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ANTI-INFLAMMATORY, ANALGESIC AND GC – MS ANALYSIS OF ESSENTIAL OIL OF *ALPINIA CALCARATA* RHIZOME

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ABSTRACT

In the present study, essential oil isolated from *Alpinia calcarata* was analyzed and also assessed for acute toxicity, anti-inflammatory and analgesic activities in animals. The essential oil isolated from *Alpinia calcarata* was analyzed by using GC-MS on a combined GC-MS instrument. For evaluation of the anti-inflammatory property "carrageenan induced paw edema model" served as acute model. "Acetic acid induced writhing response model" was used to assess analgesic activity in mice. The major components of essential oils isolated from *Alpinia calcarata* were Camphene (3.86%), Betamyrcene(4.39%), Eucalyptol(14.05%), Linalol(2.48%), Pyrazine(1.72%), L-camphor(7.90%) and Berneol(5.67%). Intraperitoneal injection of essential oil isolated from *Alpinia calcarata* significantly ($P<0.05$) suppressed the paw edema induced by carrageenan in two different dose levels studies namely 400mg/kg and 380mg/kg. Intraperitoneal injection of essential oil also significantly attenuated the acetic acid induced writhing response in three different dose levels studies namely 200 mg/kg (significant at $P<0.05$), 400mg/kg (significant at $P<0.01$) and 600mg/kg (significant at $P<0.01$). These studies suggest that essential oil isolated from *Alpinia calcarata* might possess significant anti-inflammatory activity and analgesic effect and could be a potential source for treatment of different inflammatory diseases.

KEY WORDS: ***Alpinia calcarata,*** **essential oil, Camphene, anti-inflammatory, carrageenan.**

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INTRODUCTION

The use of natural products as medicine is growing in the world especially in developing countries . Bangladesh owing to its favorable climatic influences has been blessed with immense natural resources including explored and unexplored herbal medicinal plants. *Alpinia calcarata* Rosc. (family: Zingiberaceae) is an important medicinal plant among the seven species of Alpinia that occur in Bangladesh, India, Myanmar, Indonesia, Thailand, New Guinea and the Bismark Archipelago[1, 2]. Rhizomes and leaves of *A. calcarata* are used traditionally for treatment of rheumatism, bronchial catarrh and asthma. According to Arambewela *et al.* 2004 [3], *Alpinia calcarata* is a perennial herb with non-tuberous pungent rootstock. It's rhizomes showed antinociceptive activities. The rhizome extract of *Alpinia calcarata* is used as an expectorant in the treatment of bronchitis and asthma; for purifying blood; stimulating digestion and improving voice[4]. Drugs prepared from the rhizomes of Alpinia *calcarata* are used in treatment of rheumatism, bronchial catarrh and asthma. The analysis of *A. calcarata* has revealed the presence of protocatechinic acid, quercetin, 4-O-methyl-syringic acid, vanillic acid, methyl cinnamate and several terpenes and diterpenes as constituents [5]. 1,8-Cineole had been found to be the major constituent in the oil [5-9] . Chowdhury *et al* 2003 reported the rhizome oil constituents from Bangladesh containing α-fenchyl acetate (51.4%) and 1,8-cineole (15.1%) as major constituents [9]. 1,8-cineole is used as an antiseptic (0-25 %) in dentifrices and, mixed with zinc oxide, as a temporary dental filling.

The economically important part is rhizome, which is a major constituent of many formulations of indigenous system of medicine for relieving throat inflammation, stimulating digestion, purifying blood, improving voice and marinating youthful vigor. Now day's Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used in the treatment of acute and chronic inflammation, pain and fever. But the greatest disadvantage in presently available synthetic drugs is that they cause gastrointestinal irritation and reappearance of symptoms after discontinuation. Therefore, there is a dire need for screening and development of novel, but better anti-inflammatory drugs and indigenous medicinal plants could be a logical source to find these. Hence, the present study has been planned to evaluate the phytochemical analysis of essential oil from rhizomes of *Alpinia calcarata* and effects of anti-inflammatory and antinociceptive Activities of essential oil.

MATERIAL AND METHODS

Collection of sample

Fresh rhizome of *Alpinia calcarata* was collected from the plantation area of BCSIR Laboratory, Chittagong during June 2011. One-voucher specimen (Y-112) was deposited in the herbarium of BCSIR Laboratory, Chittagong, Bangladesh.

Extraction of essential oil

Sample of rhizome was harvested from healthy, well-grown, two-year-old plants. Freshly harvested samples (500g each) were subjected to hydro distillation using a modified Clevenger-type glass apparatus for 4 h for isolation of oils [10]. The oil sample was stored at 0°C in air-tight containers after drying them over anhydrous sodium sulfate for GC-MS analysis.

