

Full Length Research Paper

Phytochemical screening, antioxidant and analgesic activities of *Croton argyratus* ethanolic extracts

N. I. Mohd Ali, H. V. Annegowda, S. M. Mansor, S. Ismail, S. Ramanathan and M. N. Mordi*

Centre for Drug Research, Universiti Sains Malaysia 11800, Penang, Malaysia.

Accepted 4 July, 2011

Series of experiments were conducted to screen phytochemical constituents, antioxidant and analgesic activities of the ethanolic extracts of the plant *Croton argyratus*. The extracts obtained from leaves, stem and root of the plant were evaluated for their antioxidant activity by means of 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, reducing power and total antioxidant capacity as well as total phenolic and flavonoid contents was studied. To determine analgesic property of the antioxidant rich extract, and formalin induced pain, hot plate and tail flick test were performed. The leaves extract showed the highest value of antioxidant activity based on DPPH radical scavenging activity, reducing power and total antioxidant capacity. The leaf extract also produced the highest total phenolic and total flavonoid content and have a significant activity in late phase of the formalin induced pain test at the dose of 200 mg/kg p.o. However, in the hot plate and tail flick tests, the extract did not show any significant analgesic effects. The results suggested the potential use of *C. argyratus* plant extracts as a natural source of antioxidant and may act peripherally to relieve pain.

Key words: DPPH, reducing power, total antioxidant capacity, total phenolic content, total flavonoid content, peripheral analgesic.

INTRODUCTION

Croton argyratus is a small or medium-sized tree that can grow up to 60 ft and can be found throughout Malaysia, Burma (Myanmar) and Bali (Burkill, 1966). *C. argyratus* is locally known as hamba raja, cheret budak, semelit mayor or akar cheret budak. This plant is also recognized as 'Silver Croton' as the undersides of the plant leaves are silvery white or silvery brown. The decoction of the leaves and stems are used by locals to cure purging and to aid recovery from childbirth. The biological and pharmacological aspects of *Croton* have been studied for anti-inflammatory (Suarez et al., 2006), anticancer (Sylvestre et al., 2006) and cytotoxicity (Morales et al., 2005) activities. Horgen et al. (2001) has carried out cytotoxicity test on human lung cancer line using the methanol extract of the leaves/twigs, roots and stem bark of *C. argyratus*. Results showed that the extracts of *C. argyratus* displayed toxicity to cancer cells with an IC₅₀

values of <5.0 µg/ml. All extracts showed selectivity of >10-fold against Lu-1 cell line compared with other cell lines tested. In addition, *C. argyratus* also showed a good antiplasmodial activity to *Plasmodium falciparum* sensitive strain D10 (Noor et al., 2007).

Free radicals are chemically reactive species bearing one or more unpaired electrons. They were found to play a potent role in affecting human health. Radicals and reactive oxygen species such as the superoxide anion (O₂⁻), hydroxyl radical (OH[•]) and peroxy radical (ROO[•]) are known as mediators for degenerative and chronic deteriorative including carcinogenesis, coronary heart disease, inflammation, arthritis, diabetes and aging (Ames et al., 1993; Heliövaara et al., 1994; Moskovitz et al., 2001).

Antioxidants are essential substances that help to protect the body from damage caused by free radicals induced oxidative stress (Orhan et al., 2009). Synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have been shown to be toxic and may cause mutagenic effect (Grice, 1986). Currently, research interest to find natural antioxidants

*Corresponding author. E-mail: mnizam@usm.my. Tel: (604)-6533272. Fax: (604)-6568669.

has risen and the present work was carried out to explore the *in vitro* antioxidant property of *C. argyratus*. An effort has also been made to evaluate analgesic activity possessed by *C. argyratus* leaves ethanolic extract (CAE) as recent studies have shown that free radicals are responsible for producing pain and inflammation (Gao et al., 2007; Koblyakov, 2001).

MATERIALS AND METHODS

Chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH), ferric chloride (FeCl₃), Folin-Ciocalteu reagent, aluminium chloride (AlCl₃), aspirin, formaldehyde, ammonium molybdate, ascorbic acid, gallic acid, and (±)-catechin hydrate standards were obtained from Sigma-Aldrich (St. Louis, MO, USA). Methanol, 95% ethanol, sulphuric acid (H₂SO₄), potassium ferricyanide [K₃Fe(CN)₆] and trichloroacetic acid (TCA) were obtained from Merck (Germany). Sodium nitrite (NaNO₂), sodium carbonate (Na₂CO₃), sodium phosphate (Na₃PO₄) and sodium hydroxide (NaOH) were purchased from Fluka (USA). Morphine was obtained from Hospital Universiti Sains Malaysia (Universiti Sains Malaysia, Kubang Kerian, Kelantan, Malaysia).

Plant material

C. argyratus was collected from Gunung Jerai, Kedah, Malaysia and identified by Dr. Rahmad Zakaria, from the School of Biological Sciences, Universiti Sains Malaysia (USM). A voucher specimen (number 11196) was kept at School of Biological Sciences, USM. The different parts of the plant were washed to remove the dirt and dried in oven at 40°C. The dried leaves, stem and root were then grinded to fine powder.

