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## Evaluation of Analgesic and Antioxidant Potency of Various Extracts of *Cinnamomum iners* Bark

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**Abstract:** The multiple traditional uses with fewer scientific investigations about *C. iners* bark made imperative to further exploit this plant for the evaluation of its therapeutic value. Analgesic and antioxidant activities of ethanolic, aqueous and alkaloid extracts prepared from *C. iners* bark was studied using both *in vivo* and spectrometric experimental models. Results of hot plate and tail flick studies show that all the screened extracts are devoid of central analgesic activity. However, promising findings regarding the peripheral analgesic and anti-inflammatory activity was revealed from the formalin induced pain method with alkaloid extract possessing significant activity followed by ethanolic and aqueous extract. Moreover, the results of total phenolic content and antioxidant activity was also confirmed the presence of higher amount polyphenolic content in ethanolic extract with significant antioxidant activity. The observed peripheral analgesic activity by ethanolic and aqueous extract might be due to the presence of higher amount polyphenolic present in them. Results of this study also supported the traditional use of this plant in the treatment of pain. Hence, it was concluded from the study that *C. iners* bark extract can be utilized as new source of peripheral analgesic in the treatment of pain.

**Key words:** *C. iners*, analgesic, anti-inflammatory, antioxidant, alkaloid extract, phenolic content

### INTRODUCTION

Pain is one of the most common reasons among individuals necessitating the medical attention (McCurdy and Scully, 2005). Though, several synthetic drugs are available the severe complications associated with NSAIDS as well drug addiction associated with opiate analgesics lead to find alternative promising analgesics from traditional medicinal plants. The plants with the traditional uses have been of great interest for researchers in the discovery of new therapeutically effective and safe drugs for the treatment of various diseases (Mohan *et al.*, 2008; Eidi *et al.*, 2011). Ethnobotanical survey shows that Malaysia has rich source of traditionally used medicinal plants which necessitates the proper scientific studies for the exploitation of these plants to develop a new drug (Abdul *et al.*, 2008; Krishnan *et al.*, 2010; Mustafa *et al.*, 2010b; Wahab *et al.*, 2009). Continuing our effort to identify the new source of antinociceptive, antioxidant and antimicrobial drug from medicinal plants, we selected the *C. iners* bark to evaluate its beneficial pharmacological activities to further exploit it as a new source of medicine (Annegowda *et al.*, 2010a; Mustafa *et al.*, 2010a).

*Cinnamomum iners* is one of the 250 species from the genus *Cinnamomum* belongs to the family Lauraceae. It is a small to average evergreen tree distributed commonly in India, Malaysia, China, Philippines, Thailand and Indonesia. Traditionally, various parts of this plant have been used as antidiarrheal, diuretic, carminative, laxative, anti-infective (Butkhup and Samappito, 2011), tonic for stomach and in the treatment of rheumatism (Nguyen *et al.*, 2004). Leaves of this plant have antimicrobial (Jantan *et al.*, 1994; Wiart *et al.*, 2004), antioxidant (Phutdhawong *et al.*, 2007), analgesic (Mustafa *et al.*, 2010a) and anticancer activities (Pang *et al.*, 2009). Hydrolysed extract of *C. iners* rind show good antioxidant as well as antimicrobial activities against few pathogenic microbes (Butkhup and Samappito, 2011). Several essential oils such as linalool, caryophyllene oxide, cardinol were identified from the leaves (Phutdhawong *et al.*, 2007) and 1, 8-cineole,  $\alpha$ -terpineol, terpinen-4-ol,  $\beta$ -pinene and caryophyllene oxide from the stem bark of *C. iners* (Baruah *et al.*, 2001). Moreover, with the synonym of wild cinnamon, the bark of this plant was used as a substitute for cinnamon in various parts of Malaysia and Thailand.

