Journal of Tropical Resources and Sustainable Science

journal homepage: jtrss.org

Brine shrimp lethality test of various *Cinnamomum iners* (Lauraceae) barks extracts

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Received 30 May 2018
Accepted 26 July 2018
Online 19 December 2018

Keywords:
Brine shrimp lethality test,
Cinnamomum iners

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Abstract

Cinnamomum iners (Lauraceae) is a traditional plant that widely used to relieve headache, breathing and appetite problems. This plant has been used over the centuries on several illnesses with bacterial symptoms such as fevers, digestive ailments and coughs. However, lack of scientific studies have been conducted to identify its toxicity. Thus, an evaluation on the toxicity of this plant extracts is crucial to support its therapeutic claims as well as to ensure that there was no side effects to consumers. Various extracts from *C. iners* barks were screened for their toxicity against brine shrimp using the brine shrimp lethality test. All extracts exhibited very high LC_{50} (50% lethal concentration) values greater than 1mg/ml (1000 µg/ml) with the hexane extracts showed the highest toxicity to the brine shrimp with LC_{50} value of 1306.79 µg/ml, while the lowest toxicity was the ethyl acetate extract at 3370.13 µg/ml. This finding corroborates the traditional uses of this plant and could be developed as another alternative natural sources in treating various diseases.

1. INTRODUCTION

Medicinal plants which form the backbone of traditional medicine, have in the last few decades been the subject for very intense pharmacological studies. This has been brought about by the knowledge by the acknowledgement of the value of medicinal plants as potential sources of new compounds of therapeutic value and as sources of lead compounds in drug development. In developing countries, it is estimated that about 80% of the population rely on traditional medicine for their primary healthcare (Taylor and Attaur, 1994; Butkhup and Samappito, 2011).

In Malaysia, the use of herbal medicines is not scientifically validated (Abdul Wahab, 2013). The knowledge on the indigenous use of plants often derived from traditional belief and passed verbally from one generation to another and has largely remained undocumented (Abdul Wahab et al., 2012). Focusing on the studied plant i.e. *Cinnamomum iners*, it is written in the Compendium of Medicinal Plants used in Malaysia (Volume I, 2002. In Malaysia, there are approximately thirteen documented *Cinnamomum* species currently being used in the Malaysian traditional system of medicine (Buru et al., 2014).

Cinnamomum iners Reinw. ex Blume 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. This plant is used as a traditional herb and for ornamental purposes (Mustaffa et al., 2013). *C. iners* is commonly known as wild cinnamon ('Kayu Manis Hutan') or 'pokok teja' as well as 'Medang Kemangi' (Ong, 1999; Choi, 2003), was used as a substitute for cinnamon in various parts of Malaysia and Thailand (Annegowda et al., 2012). In Thailand, various parts of this plant have been used traditionally as antidiarrheal, diuretic, digestive system related problem including constipation, laxative, carminative, anti-infective and stomach infection (Butkhup and Samappito, 2011), tonic for stomach and in the treatment of rheumatism (Nguyen et al., 2004; Annegowda et al., 2012), as well as to relieve fever, and to cure appetite related illness (Pengelly, 2004; Zabri Tan et al., 2011). It also traditionally claimed to be consumed by diabetic patients as natural supplements (Choi, 2003).

The leaves are widely utilized as traditional medicine to relieve fever, for digestive system problems, as carminative (Pengelly, 2004), as well as analgesic and antipyretic (Pormjit, 1989; Ghalib et al., 2012). With the daily usage of boiled leaves in bath water, it is known to be effective in treating rheumatism. The consumption of leaf juice cures headache and fever. Besides that, it has been used to heal minor wounds by applying crushed leaves on the affected area (Choi, 2003). In addition, the leaves have also been used extensively in joss stick and mosquito coils due to their fragrant nature and high mucilage content (Jantan, Ali and Goh, 1994). The non-medicinal usage of the plant leaves including raw material in food and

eISSN Number: 2462-2389

industrial products such as plastic, gum, paper, tyre and glass fibre (Mustaffa et al., 2013).