Preparation of extracts

Leaves, stem and root of *C. argyratus* (200 g each) were extracted using Soxhlet apparatus at 50°C using 95% ethanol (2.5 L). The extracts were then filtered, concentrated in a rotary evaporator and followed by freeze drying to obtain dry powder of extracts. All samples were kept in air tight glass container and stored at 4°C until further analysis.

Animals

Analgesic experiments were conducted using male Swiss Albino mice after protocol approval by the Animal Ethics Committee of USM. The mice weighed between 25 to 30 g were obtained from Animal House, USM. The animals were acclimatized to the lab conditions for one week prior to the experiments. They were kept at room temperature (37 under a light/dark cycle of 12 h and fed with free access food and tap water *ad libitum*.

Preliminary phytochemical screening

Phytochemical screening of *C. argyratus* ethanolic extracts was performed according to the method described by Sofowara (1982), Harborne (1973), and Siddiqui and Ali (1997). This experiment was carried out to detect the presence of amino acids, antraquinones, flavonoids, saponin, steroid, terpenoids, cardiac glycoside, tannins, alkaloids and reducing sugar.

Determination of total phenolic and flavonoid content

The total phenolic content of ethanolic extracts of different parts of *C. argyratus* was measured using Folin-Ciocalteu method described by Slinkard and Singleton (1977). To an aliquot of 0.4 ml sample, 2.0 ml of prediluted Folin-Ciocalteu reagent (1:10) was added. After 4 min, 1.6 ml of Na₂CO₃ solution (75 g/L) was added to the mixture and mixed well. After 1 h, the absorbance of the resulting mixture was measured at absorbance of 765 nm. Total phenolic content was calculated from the calibration curve of gallic acid standard solution and the values were expressed as mg gallic acid equivalent (GAE)/g dry extract.

The total flavonoid content was determined according to the method described by Sakanaka et al. (2005). In brief, 0.25 ml of sample was mixed with 1.25 ml distilled water followed by 75 µl of 5% NaNO₂. After 6 min, 150 µl of 10 % AlCl₃ was added and the mixture was allowed to stand for 5 min followed by addition of 0.5 ml of 1 M NaOH. Immediately, distilled water was added to the mixture to make up the final volume of 2.5 ml. The absorbance of this solution was determined at 510 nm. The total flavonoid content was interpolated from the calibration curve of catechin solution and the values were expressed in mg of catechin equivalents (CE)/g dry extract.

Determination of antioxidant activity

DPPH radical scavenging activity

DPPH radical scavenging activity *C. argyratus* plant extracts as well as ascorbic acid was determined using the method described by Brand-Williams et al. (1995). Briefly, 77 µl of each extract and ascorbic acid ranging from 0.01 to 1 mg/ml was transferred into 3 ml of 6 × 10⁻⁵ M methanolic DPPH solution. The samples were incubated in the dark for 15 min at room temperature followed by measuring the absorbance at 517 nm. The percent inhibition of radical scavenging activity was calculated using the following equation:

$$\frac{A_0 - A_e}{A_0} \times 100$$

Where; A₀ = absorbance of control, A_e = absorbance of extract
The IC₅₀ values were calculated by linear regression of plots where x-axis represented the concentration (mg/ml) and y-axis represented the scavenging effect (% inhibition).

Reducing power

The reducing power of *C. argyratus* plant extracts, ascorbic acid and gallic acid were carried out according to the method described by Oyaizu (1986). To 0.1 ml of sample solution, 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% K₃Fe(CN)₆ was added followed by mixing and then incubated at 50°C for 20 min. After 20 min, 2.5 ml of 10 % TCA, 2.5 ml of distilled water and 0.5 ml of 0.1% FeCl₃ were added. Then the absorbance of the final solution was measured at 700 nm. A higher absorbance value of the reaction mixture suggested a stronger reducing power activity.

Total antioxidant capacity

Total antioxidant capacity was determined using the method described by Dasgupta and De (2004). To the 0.3 ml of ethanolic

Table 1. Qualitative phytochemical screening of *C. argyratus* plant extracts.

Constituent	Leaf	Stem	Root
Amino acids	-	-	-
Antraquinones	-	-	-
Flavonoids	+	+	+
Saponin	-	-	-
Steroid	+	++	+
Terpenoids	+++	+++	+++
Cardiac glycosides	+	+	-
Tannins	+	+	-
Alkaloids	-	-	-
Reducing sugar	+	+	-

+++ = abundance; ++ = moderately present; + = weakly present; - = absent.

extracts of different parts of *C. argyratus*, respectively, 3 ml of reagent solution (0.6 M H₂SO₄, 28 mM sodium phosphate and 4 mM ammonium molybdate) was added. The test tubes were capped incubated at 95°C for 90 min. After the samples had cooled to room temperature, absorbance of the mixture was measured at 695 nm. The antioxidant activity was expressed as milligram ascorbic acid or gallic acid equivalent antioxidant capacity/g extract. All the experiments were conducted in triplicates.