GC-MS analysis

The essential oil from rhizome of *Alpinia calcarata* was analyzed at BCSIR laboratories, Ctittagong, Bangladesh by GC-MS electron impact ionization (EI) method on GC-17A gas chromatograph (Shimadzu) coupled to a GC-MS QP 5050A Mass Spectrometer

(Shimadzu); fused silica capillary column (column 30 m x 0.25 mm, 1 µm film thickness), coated with DB-5 ms (J&W); column temperature 100°C (2 min) to 250°C at the rate of 3°C/min; carrier gas, helium at constant pressure of 90Kpa. Acquisition parameters full scan; scan range 40-350 amu. Sample was injected by splitting method and the split ratio was 1:20. Quantitative data of the peaks were obtained and using standards peak enhancement experiments were carried out along with comparison of MS spectra with those of authentic compounds for identification.

Identification of the compounds

Compound identification was done by comparing the NIST library data of the peaks with those reported in literature, mass spectra of the peaks with literature data. Percentage composition was computed from GC peak areas on DB-5 ms column.

Animals and Diets

Swiss albino mice of both sexes weighing between 30g -35g and Wistar Albino rats of the either sex weighing between 150g-200g obtained from animal house of BCSIR laboratories, Chittagong were used for the present study. The animals were acclimatized to room temperature (28±5°C) with a relative humidity of 55±5 % in a standard wire meshed plastic cages for 4 to 5 days prior to commencement of the experiment. During the entire period of study the animals were supplied with standard pellet diet and water *ad libitum*. The animal had no access to food during the whole day of experiment. In this study, all the animal experimentation was carried out according to the guidelines of Institutional Animal Ethics Committee (IAEC).

Acute toxicity studies

Essential oil isolated from rhizomes of *Alpinia calcarata* was injected intraperitoneally in mice at various dose levels namely 250 mg/kg, 500 mg/kg, 1g/kg and 2g/kg of body weight. Two mice in each dose group were closely observed for 24 hours for any mortality and next ten days for any delayed toxic effect.

Anti-inflammatory activity

Carrageenan induced paw edema model:

According to the method designed by Winter et al. 1962 [11], the initial right hind paw volume of each rat was measured using plethysmometer (UGO Basile, Italy) and then 0.1ml of 1 % (w/v) carrageenan was subcutaneously injected into the sub-plantar region of the right hind paw in order to induce acute inflammation. The volume of right hind paw was measured at 1st, 2nd, 3rd and 4th hour after carrageenan injection and the paw edema was determined. Essential oil of *Alpinia calcarata* (100mg/kg, 380mg/kg and 400mg/kg), standard anti-inflammatory drug Dichlofenac sodium (4mg/kg) and Soya bean oil were administered orally one hour before the sub-plantar injection of carrageenan to treated, positive control and control group respectively. The inhibitory activity was calculated according to the following formula [12] : Percentage inhibition

$$\frac{(Ct - Co)\ \text{control} - (Ct - Co)\ \text{treated}}{(Ct - Co)\ \text{control}} \times 100$$

Where Ct is the right hind paw thickness volume (in mm3) at time t, Co is the right hind paw thickness volume (in mm3) before carrageenan injection. Ct−Co is paw edema. (Ct −Co) control, is edema or paw size after carrageenan injection to control rats at time t. (Ct −Co) treated, is edema or paw size after carrageenan injection to treated (reference or sample drug) rats at time t.

Analgesic Activity

Acetic acid induced writhing response test :

For writhing test 1 % (v/v) acetic acid solution (10 ml/kg body weight) was injected intraperitoneally in mice (weighing 25g-30g) and the number of writhing and stretching was counted over 20 minutes period [13,14]. The

essential oil isolated from rhizomes of *Alpinia calcarata* (200mg/kg, 400mg/kg and 600mg/kg), reference analgesic drug Dichlofenac sodium (20 mg/kg) and soybean oil were also injected intraperitoneally 30 min before acetic acid injection to treated, positive control and control group respectively.

Statistical Analysis

Data from the experiments were analyzed using the Statistical Package for social Science (SPSS) software for windows version 17 (SPSS inc., Chicago, Illionois, USA). All the experiment data were expressed as Mean ±SD as appropriate, statistical analysis of the results was performed by using the One way ANOVA (analysis of variance) followed by *Bonferroni post hoc* and Dunnett test. The limit of significance was set at >0.05.