Though, the bark of *C. iners* has multiple traditional uses and contains some essential oils, very few reports regarding therapeutic efficacy of this plant is available. Hence, it is imperative to further exploit *C. iners* bark for the biological effects. Since, *C. iners* bark is traditionally used in the treatment of painful illness, it is worthwhile to evaluate antinociceptive activity of the various extracts of *C. iners* bark in mice. An attempt also been made to evaluate polyphenolic content and antioxidant activities of the bark extracts.

## MATERIALS AND METHODS

**Plant material:** Fresh bark of *Cinnamomum iners* was collected from the campus of Universiti Sains Malaysia (USM), Penang, Malaysia in November 2009. The plant was authenticated by a botanist and the voucher specimen (No. 11014) was deposited in Herbarium, School of Biological Sciences, USM. Collected bark was then washed with running water to remove the dirt and adherent then cut into small pieces; freeze dried and grinds to get powder of 40 mesh size.

**Preparation of ethanolic and aqueous extract:** Two hundred gram of ground bark sample was refluxed with 1000 mL of ethanol (99.5%) and distilled water at their respective boiling temperature for 48 h using Soxhlet apparatus. The liquid extract obtained was separated from solid residue by vacuum filtration through Whatman filter paper (No. 1), filtered ethanolic (CIEt) and aqueous extract (CIAq) was then concentrated to small volume using rotary evaporator (Buchi Rotavapor R-215, Switzerland) followed by drying in freeze dryer (LABCONCO, Free Zone 6 Liter, USA) to get constant mass and kept in air tight container at 4°C until further analysis.

**Preparation of alkaloid extract:** The ethanolic extract (20 g) obtained was acidified to pH 3 using aqueous acetic acid and allowed the solution to stir using magnetic stirrer over night. Next day, the aqueous solution was extracted using n-hexane and the pH of the aqueous solution was adjusted to 12 using dilute ammonia. The basified solution was once again magnetic stirred for over night and partitioned with ethyl acetate followed by filtration, concentration and freeze drying of ethyl acetate solution to obtain alkaloidal extract (CIAk).

**Chemicals:** Gallic acid, butylated hydroxytoluene (BHT), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, sodium carbonate, ammonium molybdate, Folin Ciocalteu reagent, aspirin and formaldehyde were purchased from Sigma Aldrich, Germany. Morphine was obtained from Hospital USM, Kelantan, Malaysia. All solvents used

were of analytical grade and purchased from Merck KGaA, Darmstadt, Germany.

**Animals:** Male Swiss Albino mice (25-30 g) were obtained from animal house, USM. These animals were acclimatized to the laboratory condition for one week before the experimental procedure. Animals had free access to water and food pellets and were fasted overnight prior to experimental procedures. All the experimental procedures followed in this study were approved by Animal Ethics Committee, USM.

### Analgesic activity

**Tail flick test:** The central analgesic activity of the samples and standards were evaluated by measuring the response latency in tail flick test described by D'Amour and Smith (1941). A focused beam of light produced by radiant heat analgesiometer (IITC Life Science Series 8, Model 33T, Victory Blvd, Woodland Hills, CA 91367, USA) was used to induce the thermal noxious pain. The time taken by the mice (six in a group) to flick the tail in response to the heat was recorded. In order to avoid any tissue damage an automatic cut-off time of 8 sec was maintained. Prior to the administration of sample all the animals were tested to obtain the baseline latency. The different doses of extracts prepared in 20% Tween 20 were administered orally whereas, morphine was administered subcutaneously. The time taken for the flicking the tail was measured at 15, 30, 60, 90 and 120 min after the administration of samples.

**Hot plate test:** The hot plate method described by Woolfe and McDonald (1944) with slight modification (Santos *et al.*, 1998) was followed to measure the central analgesic activity of various extracts of *C. iners* bark and morphine. Mice (six in a group) were placed on the hot surface of a hot plate instrument (Hot plate analgesia meter, IITC Life Science Series, USA) maintained at 55±0.2°C. Time taken by the animals for shaking, licking or jumping due to the pain was recorded as the indicator for the response latency. Prior to the experiment, all the animals were made acquainted with the instrument and animals with response latency more than 30 sec were not used for the experiment. An automatic cut-off time of 45 sec was set in order to prevent any tissue damage of the mice paw. The bark extract samples, co-solvent (20% Tween 20) were administered orally whereas, morphine was administered subcutaneously. The response latency was measured at 15, 30, 60, 90 and 120 min after the administration of each sample.