Analgesic activity

Hot plate

The hot plate test was performed using the method of Woolfe and MacDonald (1944). Male Swiss Albino mice (n = 6) were administered with CAE (50, 100 and 200 mg/kg), negative control cosolvent (propylene glycol:tween 80:water = 4:1:4, v/v/v), and positive control morphine sulphate (5 mg/kg s.c), respectively. After 30 min treatment (except 15 min for morphine), the mice were placed on hot plate analgesia meter maintained at 55 ± 1°C. Time taken for licking paw and jumping were recorded as response latency. The animals were observed for every 15 min over 90 min periods. The cut off time 45 s was chosen to prevent tissue damage.

Tail flick

The experiment was carried out as described by D'Amour and Smith (1941). Mice were administered orally with CAE (50, 100 and 200 mg/kg), cosolvent (propylene glycol: tween 80: water = 4:1:4, v/v/v) as control and morphine sulphate (5 mg/kg, s.c) respectively. The tail flick response of the mice was carried out by gently placing the mice tail at the central position of the light beam and mice's response to the light by flicking or removing its tail was referred as latency time (s). The cut off time of 10 s was maintained to prevent tissue injury to the mice tail.

Formalin induced pain

The method used was similar to that described previously by Hunskaar and Hole (1987) was followed to study formalin induced pain Mice, which were treated with *C. argyratus* leaves extract (50, 100 and 200 mg/kg), cosolvent (propylene glycol: tween 80: water = 4:1:4, v/v/v) and morphine sulphate (5 mg/kg. s.c)

respectively, 30 min before formalin injection (15 min for morphine). After 30 min, 20 µl of 2.5% formalin in saline was injected to the right hind paw of mice. The mice were placed in a glass cylinder and the time spent (s) licking and biting the injected paw in the early phase (0 to 5 min) and late phase (15 to 30 min) was recorded as indicative of pain.

Statistical analysis

The results of antioxidant and analgesic activity were presented as mean ± S.D from triplicate determination. Analysis of variance (ANOVA) followed by Tukey's test and Dunnet's test were performed to determine the significant difference between samples (p < 0.05) using SPSS version 18 and Sigmaplot version 11.

RESULTS

Preliminary phytochemical screening

The result of qualitative phytochemical screening is presented in Table 1. All extracts contained flavonoids, terpenoids and steroids. Cardiac glycosides, tannins and reducing sugar were detected in leaves and stem but absent in root extract. However, amino acids, antraquinones, saponin and alkaloids were completely absent in all extracts.

Determination of total phenolic and total flavonoid content

Table 2 shows the total phenolic and flavonoid contents in different parts of *C. argyratus*. Total phenolic contents was determined using the Follin-Ciocalteu reagent and reported as gallic acid equivalents (GAE) by reference to a standard curve ($y = 8.470x + 0.285$, $r^2 = 0.993$). Meanwhile, total flavonoid content was calculated in comparison with standards of catechin equivalent ($y = 3.093x + 0.0558$, $r^2 = 0.995$) and the result expressed as in terms of catechin equivalent/g (CE/g) dry sample. The results showed that leaves contained highest phenol content followed by stem and root with the values of 40.62, 17.95 and 12.0 1mg GAE/g dry sample, respectively. The highest flavonoid content was found in leaves extract followed by stem and root with total flavonoid content of 17.78, 14.29 and 7.08 mg CE/g dry extract.

DPPH radical scavenging activity

As shown in Figure 1, the highest activity was observed in the leaves extract followed by stem and root. The scavenging abilities on DPPH radicals at 1 mg/ml of extracts were leaves (62.54%), stem (30.48%) and root (25.02%), respectively. Ascorbic acid (a standard antioxidant) showed 96.04% inhibition of DPPH radical at a concentration of 1 mg/ml. The IC₅₀ value of *C.*

Table 2. Total phenolic and flavonoid content and IC₅₀ values of *C. argyratus* plant extracts.

Extract	Total phenolic content mg GAE/g extract	Total flavonoid content mg CE/g extract	DPPH assay (IC ₅₀ mg/ml)
Leaves	40.62 ± 3.12	17.78 ± 0.34	1.45
Stem	17.95 ± 0.67	14.29 ± 0.13	ND
Root	12.01 ± 0.55	7.08 ± 0.73	ND
Ascorbic acid	-	-	0.09

Data shown as mean ± SD, n = 3. ND, could not be determined.

