antioxidant activity.

Analgesic activity

Writhing assay

The results obtained for the effect of CLE and CSE on the writhing counts are shown in Figure 5 below. The results show that both extracts inhibited writhing movements induced by acetic acid in mice in dose depended manner. CLE and CSE produced statistically significant ($p <$

0.05) analgesic activity similar to the analgesic activity of diclofenac sodium in this study. The highest analgesic activity of CLE, CSE (200 mg/kg p.o.) and diclofenac sodium (10 mg/kg p.o.) were calculated to be 26.77, 35.69 and 36.25% respectively. CSE possessed higher analgesic activity than CLE in the writhing assay.

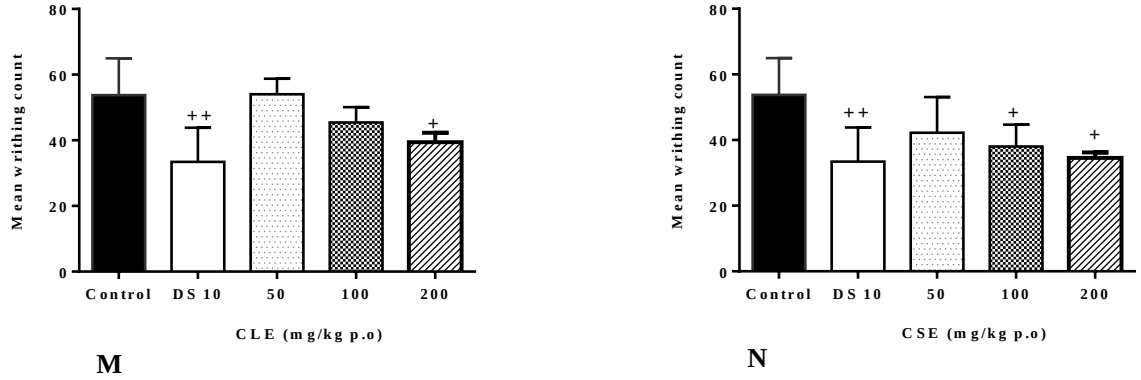

Figure 5: Inhibitory effect of CLE or CSE (50-200 mg/kg p.o.) and diclofenac sodium (10 mg/kg p.o.) on acetic acid-induced writhing counts in mice. Each column denotes mean ± SEM (n = 5). $^{+}p < 0.05$, $^{++}p < 0.01$ compared to vehicle treated control group (One-way ANOVA followed by Dunnett's post hoc test)

The writhing responses generated in the acetic acid-induced writhing assay are mediated by the release of arachidonic acid (AA) by means of cyclooxygenase enzyme and prostaglandin biosynthesis [38]. The results show that CLE and CSE produced significant ($p<0.05$) analgesic activity in the writhing assay. Hence, the analgesic activity of the extracts may be due to their anti-inflammatory constituents which are able to inhibit AA.

Hot plate assay

The results of the analgesic activity of CLE and CSE are shown below on the TCC and overall analgesic activity in Figure 6. CLE and CSE showed similar patterns of analgesic activity in the hot plate assay. Both CLE and CSE produced effective analgesia in the hot plate assay by spontaneously increasing the latency of mice to respond to thermally-induced hyperalgesia in inverse dose-depended manner. The analgesic activity of CLE and CSE were short lived on the TCC as compared to that of tramadol (Figures 6: O and Q).