The bark of *C. iners* has multiple traditional uses and contains some essential oils. The bark is used as detoxifying agent by drinking the juice as herbal tea. The bark sold as 'mesni' in Malaysia is used as medicinal tea and curry flavor. A decoction of the bark is drunk after child-birth as post-partum medicine (Ong, 1999). A decoction of the roots is drunk against fever (Ong, 1999) and also very popular in postpartum treatment to regain energy, to repair blood flows and womb contraction. Meanwhile, the wood is being used to build houses, household cabinet and furniture (Choi, 2003; Mustaffa et al., 2013). In addition, it is still lacking reports regarding its ability to repel insects such as mosquitoes. It has been explained verbally by local people that Chinese community uses the barks by burning them which could act as mosquitoes and houseflies repellents (Annegowda et al., 2012).

Recent studies by Mustaffa et al. (2011) reported the presence of α-caryophyllene, stigmasterol, cardiac glycoside, flavonoid, polyphenol, saponin, sugar, tannin and terpenoid in the tree (Espineli et al., 2013). Several essential oils such as linalool, caryophyllene oxide, cardinol were identified from the leaves while 1, 8-cineole, α-terpineol, terpinen-4-ol, β-pinene and caryophyllene oxide from the stem bark of *C. iners* (Baruah et al., 2001; Phutdhawong et al., 2007; Annegowda et al., 2012). Its extract was found to contain tannins (Butkhup & Samappito, 2011), cardiac glycoside, flavonoid, polyphenol, saponin, sugar, tannin dan terpenoid (Mustaffa et al., 2010; Zabri Tan et al., 2011).

The latest study by Espineli et al. (2013) using barks and leaves of *C. iners* collected from Guimaras, Philippines showed the presence of seven compounds in the dicholoromethane extract. After several series of chromatography of the bark extract, the isolated compounds were identified as 5,7-dimethoxy-3c,4c-methylenedioxyflavan-3-ol and β-sitosterol, 4-(4-hydroxy-3-methoxyphenyl)but-3-en-2-one, cinnamaldehyde, linoleic acid and vanillin. The leaves of *C. iners* afforded eugenol, linoleic acid, and β-sitosterol.

However, there should be a vital requirement to determine the toxic effects of the substances contained in the plant. Toxicity is an expression of being poisonous, indicating the state of adverse effects led by the interaction between toxicants and the cell membrane, as it may occur on the cell surface, within the cell body, or in the tissues beneath as well as at the extracellular matrix (Abdul Rani et al., 2010). Brine shrimp lethality test (BSLT) is the general bioassay which capable to screen and evaluate the toxicity effect which commonly used in the medicines especially natural plants extracts, the evaluation of toxicity of heavy metals and pesticides. It's a basic toxicity test for further experiments on mammalian animal models (Zhao et al., 1992). Due to extensive use of its extract, hence, this study was designed to screen the toxicity effects of various *C. iners* extracts using the brine shrimp lethality test.

2. MATERIALS AND METHODS

2.1. Plant materials

The plant samples were collected in Machang, Kelantan in September 2015 which later were identified and its voucher specimens (UKM B40385) was deposited at the Herbarium, Institute of Bioscience, Universiti Putra Malaysia (UPM). The barks were oven-dried at 40 °C and ground into powder form using a grinder and kept in a chiller until further use.

2.2. Extraction

The solvents used for the extraction were analysis grade always, exception to ethanol, which was the commercial grade 96º GL. Activated charcoal (Sigma) was also used to remove chlorophyll, when needed. The extraction of 634.75 g dried barks of *C. iners* was performed using Soxhlet apparatus with ethanol for 72 h and concentrated under reduced pressure to get the crude extract. The obtained crude ethanol extract was then submitted to a liquid-liquid partition using hexane, dichloromethane, ethyl acetate and butanol yielding various partitions of extracts.

2.3. Toxicity testing against the brine shrimp

2.3.1. Hatching shrimp

Brine shrimp eggs, *Artemia salina* leach were hatched in artificial seawater prepared by 3.8 g of sea salt (Sigma) in 1 L of distilled water. The vessel was kept under an inflorescent bulb and facilitated with good aeration for 24h at room temperature (25-27 °C). After hatching, the larvae (nauplii) were attracted to one side of the vessel near the light source and collected using a micropipette. Nauplii were isolated by aliquoting those three times in a beaker containing the seawater.