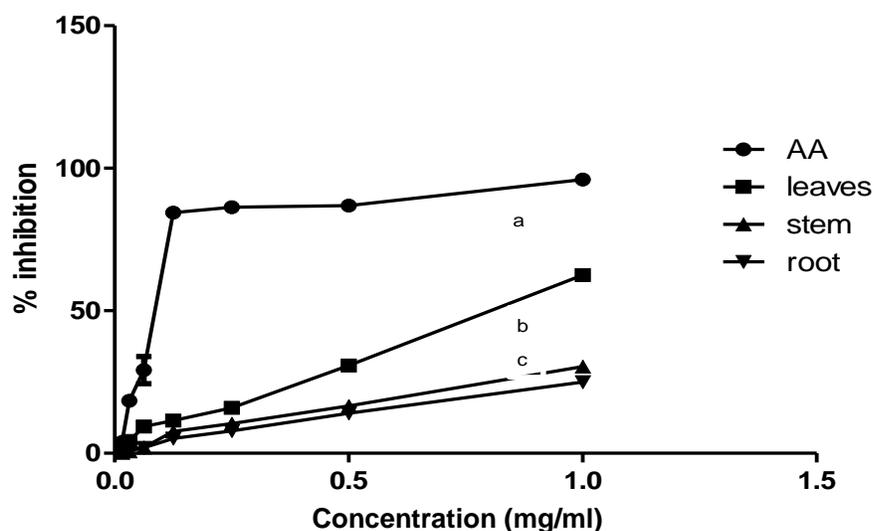


Figure 1. Free radical scavenging activity of ethanolic extracts from different parts of *C. argyratus* measured by DPPH assay. Results are mean ± SD (n = 3). ^a indicates significant differences as compared to the control ascorbic acid at p < 0.05; ^b indicates significant differences as compared to the control ascorbic acid at p < 0.01; ^c indicates significant differences as compared to the control ascorbic acid at p < 0.01.

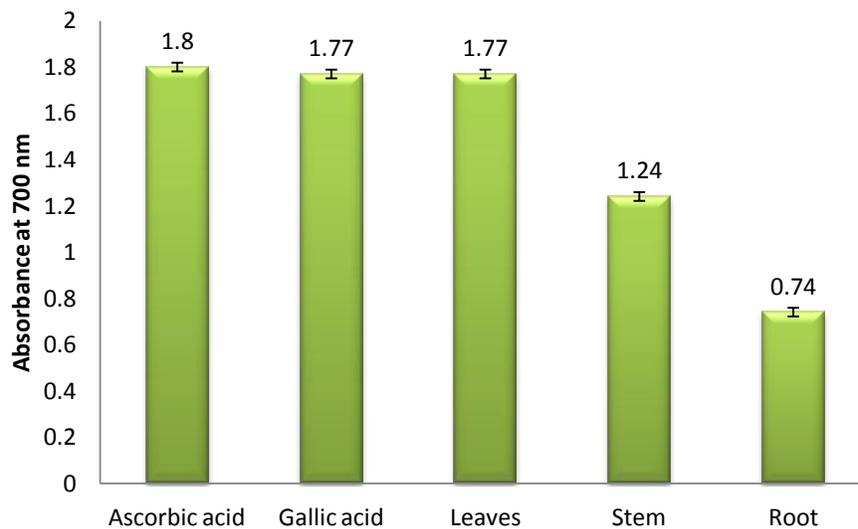


Figure 2. Reducing power of the extracts from *C. argyratus* plant. Ascorbic acid and gallic acid were used as reference antioxidants. Values are mean ± SD (n = 3).

Table 3. Total antioxidant capacities of different parts of *C. argyratus*.

Sample	Equivalent to ascorbic acid (mg)/g of extract	Equivalent to gallic acid (mg)/g of extract
Leaf	0.087 ± 0.0020 ^c	0.14 ± 0.0030 ^c
Stem	0.046 ± 0.0042 ^b	0.074 ± 0.0066 ^b
Root	0.032 ± 0.0013 ^a	0.023 ± 0.0010 ^a

Data expressed as mean ± SD, n = 3. Means with different superscript letters were significantly different at the level $p < 0.05$.

Table 4. Effect of *C. argyratus* leaves extract (CAE) and morphine on pain induced by hot plate test.

Treatment	Dose (mg/kg)	Latency period (min)				
		30	45	60	75	90
Control	0	15.24 ± 4.25	11.04 ± 3.12	14.68 ± 5.75	15.34 ± 6.83	17.3 ± 2.51
CAE	50	16.56 ± 6.57	19.95 ± 13.46	14.25 ± 3.50	15.19 ± 3.19	21.06 ± 10.60
CAE	100	16.03 ± 8.62	19.1 ± 7.10	23.17 ± 6.41	23.44 ± 5.31	20.96 ± 9.98
CAE	200	16.13 ± 9.28	16.03 ± 6.79	16.73 ± 5.64	18.64 ± 7.11	19.13 ± 10.33
Morphine	5	31.01 ± 7.61*	34.46 ± 7.55*	30.71 ± 11.10*	27.41 ± 10.26*	29.47 ± 11.08

Value expressed as mean ± SD (n = 6). * $p < 0.05$ when compared with control values.

Table 5. Effect of *C. argyratus* leaves extract (CAE) and morphine on pain induced by tail flick test.