**Formalin induced pain:** The potency of the samples to reduce the neurogenic and anti-inflammatory pain induced

by the injection of formalin was studied by the modified method described by Santos and Calixto (1997). To the right hind paw of mice, 20  $\mu$ L of 2.5% of formalin (formaldehyde 40%) in saline was injected and the time spent in licking and biting the injected paw was recorded from 0 to 5 min (first phase) and 15 to 30 min (second phase). The various bark samples (200 mg  $\text{kg}^{-1}$ ) in 20% Tween 20, aspirin (100 mg  $\text{kg}^{-1}$ ) were administered orally and morphine (5 mg  $\text{kg}^{-1}$ ) subcutaneously 30 min prior to the injection of formalin. The percentage of pain inhibition by mice was obtained as follows:

$$\text{Percentage inhibition} = \frac{A_0 - A_1}{A_0} \times 100$$

where,  $A_0$  is the reading of co-solvent (control) and  $A_1$  is the reading of samples and standards.

**Evaluation of total phenolic content (TPC) and antioxidant activity:** The total phenolic content of the various extracts of *C. iners* bark was determined by following the Folin Ciocalteu method described by Singleton and Rossi (1965). The TPC values were expressed as mg gallic acid equivalent (mg GAE)/g dry extract. The DPPH radical scavenging capacity of the prepared extracts and standards was determined by Blois (1958) method with slight modifications (Annegowda *et al.*, 2011) and the values were expressed as mg vitamin C equivalent antioxidant capacity (mg VCEAC)/g sample (Annegowda *et al.*, 2010b). The reducing ability of the samples and standards was studied using the phosphomolybdenum UV spectrometric method described by Prieto *et al.* (1999) and the antioxidant values were expressed as mg VCEAC/g sample.

**Statistical analysis:** The results of analgesic and antioxidant activity were expressed as Mean $\pm$ SEM and Mean $\pm$ SD, respectively. Statistical analysis of variance was performed with one way ANOVA, followed by Tukey's HSD (Honestly Significant Difference) using SPSS 17 (SPSS Inc, Wacker Drive, Chicago, USA). The

$p < 0.05$  was considered to be statistically significant when compared to control for analgesic activity.

## RESULTS AND DISCUSSION

### Analgesic activity

**Tail flick and hot plate tests:** Both these methods are the classical methods used to evaluate the central analgesic effects of the sample. Tail flick method is normally used to discriminate the opiates from non opiate analgesics (Vogel, 2002). In this study various doses of CIET, CIAq and CIAk (100, 200, 500 mg  $\text{kg}^{-1}$ , p.o.) were studied for the analgesic activity in tail flick method. The dose for this study was based on result of toxicity study published recently from our research group where in they reported the absence of any toxic symptoms even at the oral dose of 5000 mg  $\text{kg}^{-1}$  (Mustaffa *et al.*, 2010a). It is evident from the Table 1, none of the dose of the tested sample shown significant analgesic activity in comparison with control at all the time intervals tested confirming the absence of central analgesic activity. However, as expected morphine exhibited significant central analgesic activity ( $p < 0.05$ ) at the intervals of 15, 30, 60 min. Though the values of some of the samples (Table 1) looks higher than the control but it was almost similar to that of their initial reading took 30 min prior to the administration of samples.