2.3.2. Brine shrimp assay

The bioactivity of the extracts was monitored by the brine shrimp lethality test (Meyer et al., 1982). Samples were dissolved in dimethyl sulphoxide (DMSO) and diluted with artificial sea salt water to achieve final concentration of DMSO at 1% and extracts at 1 mg/ml. From this stock solution, a series of concentration of extract at 1000 µg/ml, 750 µg/ml, 500 µg/ml, 250 µg/ml, 100 µg/ml and 50 µg/ml was prepared.

Fifty microlitre of sea salt water was placed in all the wells of 24-well cell culture plate in triplicates. DMSO was used as the drug free control while potassium dichromate used as a positive control. Negative test was done and exhibited that 1% of DMSO did not affect the mortality of the shrimps. Extracts at various concentrations were put in the wells, suspensions of nauplii containing about 10 larvae was added and incubated for 48 h. The

eISSN Number: 2462-2389

plates were then examined under a microscope and the number of dead nauplii in each well was counted percentage of mortality using Equation 1 (Abbott, 1987).

$$\text{Mortality}(\%) = \frac{\text{total nauplii-alive nauplii}}{\text{Total nauplii}} \times 100 \quad (1)$$

The percentage of living brine shrimp was also then calculated. Based on the percentage of the mortality, the concentration that led 50% lethality (LC50) to the nauplii was determined by using the graph of mean percentage mortality versus the concentration (Islam et al., 2009).

2.4. Statistical analysis

Data were collected in triplicate and expressed as mean ± standard deviation. Statistical Package for the Social Science (SPSS) version 20 was used to analyse the data. The significance differences within groups were determined by One-way ANOVA and post hoc tests which are Duncan Multiple Range Test and Dunnett-test. Duncan test were performed to determine the differences between partitions while Dunnett t-tests treat potassium dichromate as a control, and compare all other samples against it. These equations were later used to calculate LC_{50} values for the samples tested with consideration of value greater than 1.0 mg/ml (1000 µg/ml), suggesting that the extract is non-toxic (Meyer et al., 1982; Clarkson et al., 2004).

3. RESULTS AND DISCUSSION

The extraction of *C. iners* barks by means of Soxhlet apparatus with ethanol for 72 h yielded 8.84% (56.09 g) of crude ethanol extracts and further partition process yielded 2.8%, 31.6%, 10.8% and 29.8% of hexane, dichloromethane, ethyl acetate and butanol extracts, respectively.

Results of the toxicity against brine shrimp of the extracts are shown in Table 1. The Brine Shrimp Lethality Test (BSLT) was done by counting the number of brine shrimps dead in the 24-well cell culture plates after 48 h.

Table 1: Brine shrimp toxicity of various extracts from *C. iners* barks expressed as LC_{50} value.

Plant extracts	LC_{50} ± S.D. (µg/ml)
Crude ethanol extract	1623.41 [a] ± 0.50
Hexane	1306.79 [a] ± 0.53
Dichloromethane	1988.35 [a] ± 0.54
Ethyl acetate	3370.13 [a] ± 0.55
Butanol	2583.10 [a] ± 0.55
***potassium dichromate (positive control)**	255.50 [b] ± 0.20

In accordance to Meyer et al. (1982) and Clarkson et al. (2004) with respect to brine shrimp lethality test, the criterion of toxicity for plant remedies is as follows; the plant extract showing LC_{50} values greater than 1000 µg/ml (1 mg/ml) are considered non-toxic, LC_{50} values equal/greater than 500 µg/ml (0.5 mg/ml) but not greater than 1000 µg/ml are considered to have weak toxicity while those having LC_{50} values less than 500 µg/ml are considered toxic. The finding form this study showed that all extracts exhibited the 50% lethality more than 1000 µg/ml which considered as non-toxic or very low toxicity after 48 h exposure to the brine shrimp (Table 1).