Treatment	Dose (mg/kg)	Latency period (min)				
		30	45	60	75	90
Control	0	2.26 ± 0.54	1.77 ± 0.61	1.95 ± 0.29	2.08 ± 0.41	2.52 ± 1.12
CAE	50	1.85 ± 0.44	1.60 ± 0.31	2.24 ± 0.85	2.45 ± 1.46	2.53 ± 0.56
CAE	100	2.64 ± 0.23	2.28 ± 0.63	2.47 ± 0.81	3.36 ± 1.33	2.42 ± 1.11
CAE	200	3.42 ± 2.02	2.10 ± 0.71	1.73 ± 0.69	3.34 ± 1.53	1.54 ± 1.54
Morphine	5	9.71 ± 0.46*	10.00 ± 0.00*	10.00 ± 0.00*	10.00 ± 0.00*	9.62 ± 0.59*

Value expressed as mean ± SD (n = 6). * $p < 0.05$ when compared with control values.

argyratus leaves extract was 1.45 mg/ml and is higher than IC_{50} for ascorbic acid, 0.09 mg/ml (Table 2).

Reducing power

Figure 2 shows the reducing capability of different parts of *C. argyratus*. Leaves exhibited the stronger reducing power (1.77) than stem (1.24) and root (0.74) respectively, while ascorbic acid (1.80) had the strongest reducing power among all the samples tested.

Total antioxidant capacity

Results of total antioxidant capacity method were present in Table 3. Similar trend of antioxidant activity was also found in this method as leaves extract of *C. argyratus* had a higher capacity followed by stem and root.

Hot plate and tail flick tests

C. argyratus leaves extract did not show any significant

increase in the reaction time in both tests. In contrast, morphine showed significant increase in reaction time ($p < 0.05$) as compared with control group (Tables 4 and 5).

Formalin test

C. argyratus leaves extract did not show any effect in the early phase of the formalin test (0 to 5 min). However, in the late phase (15 to 30 min), the extract at the higher dose of 200 mg/kg showed significant reduction in the licking and biting of paw. Even aspirin was also significantly active ($p < 0.05$) in the second phase however morphine was active in both phases of formalin induced pain (Figure 3).

DISCUSSION

Phenolic compounds are known to possess biological effects such as antioxidants, anti-aging, cardioprotection and anti-cancer activities (Han et al., 2007). In this study,

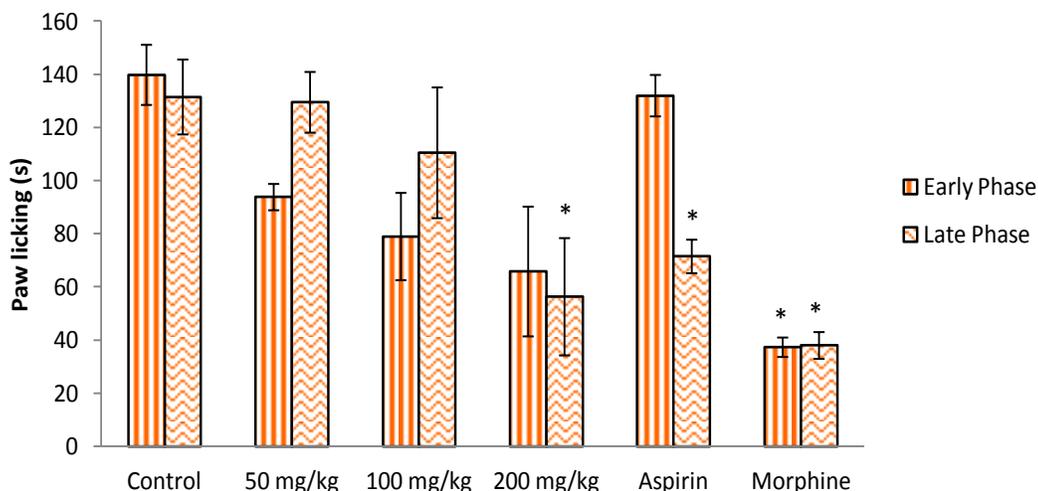


Figure 3. Effect of *C. argyratus* leaves extract, morphine and aspirin on formalin test. Value expressed as mean \pm SD (n = 6). *p < 0.05 when compared with control values.

C. argyratus leaves contain higher phenols and polyphenolic compounds than its stem and root. This is in agreement with previous studies which reported that total phenolic content in leaf was higher than other parts of the plant for *Cucumis melo*, *Petroselinum crispum*, *Coriandrum sativum* and *Beta vulgaris* (Ismail et al., 2010; Pyo et al., 2004; Wong and Kitts, 2006). In this study, leaves extract of *C. argyratus* also exhibited the highest total flavonoid content, followed by stem and root (Table 1). This is not surprising as flavonoids are the most common and widely distributed group of plant phenolic compounds which are very effective antioxidants (Pokorny et al., 2001). Flavonoids are also responsible for the free radical scavenging activity in plants (Das and Pereira, 1990).