RESULTS

Phytochemical analysis of essential oil GC-MS Chromatogram and GC-MS peak report obtained from GC-MS instrument are shown in Figure 1 and 2. The chemical constituents of the essential oil isolated from rhizomes of *Alpinia calcarata* is shown in Table 1.

Figure 1

GC-MS chromatogram of the essential oil isolated from rhizomes of Alpinia calcarata

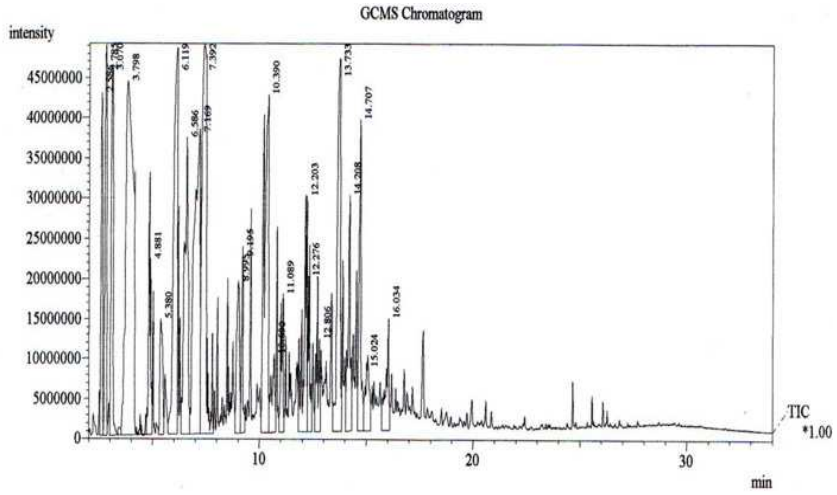

Figure 2
GC-MS peak report of the essential oil isolated from rhizomes of Alpinia calcarata

Peak#	R.Time	I.Time	F.Time	Area	Height	A/H	Mark	%Total
1	2.586	2.533	2.683	213279274	42692059	4.99	V	2.84
2	2.785	2.683	2.875	289898345	48578143	5.96	V	3.86
3	3.070	2.975	3.517	329848624	46006726	7.16	SV	4.39
4	3.798	3.517	4.700	1054468979	44090142	23.91	SV	14.05
5	4.881	4.700	4.975	186509258	21932799	8.50	V	2.48
6	5.380	5.283	5.533	128824084	14413863	8.93	V	1.72
7	6.119	5.725	6.158	593055380	48065331	12.33	V	7.90
8	6.586	6.325	6.725	425886161	35660010	11.94	V	5.67
9	7.169	6.725	7.225	718328907	37668913	19.06	V	9.57
10	7.392	7.225	7.842	740811187	48470964	15.28	SV	9.87
11	8.995	8.858	9.108	180341122	18927410	9.52	V	2.40
12	9.195	9.108	9.308	106529448	21836380	4.87	V	1.42
13	10.390	10.075	10.442	596639221	41953290	14.22	V	7.95
14	10.690	10.442	10.758	105732407	9615933	10.99	V	1.41
15	11.089	10.908	11.142	118711372	17230678	6.88	V	1.58
16	12.203	11.825	12.242	276162408	28680927	9.62	V	3.68
17	12.276	12.242	12.442	121254076	18428368	6.57	V	1.62
18	12.806	12.575	12.842	130944589	11231135	11.65	V	1.74
19	13.733	13.425	13.842	487479313	46310533	10.52	V	6.49
20	14.208	14.042	14.325	209141592	28660740	7.29	V	2.79
21	14.707	14.592	14.875	251514061	37984866	6.62	V	3.35
22	15.024	14.875	15.208	116264268	8120939	14.31	V	1.55
23	16.034	15.725	16.092	124929267	13288706	9.40	V	1.66
				7506553343	689848855			100.00