Hot plate test was normally used to evaluate the centrally acting analgesics (Vogel, 2002). CIET and CIAq at the tested dose (200 mg  $\text{kg}^{-1}$ , p.o.) did not show any promising analgesic activity (Table 2). Moreover, the various doses of CIAk (100, 200, 500 and 1000 mg  $\text{kg}^{-1}$ , p.o.) too did not exhibited analgesic activity. However, as expected morphine the opiate central analgesic displayed significant activity ( $p < 0.05$ ) at the tested time intervals of 15, 30 and 60 min. Hence, the results of hot plate test support the results of tail flick and affirm the absence of central analgesic activity. Aqueous juice of the bark was prepared and orally administered to mimic the traditional way of use. Unfortunately, even the bark juice also did not show any potential central analgesic activity.

Table 1: Effect of various extracts of *C. iners* bark and morphine on pain in tail flick test

Samples	Dose (mg $\text{kg}^{-1}$ )	Initial reading	Latency period (min)				
			15	30	60	90	120
Control	0	2.23 $\pm$ 0.13	2.32 $\pm$ 0.16	2.55 $\pm$ 0.22	2.65 $\pm$ 0.17	2.90 $\pm$ 0.09	2.77 $\pm$ 0.18
CIAk	100	2.57 $\pm$ 0.01	2.70 $\pm$ 0.09	2.64 $\pm$ 0.09	2.82 $\pm$ 0.13	2.91 $\pm$ 0.17	2.91 $\pm$ 0.06
CIET	100	1.76 $\pm$ 0.01	2.09 $\pm$ 0.21	1.98 $\pm$ 0.13	2.35 $\pm$ 0.20	2.58 $\pm$ 0.11	2.29 $\pm$ 0.16
CIAq	100	1.65 $\pm$ 0.25	2.06 $\pm$ 0.14	2.37 $\pm$ 0.16	2.55 $\pm$ 0.18	2.36 $\pm$ 0.16	2.22 $\pm$ 0.14
Morphine	5	2.04 $\pm$ 0.18	4.80 $\pm$ 0.62*	5.31 $\pm$ 0.83*	6.51 $\pm$ 0.48*	2.96 $\pm$ 0.58	2.17 $\pm$ 0.17
CIAk	200	2.51 $\pm$ 0.21	2.66 $\pm$ 0.15	2.85 $\pm$ 0.20	3.12 $\pm$ 0.16	2.85 $\pm$ 0.09	2.75 $\pm$ 0.12
CIET	200	2.38 $\pm$ 0.22	2.27 $\pm$ 0.12	2.71 $\pm$ 0.26	2.90 $\pm$ 0.16	2.58 $\pm$ 0.01	2.59 $\pm$ 0.15
CIAq	200	2.54 $\pm$ 0.33	2.34 $\pm$ 0.17	2.74 $\pm$ 0.25	3.51 $\pm$ 0.52	2.89 $\pm$ 0.11	3.35 $\pm$ 0.24
CIAk	500	2.90 $\pm$ 0.24	3.21 $\pm$ 0.28	3.62 $\pm$ 0.25	3.65 $\pm$ 0.25	3.24 $\pm$ 0.12	3.09 $\pm$ 0.12
CIET	500	2.34 $\pm$ 0.31	2.57 $\pm$ 0.13	2.69 $\pm$ 0.01	2.83 $\pm$ 0.14	2.91 $\pm$ 0.09	2.79 $\pm$ 0.10
CIAq	500	2.23 $\pm$ 0.23	2.50 $\pm$ 0.12	2.45 $\pm$ 0.01	3.00 $\pm$ 0.14	2.88 $\pm$ 0.10	3.28 $\pm$ 0.10

Each values represents mean $\pm$ SEM in seconds for six mice treated with *C. iners* extracts and morphine, \* $p < 0.05$ , significant from control

Table 2: Effect of various extracts of *C. iners* bark and morphine in pain induced by hotplate