DPPH assay has been widely used for screening antioxidant activity and is sensitive enough to detect the active ingredients at low concentrations (Sánchez-Moreno, 2002). The DPPH radical scavenging activity of 1 mg/ml ascorbic acid was the highest, followed by *C. argyratus* leaves, stem and root respectively. The quality of the antioxidants potency in the extracts was determined by the IC_{50} value whereby, a low IC_{50} value indicates strong antioxidant activity. The IC_{50} value of leaves extract was determined at 1.45 mg/ml. On the other hand, the IC_{50} value of stem and root extract could not be determined due to the low percentage of inhibition (<50 %) even at high concentration of 5 mg/ml. Even though antioxidant activity of *C. argyratus* leaves extract (IC_{50} = 1.45 mg/ml) was moderate compared to ascorbic acid (IC_{50} = 0.09 mg/ml), it is comparable to other fruits which are considered to have good antioxidant activity (orange IC_{50} = 5.40 \pm 1.30 mg/ml, guava IC_{50} = 2.11 \pm 0.63 mg/ml, star fruit IC_{50} = 3.80 \pm 2.10 mg/ml) (Pin-Der-Duh, 1998).

C. argyratus leaves extract showed the most reducing

power than stem and root. The reducing ability of a compound is generally correlated with the presence of reductones (Duan et al., 2007) which exert antioxidant action by breaking the free radical chain via donating a hydrogen atom (Shimada et al., 1992). In this assay, Fe^{3+} /ferricyanide complex was used as an indicator of electron-donating activity. The presence of reductants (antioxidants) in the sample caused the reduction of the Fe^{3+} /ferricyanide complex to its ferrous form. The Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. The ability to reduce Fe^{3+} might be due to hydrogen donation from phenolic compounds (Prieto et al., 1999).

Total antioxidant capacity was estimated using phosphomolybdenum method based on the reduction of Mo (VI) to Mo (V) by the formation of a green phosphate/Mo (V) complex at acidic pH (0.6 to 1.0) with a maximum absorbance at 695 nm (Nagendra et al., 2009). This assay is quantitative since the antioxidant activity is expressed as the number of equivalents of ascorbic acid and gallic acid. Leaves of *C. argyratus* had the higher capacity than stem and root (Table 3). The total antioxidant capacity of plant extract could be attributed to their chemical composition and phenol content. Free radicals have been implicated to be a contributing factor in modulation of pain and tissue injury (Khalil et al., 1999). Based on results, *C. argyratus* leaves extract (CAE) gave the best antioxidant result and was chosen for analgesic study. The analgesic effect of CAE was examined using thermal models (hot plate and tail flicks) and chemical model (formalin induced pain). Hot plate and tail flicks methods are usually applied to discover the involvement of central analgesic effect. The hot plate test is thought to involve supraspinal reflex (Pini et al., 1997); whereas, tail flick test involve spinal motor reflex (D'amour and Smith, 1941). In this study, CAE did

did not show any significant analgesic effect on pain induced by both hot plate and tail flick tests in animals. On the other hand, morphine (5 mg/kg), a centrally acting analgesic produced significant effect on both tests. Based on this observation, it can be suggested that CAE does not act on the central analgesic mechanism.

The formalin injection causes an immediate and intense increase in the spontaneous activity of afferent C fibers and induces distinct quantifiable behavior indicate of pain, for example, licking of the injected paw (Heapy et al., 1987). The formalin test produces a distinct biphasic nociceptive response that is neurogenic and inflammatory phase. It has been reported that the early phase is caused by a direct effect of formalin on nociceptors while the late phase is a tonic response in which inflammatory processes are involved and neurons in the dorsal horns of the spinal cord are activated (Tjølsen et al., 1992). CAE produced a significant reduction of licking activity in the late phase at dose 200 mg/kg but did not give any responses in the early phase. Centrally acting drugs such as morphine (opioids) inhibited both phases whereas peripherally acting drugs, aspirin (NSAIDs) inhibited the late phase of formalin test (Santos et al., 1994; Shibata et al., 1989). Thus, it can be deduced that CAE acts peripherally and not centrally. Similar findings also have been reported by using methanolic extract of *Cinnamomum iners* and ethanolic extract of *Curcuma xanthorrhiza* Roxb (Mustafa et al., 2010; Devaraj et al., 2010).

Previous work with the root of *C. argyratus* identified clerodane type diterpene, (-)-junceic acid (Norizan et al., 2007) which has an anti-inflammatory effect (Bruno et al., 1993). Although, (-)-junceic acid is a potential compound to justify the analgesic effect of *C. argyratus*, this compound was isolated from the root of *C. argyratus* and not from leaves, the material used in this study. As phytochemical screening revealed the presence of flavonoids, terpenoids and steroids in *C. argyratus*, these constituents might also be responsible for the antioxidant and analgesic activities (Galati et al., 1994; Saeed et al., 2010).

Based on the results, *C. argyratus* has a natural antioxidant capacity as an alternative to synthetic antioxidants. A bio-assay guided fractionation of this extract is now in progress to identify the bioactive substance(s) in the ethanol extract of *C. argyratus* as well as the mechanisms of action involved in the effects described in this work.

ACKNOWLEDGEMENTS

This project was funded by Research University Grant from Universiti Sains Malaysia. N. I. Mohd Ali was supported by Ministry of Higher Education, Malaysia and Institute of Postgraduate Studies, Universiti Sains Malaysia.