Among all, hexane extracts (Figure 2) showed the highest toxicity with the LC_{50} value of 1306.79 µg/ml, followed by crude ethanol (Figure 1), dichloromethane (Figure 3), butanol (Figure 5) and ethyl acetate (Figure 4) extracts with the LC_{50} values of 1623.41 µg/ml, 1988.35 µg/ml, 2583.10 µg/ml and 3370.13 µg/ml, respectively. Plotting of lethality percentage versus concentration for the test demonstrates an approximate linear correlation. Furthermore, there is a direct proportional relation between the concentration of the extracts and the degree of lethality. The LC_{50} value for the positive control (data not shown) after 48 h exposure was 255.50 µg/ml and as expected, has shown that it exhibited toxic expression (The LC_{50} value less than 1000 µg/ml) against the brine shrimp. The mean statistical analysis of mean comparison using Duncan Multiple Range Test confirmed that, there were no significant differences between all extracts ($p \leq 0.05$) for the value of LC_{50}.

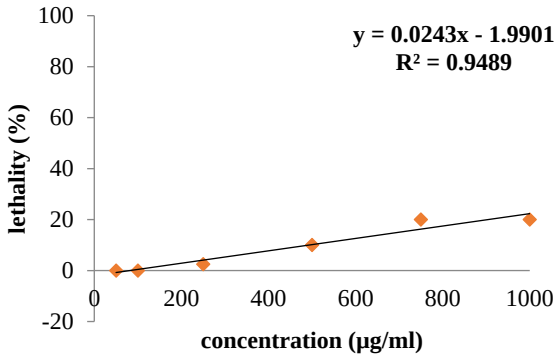

Figure 1: Brine shrimp lethality of *C. iners* crude ethanol extract after 48 h treatment period.

Brine shrimp bioassay is considered as a rapid preliminary screening for the presence of biochemical activity and was used to determine the crude extract's toxicity (Abdul Rani et al., 2010). Eventhough the screening using brine shrimp could result in limited information, the findings could support the therapeutic claims by the local community.

This test is based on the potential of *C. iners* barks extracts to become lethal to *A. salina* nauplii due to its toxic expression. According to Meyer et al. (1982), extracts derived from natural products which have $LC_{50} \leq 1.0$ mg/ml (1000 µg/ml) are known to possess toxic effects. In this study, the plotted graphs show that the LC_{50} values of all five tested extracts were greater than 1000 µg/ml which indicating to very low toxicity effects. The findings also

eISSN Number: 2462-2389

support previous reports on toxicity effects of this plant extracts.

A finding from cytotoxic activity study of *C. iners* extracts showed only weak activity against HUVEC, HL-60 and MCF-7 cells with EC50 values of >100 µg/ml while only 95.08 µg/ml against HL-60 cells (Lin et al., 2007). Later in 2010, Mustafa and co-workers proved that this plant was non-toxic for consumption. The very weak toxicity activity on the brine shrimp could be due to non-toxic compounds or mixture of compounds present in the barks extracts. On the other hand, a report on *C. iners* essential oils showed different toxicity results. Brine shrimp lethality assay on the *C. iners* leaves oils showed very toxic to the brine shrimp with LC50 value of 5.1 µg/ml (Jantan, Ali and Goh, 1994).

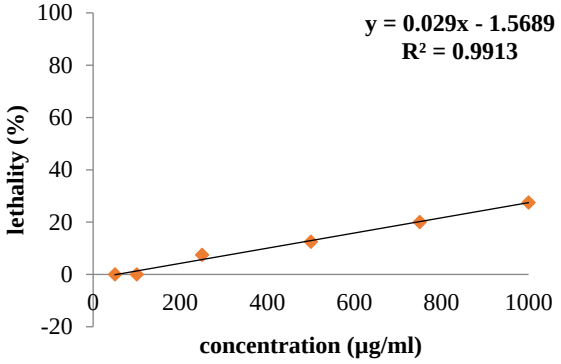

Figure 2: Brine shrimp lethality of *C. iners* hexane extracts after 48 h treatment period.