Samples	Dose	Initial reading	Latency period (min)				
			15	30	60	90	120
Control	0 mg kg <sup>-1</sup>	12.49±1.14	13.28±2.69	15.01±2.05	15.26±2.49	13.35±3.19	11.72±0.92
Morphine	5 mg kg <sup>-1</sup>	11.89±5.32	27.40±8.43*	36.38±6.17*	38.43±7.28*	18.57±3.52	16.47±4.04
CIaK	100 mg kg <sup>-1</sup>	11.87±2.18	13.25±1.28	11.19±2.32	11.53±1.66	10.73±2.92	13.79±1.58
CIaK	200 mg kg <sup>-1</sup>	6.28±0.94	7.24±1.65	8.58±0.62	10.64±1.66	6.73±1.04	9.33±1.03
CIeT	200 mg kg <sup>-1</sup>	10.00±1.35	12.41±0.90	12.65±2.22	13.95±2.55	15.78±2.46	13.45±1.50
CIaQ	200 mg kg <sup>-1</sup>	11.56±1.24	9.79±0.84	9.94±1.66	11.39±2.11	10.57±1.71	11.12±1.94
CIaK	500 mg kg <sup>-1</sup>	6.73±1.21	5.84±0.61	7.09±1.43	9.39±0.52	9.60±1.47	8.07±1.32
CIaK	1000 mg kg <sup>-1</sup>	6.48±1.02	7.67±0.79	6.32±1.10	6.43±0.63	7.06±0.71	6.73±1.66
Juice	10 mL kg <sup>-1</sup>	8.47±1.56	6.56±1.27	7.67±2.88	8.11±2.72	11.85±3.92	11.27±3.81

Each values represents mean±SEM in seconds for six mice treated with *C. iners* extracts and morphine, \*p<0.05, significant from control

Table 3: Effect of various extracts of *C. iners* bark, morphine and aspirin in formalin-induced pain

Treatment	Dose (mg kg <sup>-1</sup> )	Total time spent in licking (sec)			
		0-5 min	Inhibition (%)	15-30 min	Inhibition (%)
Control	0	65.00±3.71 <sup>a</sup>		147.00±4.29 <sup>a</sup>	
CIeT extract	200	53.17±4.88 <sup>b</sup>	18.21	54.83±2.39 <sup>a</sup>	62.70
CIaQ extract	200	55.16±3.99 <sup>b</sup>	15.13	72.17±8.77 <sup>b</sup>	50.91
CIaK extract	200	50.33±3.61 <sup>b</sup>	22.56	44.67±4.01 <sup>cd</sup>	69.61
Morphine	5	20.00±3.57 <sup>c</sup>	69.23	30.00±2.72 <sup>d</sup>	79.59
Aspirin	100	57.67±1.17 <sup>b</sup>	11.28	51.50±3.15 <sup>c</sup>	64.97

Each values represents mean±SEM in seconds for six mice treated with *C. iners* extracts or reference drugs, Values followed with different alphabets in the same column were significantly different from each other (p<0.05)

**Formalin induced pain:** Formalin is used as chemical noxious stimuli to trigger the pain. This test was normally used to study both central as well as peripheral analgesic activity (Tjolsen *et al.*, 1992). Injection of formalin is associated with the neurogenic pain during early phase followed by the pain due to the inflammation during the late phase (Hunskar and Hole, 1987; Santos *et al.*, 1998). It is evident from the Table 3, the antinociceptive activity in the early phase (0 to 5 min) for all the samples at the dose tested (200 mg kg<sup>-1</sup>, p.o.) and aspirin (100 mg kg<sup>-1</sup>, p.o.) was not significant (p≤0.05) in comparison with the control. However, all the samples and aspirin at the same dose inhibited the pain induced during the late phase (15 to 30 min) of the formalin test. The found analgesic activity in late phase was significantly different (p<0.05) from each other and it was in the order of morphine>aspirin>CIaK>CIeT>CIaQ. During the late phase of formalin assay the observed inflammatory pain is due to the release of citrulline, PGE<sub>2</sub>, glutamate and aspartate (Malmberg and Yaksh, 1995). Earlier, several studies shown that plant phenols such as flavonoids are responsible for the anti-inflammatory and antinociceptive activity (Kim *et al.*, 2004; De Melo *et al.*, 2009; Toker *et al.*, 2004; Kumar and Rajami, 2011; Makambila-Koubemba *et al.*, 2011). Hence, the presence of higher phenolic content in CIeT and CIaQ might be responsible for the found activity by inhibiting the release of these inflammatory mediators. However, the anti-inflammatory activity displayed by CIaK might be due to presence of other than phenolic compounds.