REFERENCES

- Ames BN, Shigenaga MK, Hagen TM (1993). Oxidants, antioxidants, and the degenerative diseases of aging. *Proc. Natl. Acad. Sci. USA*, 90: 7915-7922.
- Brand-Williams W, Cuvelier ME, Berset C (1995). Use of free radical method to evaluate antioxidant activity. *Food Sci. Technol.*, 28: 25-30.
- Bruno M, De LT, Rodrigues MCB, Omar AA (1993). Guaiane sesquiterpenes from *Teucrium leucocladum*. *Phytochemistry*, 34: 245-247.
- Burkill IH (1966). *A Dictionary of the Economic Product of the Malay Peninsula*. Kuala Lumpur, 1: 698-699.
- D'Amour EE, Smith DL (1941). A method of determining loss of pain sensation. *J. Pharmacol. Exp. Ther.*, 72: 74-79.
- Das NP, Pereira TA (1990). Effects of flavonoids on thermal autooxidation in Palm oil: structure-activity relationship. *J. Am. Oil Chem. Soc.*, 67: 255-258.
- Dasgupta N, De B (2004). Antioxidant activity of *Piper betle* L. leaf extract *in vitro*. *Food Chem.*, 88: 219-224.
- Devaraj S, Esfahani AS, Ismail S, Ramanathan S, Yam MF (2010). Evaluation of Antinociceptive Activity and Acute Oral Toxicity of Standardized Ethanolic Extract of the Rhizome of *Curcuma Xanthorrhiza* Roxb. *Molecules*, 15: 2925-2934.
- Duan X, Wu G, Jiang Y (2007). Evaluation of phenolics from litchi fruit in relation to pericarp browning prevention. *Molecules*, 12: 759-771.
- Galati EM, Monforte MT, Kirjavaine S, Forestieri AM, Trovato A, Tripodo MM (1994). Biological effects of hesperidin, a citrus flavonoid. (Note I): anti-inflammatory and analgesic activity. *IL Farmaco.*, 40: 709-712.
- Gao X, Kim HK, Chung JM (2007). Reactive oxygen species (ROS) are involved in enhancement of NMDA-receptor phosphorylation in animal models of pain. *Pain*, 131: 261-271.
- Grice HC (1986). Safety evaluation of butylated hydroxytoluene (BHT) in the liver, lung and gastrointestinal tracts. *Food Chem. Toxicol.*, 24: 1127-1130.
- Han X, Shen T, Lou H (2007). Dietary Polyphenols and Their Biological Significance. *Int. J. Mol. Sci.*, 8: 950-988.
- Harborne JB (1973). *Phytochemical Methods*; Chapman and Hall: London, UK, pp. 49-188.
- Heapy CG, Jamieson A, Russell NJW (1987). Afferent C-fiber and A-delta activity in models of inflammation. *Br. J. Pharmacol.*, 90: 164.
- Heliövaara M, Knekt P, Aho K, Aaran RK, Alftan G, Aromaa A (1994). Serum antioxidants and risk of rheumatoid arthritis. *Ann. Rheum. Dis.*, 53: 51-53.
- Horgen FD, Edrada RA, Reyes G, Agcaoili F, Madulid DA, Wongpanich V, Angerhofer CK, Pezzuto JM, Soejarto DD, Farnsworth (2001). Biological screening of rain forest plot trees from Palawan Island (Philippines). *Phytomedicine*, 8(1): 71-81.
- Hunskar S, Hole K (1987). The formalin test in mice: Dissociation between inflammatory and inflammatory pain. *Pain*, 30: 103-144.
- Ismail HI, Chan KW, Mariod AA, Ismail M (2010). Phenolic content and antioxidant activity of cantaloupe (*cucumis melo*) methanolic extracts. *Food Chem.*, 119: 643-647.
- Khalil Z, Liu T, Helme RD (1999). Free radicals contribute to the reduction in peripheral vascular responses and the maintenance of thermal hyperalgesia in rats with chronic constriction injury. *Pain*, 79: 31-37.
- Kobylakov VA (2001). Free radicals and inflammation (progress in inflammation research series). *Biochemistry*, 66: 937-938.
- Morales A, Perez P, Mendoza R, Compagnone R, Suarez AI, Arvelo F, Ramirez JL, Castro IG (2005). Cytotoxic and proapoptotic activity of ent-16 β -17a-dihydroxykaurane on human mammary carcinoma cell line MCF-7. *Cancer Lett.*, 218(1): 109-116.
- Moskovitz J, Yim KA, Choke PB (2001). Free radicals and disease. *Arch. Biochem. Biophys.*, 397: 354-359.
- Mustafa F, Indurkar J, Ismail S, Mordi MN, Ramanathan S, Mansor SM (2010). Analgesic activity, toxicity study, and phytochemical screening of standardized *Cinnamomum iners* leaves methanolic extract. *Phcog. Res.*, 2(2): 76-81.
- Nagendra KP, Bao Y, Xinhong D, Guoxiang J, Haiyan Z, Haihui X, Yueming J (2009). Flavonoid contents and antioxidant activities from *Cinnamomum* species. *Innov. Food Sci. Emerg. Technol.*, 10: 627-