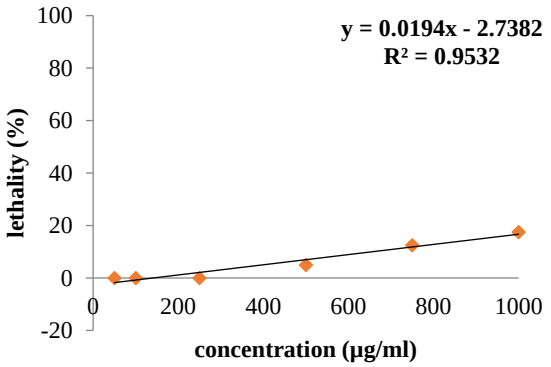

Figure 3: Brine shrimp lethality of *C. iners* dichloromethane extracts after 48 h treatment period.

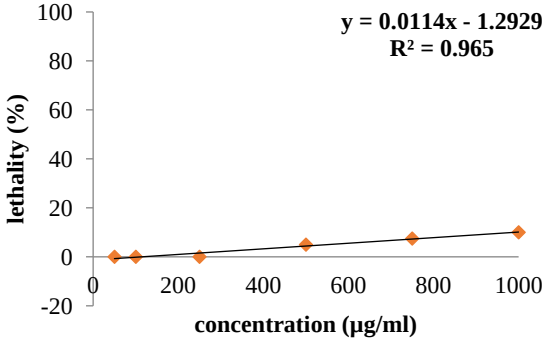

Figure 4: Brine shrimp lethality of *C. iners* ethyl acetate extracts after 48 h treatment period.

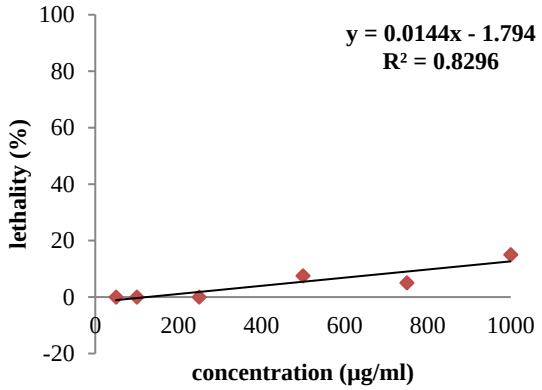

Figure 5: Brine shrimp lethality of *C. iners* butanol extract after 48 h treatment period.

Furthermore, by comparing with the previous phytochemical data, the presence of major compounds in this plant extract which identified as β-caryophyllene, linalool, β-sitosterol and cinnamaldehyde were also reported as safe. Oliveira et al. (2018) reported that β-caryophyllene only showed toxicity at doses higher than 2000 mg/ kg body weight of mice which corroborate to its use as a food additive and has broad pharmacological potential. Linalool also identified as not a developmental toxicant in rats at maternal doses of up to 1000 mg/ kg/ day (Politano et al., 2008). In addition, a chronic administration of β-sitosterol subcutaneously to rats for 60 days showed no clear cut evidence of any gross or microscopic lesions either in the liver or kidney (Malini and Vanithakumari, 1990). Besides, Hebert et al. (1994) also found that there were no deaths in animals receiving microencapsulated of cinnamaldehyde, even though the rats and mice were administered at doses of 0–3000 mg cinnamaldehyde/ kg body weight and 0–10,000 mg cinnamaldehyde/ kg body weight, respectively.

The barks extracts could be further explored for the development of natural-based products such as natural insect repellent as well as pharmaceutical products.

4. CONCLUSION

All barks extracts showed no toxic towards the brine shrimp which could suggest that this plant can be an alternative especially (in known dosage) particularly in rural area where the modern drugs are unavailable, unaffordable or the health facilities are inaccessible. Further investigation of these extracts should be persued by using *in vivo* as well as *in vitro* cytotoxicity methods to clarify the claims made by community locally as well as for the development of plant-based pharmaceutical drugs.

ACKNOWLEDGEMENT

The authors are grateful to Dr. Shamsul Khamis (UPM) for assisting in the identification and verification of plant materials.

eISSN Number: 2462-2389

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eISSN Number: 2462-2389