Table 1
The chemical constituents of rhizomes of Alpinia calcarata

SL. NO	Name of Chemical constituents	%
1.	Bicyclo[3.1.1]hept-2-ene, 2,6,6-trimethyl-,	2.84
2.	Camphene	3.86
3.	.beta.-Myrcene	4.39
4.	Eucalyptol	14.05
5.	Linalol	2.48
6.	Pyrazine	1.72
7.	L-camphor	7.90
8.	Borneol	5.67
9.	Terpinyl acetate	9.57
10.	Fenchyl acetate	9.47
11.	1,2-Cyclohexanediol, 1-methyl-4-(1-methylethenyl)-	2.40
12.	Myrtenol	1.42
13.	Methyl cinnamate	7.95
14.	trans-p-Mentha-2,8-dienol	1.41
15.	4H-1,3-Benzodioxin-4-one, hexahydro-4a,5-dimethyl-, [4as-(4a.alpha.,5.beta.,8a.beta.)]-	1.58
16.	Caryophyllene oxide	3.68
17.	Naphthalene, 1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl)-, [1R-(1.alpha.,7.beta.,8a.alpha.)]-	1.62
18.	Oxacyclotetradecan-2-one, 14-methyl-	1.74
19.	Carotol	6.49
20.	Daucol	2.79
21.	6.beta.Bicyclo[4.3.0]nonane, 5.beta.-iodomethyl-1.beta.-isopropenyl-4.alpha.,5.alpha.-dimethyl-,	3.35
22	Caryophyllene	1.55
23.	2,5-Disec-butylphenol	1.66

Acute toxicity test

In the acute toxicity assay of essential oil isolated from *Alpinia calcarata*, no death was observed at higher (2g /kg) dose level. But at higher (1 g/kg and 2 g/kg) doses some abnormalities (balance less walking, drowsiness etc.) are observed within 10 to 20 minutes of injection. The lower doses such as 250 mg/kg or 500 mg/kg did not show any abnormalities.

Anti-inflammatory activity

The essential oil isolated from rhizomes of *Alpinia calcarata* was evaluated for anti-inflammatory activity in animal models and the results are depicted in Table 2.

Table 2

Effect of essential oil isolated from Alpinia calcarata plant on carrageenan induced paw edema at three different dose levels

Group	Dose (i.p)	Paw edema (mm^3) (Ct -Co)				% Inhibition $\frac{(Ct-Co)\ control - (Ct-Co)\ treated}{(Ct-Co)\ control} \times 100$			
		1st hr	2nd hr	3rd hr	4th hr	1st hr	2nd hr	3rd hr	4th hr
Control	Soya bean oil	0.37 ± 0.08	0.91± 0.06	0.94± 0.1	0.96± 0.13	-	-	-	-
Diclophenac Na	4 mg/kg	0.24± 0.05*	0.22± 0.07*	0.35± 0.02*	0.33± 0.07*	36.1%	75.8%	62.8%	66.3%
Essential oil mixed with Soybean oil	100 mg/kg	0.33± 0.06	0.66± 0.2	0.67± 0.14	0.63± 0.17	9.5%	27.7%	29.3%	34.7%
Essential oil mixed with Soybean oil	400 mg/kg	0.26 ± 0.03	0.33± 0.11*	0.53± 0.06*	0.74± 0.05	29.3%	63.7%	43.6%	23.8%
Essential oil (Given directly)	380 mg/kg	0.215 ± 0.07*	0.36 ± 0.09*	0.34 ± 0.08*	0.46 ± 0.1*	41.5%	60.4%	64.1%	52.3%

***All values are expressed as mean ± SEM (n=5) *P<0.05 significant compared to control (Student's t test) Here; Co is the paw thickness volume (in mm^3) before carrageenan injection. Ct is the paw thickness volume (in mm^3) at time and (Ct -Co) is p*aw edema**

Analgesic Activity

Table 3 shows the pain behavior of writhing response, which was presented as cumulative abdominal stretching response. Table 3 also indicates the % Analgesic activity of essential oil isolated from *Alpinia calcarata* on acetic acid induced wreathing response. The treatment of mice with the essential oil (400 mg/kg and 600 mg/ kg) produced a significant ($P<0.01$) inhibition and even a lower dose of essential oil (200 mg/kg) also produced a significant ($P<0.05$) inhibition in abdominal writhes produced by acetic acid.

Table 3
Effect of Essential oil of Alpinia calcarata on acetic acid induced wreathing response at three different dose levels

Treatment	Dose	No. of Wreathing (Counts/20 min)	% Analgesic activity
Control	Soybean oil	52.5 ± 2.53	-
Diclofenac Sodium	20 mg/ kg	14.0 ± 1.68 ***	73.3 %
Essential oil mixed with Soybean oil	200 mg/kg	41.8 ± 3. 06 *	41.8 ± 3. 06 *
Essential oil mixed with Soybean oil	400 mg/ kg	29.0 ± 3.80**	44.8%
Essential oil mixed with Soybean oil	600 mg/ kg	31.3 ± 2.62**	40.5%

All values are expressed as mean ± SEM (n=5)
****P<0.05 and ** P< 0.01 significant compared to control *** P< 0.001 significant compared to control (Student's t test)***