- 632.
- Noor RA, Khozirah S, Mohd RMAR, Ong BK, Rohaya C, Rosilawati M, Hamdino I, Badrul A, Zakiah I (2007). Antiplasmodial properties of some Malaysian medicinal plants. *Trop. Biomed.*, 24(1): 29-35.
- Norizan AAH, Ikram MS, Jalifah L, Laily BD, Yana MS, Euis HH (2007). Stryldehydropyrone and Clerodane-Type Diterpene from *Croton argyratus*. *Malays. J. Anal. Sci.*, 1: 189-192.
- Orhan I, Kartal M, Abu-Asaker M, Senol FS, Yilmaz G, Sener B (2009). Free Radical scavenging properties and phenolic characterization of some edible plants. *Food Chem.*, 114: 276-281.
- Oyaizu M (1986). Studies on product of browning reaction prepared from glucose amine. *Japan J. Nutr.*, 44: 307-315.
- Pin-Der-Duh X (1998). Antioxidant activity of burdock (*Arctium lappa Linne*); its scavenging active oxygen. *J. Am. Oil Chem. Soc.*, 75: 455-461.
- Pini LA, Vitale G, Ottani A, Sandrini M (1997). Naloxonereversible antinociception by paracetamol in the rat. *J. Pharmacol. Exp. Ther.*, 280: 934-940.
- Pokorny J, Yanishlieva N, Gordon M (2001). Antioxidants in food, Practical Applications, Cambridge. Woodhead Publishing Limited. pp. 1-3.
- Prieto P, Pineda M, Aguilar M (1999). Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. *Anal. Biochem.*, 269: 337-341.
- Pyo YH, Lee TC, Logendra L, Rosen RT (2004). Antioxidant activity and phenolic compounds of Swiss chard (*Beta vulgaris* subspecies *cykla*) extracts. *Food Chem.*, 85(8): 19-26.
- Saeed MK, Deng Y, Dai R, Li W, Yu Y, Iqbal Z (2010). Appraisal of antinociceptive and anti-inflammatory potential of extract and fractions from the leaves of *Torreya grandis* Fort Ex Lindl. *J. Ethnopharmacol.*, 127: 414-418.
- Sakanaka S, Tachibana Y, Okada Y (2005). Preparation and antioxidant properties of extracts of Japanese persimmon leaf tea (kakinoha-cha). *Food Chem.*, 89: 569-575.
- Santos ARS, Filho VC, Niero R, Viana AM, Morenof N, Campos MM, Yunes RA, Calixto JB (1994). Analgesic effect of Callus culture extracts from selected species of *Phyllanthus* in mice. *J. Pharm. Pharmacol.*, 46: 755-759.
- Sánchez-Moreno C (2002). Methods used to evaluate the free radical scavenging activity in foods and biological systems. *Food Sci. Technol. Int.*, 8: 121-137.
- Shibata M, Ohkubu T, Takahashi H, Inoki R (1989). Modified formalin test: characteristics biphasic pain response. *Pain*, 38: 347-352.
- Shimada K, Fujikawa K, Yahara K, Nakamura T (1992). Antioxidative properties of xanthan on the autoxidation of soyabean oil in cyclodextrinemulsion. *J. Agric. Food. Chem.*, 40: 945-948.
- Siddiqui AA, Ali M (1997). *Practical Pharmaceutical Chemistry*, 1st ed. CBS Publishers and Distributors: New Delhi, India, pp. 126-131.
- Slinkard K, Singleton VL (1977). Total phenol analyses: Automation and comparison with manual methods. *Am. J. Enol. Vitic.*, 28: 49-55.
- Sofowara A (1982). *Medicinal Plants and Traditional Medicine in Africa*; John Wiley and Sons, Ltd: New York, NY, USA.
- Suarez AI, Blanco Z, Compagnone RS, Salazar-Bookaman MM, Zapata V, Alvarado C (2006). Antinflammatory activity of *Croton cuneatus* aqueous extract. *J. Ethnopharmacol.*, 105 (1-2): 99-101.
- Sylvestre M, Pichette A, Longtin A, Nagau F, Lagault J (2006). Essential oil analysis and anticancer activity of leaf essential oil of *Croton flavens* L. from Guadeloupe. *J. Ethnopharmacol.*, 103(1): 99-102.
- Tjølsen A, Berge OG, Hunskaar S, Rosland JH, Hole K (1992). The formalin test: an evaluation of the method. *Pain*, 51: 5-17.
- Wong PYY, Kitts DD (2006). Studies on the dual antioxidant and antibacterial properties of parsley (*Petroselinum crispum*) and cilantro (*Coriandrum sativum*) extracts. *Food Chem.*, 97(3): 505-515.
- Woolfe G, MacDonald AD (1944). The evaluation of analgesic action of pethidine hydrochloride (DEMEROL). *J. Pharmacol. Exp. Ther.*, 80: 300-307.