Nevertheless, morphine in exhibited significant analgesic activity (p<0.05) in both early and late phase of formalin induced pain. Present results are in agreement with the findings of Mustaffa *et al.* (2010a), where in they reported the peripheral analgesic activity from the leaves of *C. iners* methanolic extract.

**Evaluation of total phenolic content (TPC) and antioxidant activity:** Folin Ciocalteu method is normally used to determine the total amount of polyphenolic compounds present in the sample to be tested. It is evident from the Table 4, CIeT possessed significant (p<0.05) amount of phenolic content followed by CIaQ and CIaK. The presence of significant amount of polyphenolic content in CIeT might be due to the higher diffusive ability and medium polarity of the solvent ethanol in comparison with water. Pain and inflammation are always associated with release of several free radicals (Cuzzocrea *et al.*, 1998). Analgesics used will only take care of the pain but not the several complications associated with pain whereas presence of several phytoconstituents in medicinal plant extracts may address this issue. Hence, in this study we evaluated the antioxidant activities of these bark samples by DPPH assay and phosphomolybdenum assay. As seen in the Table 4, DPPH radical scavenging activity of tested samples and standards were significantly different from each other with gallic acid exhibiting higher radical scavenging activity followed by CIeT, BHT, CIaQ and CIaK. The reducing ability of samples and standards

Table 4: Total phenolic content and antioxidant activity of various extracts of *C. iners* bark and standards (n = 3)

Treatment	Total phenolics (mg GAE/g dry extract)	DPPH assay (mg VCEAC/g sample)	Phosphomolybdenum assay (mg VCEAC/g sample)
CIEt extract	511.8±4.33 <sup>c</sup>	384.9±0.50 <sup>d</sup>	642.6±14.62 <sup>d</sup>
ClAq extract	291.8±5.60 <sup>b</sup>	248.4±7.93 <sup>b</sup>	371.3±8.29 <sup>c</sup>
ClAk extract	100.6±3.43 <sup>a</sup>	92.5±4.30 <sup>e</sup>	307.1±5.54 <sup>b</sup>
BHT	NT	322.6±3.49 <sup>c</sup>	216.6±4.35 <sup>a</sup>
Gallic acid	NT	2805.3±45.25 <sup>e</sup>	3240.3±28.99 <sup>c</sup>

Each value was represented as mean±SD, NT: Not tested. Values followed with different alphabets in the same column were significantly different from each other (p<0.05)

tested by phosphomolybdenum assay also displayed similar pattern of antioxidant activity as that of DPPH assay. Even, in this assay the reducing capacity of gallic acid was significantly higher followed by CIEt, ClAq, ClAk and BHT. The found significant antioxidant activity of the samples might be due to the presence of higher amount of polyphenolic content in them (Annegowda *et al.*, 2011). Though, alkaloid extract contained significantly less amount of polyphenolic content but still exhibited significant reducing ability in comparison with BHT which might be due to the presence of other than polyphenolic compounds present in the sample.

### CONCLUSION

The results of the present study show that all the samples prepared from *C. iners* bark exhibited antinociceptive activity against the inflammatory pain caused by formalin. Hence, these samples can be therapeutically employed as peripheral analgesics. Present results also support the traditional use of *C. iners* in the treatment of pain. The observed peripheral analgesic activity by the ethanolic and alkaloid extract might be due to the presence of significant amount of polyphenolic compounds as well as non phenolic compounds, respectively. In contrast, in both the hot plate and tail flick test none of these samples shown promising antinociceptive activity confirming the absence of central analgesic activity. Further studies are in progress to find the possible bioactives in *C. iners* bark ethanolic and alkaloid extracts.

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