DISCUSSION

The results of the present study revealed that the essential oil isolated from rhizome of *Alpinia calcarata* might possess significant anti-inflammatory and analgesic activity. The acute toxicity studies showed that no death is observed at high dose level (2g/kg) in mice. Although some abnormalities (such as balance less walking, drowsiness etc) are observed at higher dose level (1g/kg or 2g/kg), there is no abnormality in effective dose level (400 mg/ kg or 600 mg/kg). So the essential oil isolated from *Alpinia calcarata* may be a source of anti-inflammatory and analgesic drug without any lethal side effect. In the essential oil isolated from rhizomes of *Alpinia calcarata,* twenty three compounds were identified. The major components were Camphene (3.86%), Betamyrcene (4.39%), Eucalyptol (14.05%), Linalol (2.48%), Pyrazine (1.72%), L-camphor (7.90%), Berneol (5.67%), Terpinyl acetate (9.57%), Fenchyl acetate (9.47%), Myrtenol (1.42%), Methyl cinnamate (7.95%), Caryophyllene oxide (3.68%), Carotol (6.49%), and Daucol (2.79%) (Table 1). Carrageenan injection into the rat paw provokes a local, acute inflammatory reaction that is a suitable criterion for evaluation of anti-inflammatory agents [15]. The inflammation consists of two phases, early phase, which is related to the production of histamine,5- hydroxytryptamin, bradykinins and cyclooxygenase products and delayed phase, which has been linked to neutrophil infiltration, as well as production of arachidonic acid metabolites [16,17,18]. Carrageenan induced paw edema model is a suitable test for evaluating anti-inflammatory properties for natural drugs because of its sensitivity in detecting orally active anti-inflammatory agents particularly in the acute phase of inflammation [19]. Development of edema in the paw of rat after injection of carrageenen is a biphasic event [20]. The initial phase observed during the first hour is attributed to the release of histamine and serotonin. The second phase of edema is due to the release of prostaglandins, protease, and lysosome [21]. The results of the present study demonstrate that the essential oil (380mg/kg) isolated from *Alpinia calcarata* exhibited significant anti-inflammatory activity at 1, 2,3 and 4 hr after carrageenan injection. (Table 2).Therefore it is possible that the essential oil isolated from *Alpinia calcarata* contains the

active constituents that exhibits its anti-inflammatory action probably by means of either inhibiting the synthesis, release or action of inflammatory mediators e.g. histamine, serotonin and prostaglandins. But actually in which mechanism the essential oil isolated from *Alpinia calcarata* act as anti- inflammatory agent is not clear, which may be the subject of further study. In our study, we injected the essential oil of *Alpinia calcarata* into rat in two ways that is direct and indirect injection. Direct injection means the injection of only essential oil of the plant. Indirect injection means the injection of essential oil of *Alpinia calcarata* mixed with soybean oil. The result of the current study revealed that the dose (400 mg/kg) of essential oil of *Alpinia calcarata* mixed with soybean oil (Indirect injection) exhibited anti inflammatory activity by significant ($P<0.05$) suppression of the paw edema induced by carrageenan only at 2, 3 and 4 hr after carrageenan injection as shown in Table 2. But direct injection of essential oil (380 mg/kg) exhibited anti-inflammatory activity by significant ($P<0.05$) suppression of the paw edema induced by carrageenan at 1, 2, 3, and 4 hr after carrageenan injection. The result also showed that % inhibition of carrageenan induced paw edema by direct injection (only essential oil) is greater than that of indirect injection (mixed with soybean oil), although the dose level was almost the same. This observation indicates that the soybean oil might have a little influence on the result. We evaluated the anti-inflammatory activity of essential oil of *Alpinia calcarata* by "Carrageenan induced paw edema model". It is an established method for evaluating Anti-inflammatory activity.

The analgesic model used in the present study was chosen in order to test nociceptive stimuli, namely chemical visceral (writhing) stimuli. In acetic acid induced abdominal writhing model, which is the visceral pain model, the release of arachidonic acid plays a role in the nociceptive mechanism. Result of the present study shows that the essential oil of *Alpinia calcarata* (400 mg/kg) produces significant ($P<0.05$) antinociceptive effect (Table 3)and this effect may be due to inhibition of the synthesis of the arachidonic acid metabolite [22]. Finally, it can be concluded that the essential oil isolated from rhizome of *Alpinia calcarata* shows significant anti-inflammatory and analgesic effects on experimental animal models and might be an alternative source to treat inflammation, arthritis and pain. These observations strongly suggest that essential oil isolated from rhizome of *Alpinia calcarata* may possess significant analgesic effect.

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