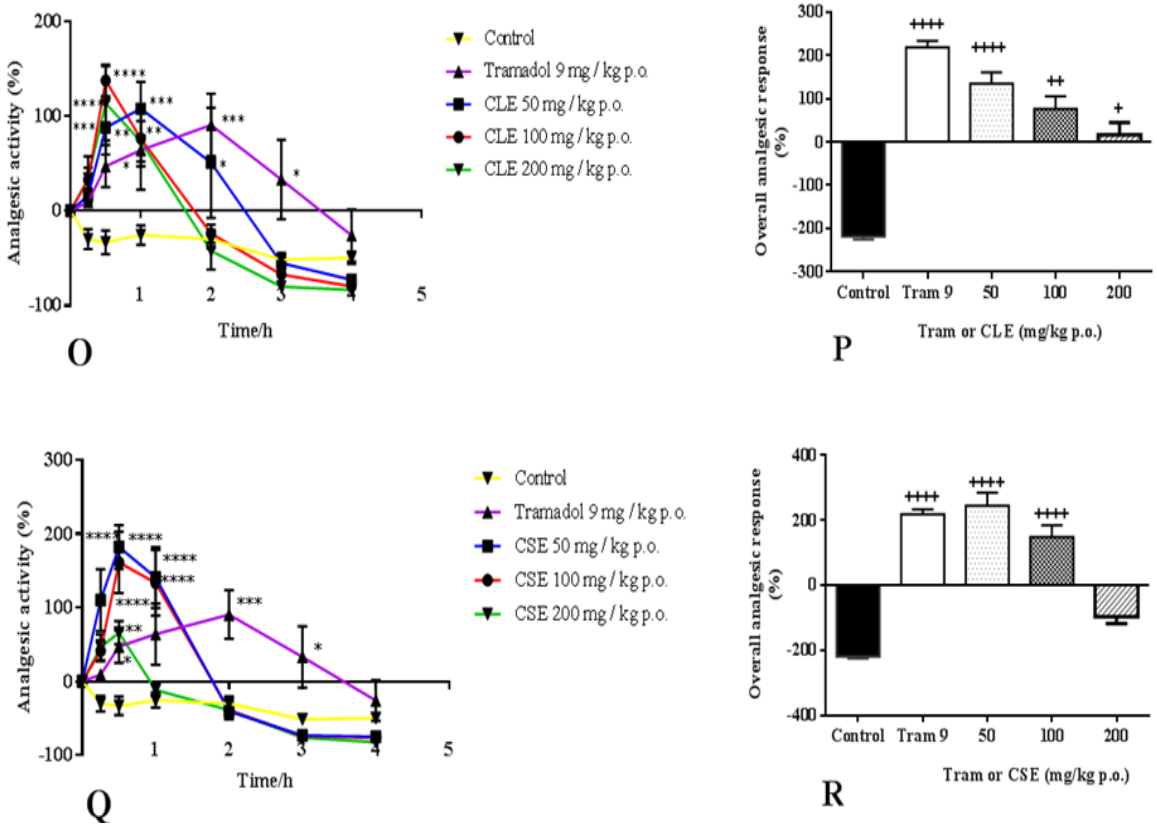

Figure 6: Analgesic activity of CLE, CSE and tramadol in the hot plate assay on TCC (O and Q) and Overall analgesic activity calculated as summation of analgesic activities on TCC for each dose (P and R). Each point on TCC and bar chart represents mean ± S.E.M or summation ± S. E. M. (n = 5). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$ compared with control. (Two – way ANOVA followed by effect within row Bofferonis' post hoc test). $^{+}p < 0.05$; $^{++}p < 0.01$; $^{++++}p < 0.0001$ compared with control (One-way ANOVA followed by Dunnett's post hoc test)

The analgesic effect of CLE (at 100 mg/kg p.o.) become statistically significant ($p<0.0001$) at 30 min after it was administered with peak analgesic effect of 137.6% at the same time and then become impotent at about 1 h and 50 min on the TCC. Similarly, the analgesic effect of CSE (at 50 mg/kg p.o.) appeared ($p<0.0001$) just 15 min after it was administered and peaked at 30 min with maximum analgesic activity of 182.2% and then become impotent at 2 h 50 min on the TCC (Figure 6Q). Conversely, the standard drug, tramadol produced gradual analgesic effect which appeared ($p < 0.05$) at 30 min and attained its peak effect with analgesic activity of 90.63% at 3 h period. The peak analgesic activity of tramadol is 40 and 100% lower than that of CLE and CSE respectively. Moreover, the extracts attained their peak analgesic effect quicker than tramadol. Therefore, the extracts may provide quicker pain relief than tramadol.

Although, the extract lost their activities within 1: 50-2: 30 h after their administration, the analgesic effect they produced within that period was so high that it abolished the loss of activity in the last 2 h or so. Hence the overall analgesic effect was very significant ($p < 0.0001$) and positive. The least dose of 50 mg/kg p.o. was the most potent of both extracts with overall analgesic activity of 134.90 and 244.50% for CLE and CSE respectively. The overall analgesic activity of tramadol was 218.50%. The results also showed that CSE possessed higher analgesic activity than CLE in the hot plate assay.

Narcotic or opioid analgesics, like tramadol, produce analgesic effect by inhibiting the transfer of pain impulses to the central nervous system [39]. The hot plate assay is a thermally-induced pain model, to evaluate the therapeutic efficacy of analgesic agents that act through the central nervous system [32]. CLE and CSE produced significant analgesic action in the hot plate assay comparable to tramadol. This suggests that the extracts may possess central analgesic mechanism of action.

CONCLUSION

CLE and CSE possessed significant anti-inflammatory and analgesic activities and weak anti-oxidant activity. However, CSE demonstrated higher analgesic, anti-inflammatory and anti-oxidant activities than CLE. Flavonoids and phenolic content of CSE were higher than CLE. This may partly explain where CSE was more active than CLE in all the assays. These results lend credence to the ethnopharmacological utilization of leaf and stem of *C. anisata*.

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