Brine Shrimp Lethality Activity of Thai Medicinal Plants in the Family Meliaceae

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Abstract

Brine shrimp larvae have been used as a bioassay for a variety of toxic substances. The method has also been applied to plant extracts in order to facilitate the isolation of biologically active compounds. In this study, the plants in the family Meliaceae have been selected to test for brine shrimp lethality activity based on taxonomic approach including *Azedirachta indica, Azedirachta indica* var. *siamensis, Melia azedarach, Sandoricum indicum* and *Swietenia macrophylla.* The stem bark and leaf were separately collected and investigated for their activities. All of the stem bark extracts, except *S. macrophylla*, showed significant toxicity whereas all of the leaf extracts were inactive. The stem bark of *Annona squamosa*, which exhibited brine shrimp toxicity, has been used as positive control and its leaf and seed were also examined. The result showed that the leaf and seed of this plant exhibited higher potency than the stem bark.

Keywords : brine shrimp lethality activity, Meliaceae

Introduction

A general bioassay that appears capable of detecting a board spectrum of bioactivity present in crude extracts is the brine shrimp lethality bioassay (BSLT). The technique is easily mastered, costs little, and utilizes small amount of test material. The aim of this method is to provide a front-line screen that can be backed up by more specific and more expensive bioassays once the active compounds have been isolated. It appears that BSLT is predictive of cytotoxicity and pesticidal activity (Ghisalberti, 1993). Since its introduction in 1982 (Meyer et al., 1982), this *in vivo* lethality test has been successively employed for bioassay-guide fractionation of active cytotoxic and antitumor agents such as trilobacin from the bark of *Asimina triloba* (Zhao et al., 1992), *cis*-annonacin from *Annona muricata* (Rieser et al., 1996) and ent-kaur-16-en-19-oic acid from *Elaeoselinum foetidum* (Mongelli et al., 2002).

According to the distribution of cytotoxic agents in various plants, five members of Thai medicinal plants in the Family Meliaceae were selected based on toxonomic approach. The samples composed of *Azedirachta indica, Azedirachta indica* var. *siamensis*, *Melia azedarach, Sandoricum indicum* and *Swietenia macrophylla* (Cordell et al., 1993). Among these, only the root of *Melia azedarach* (Fukuyama et al., 2000) has ever been evaluated by BSLT. In this study, two parts of plants including the stem bark and leaf were investigated. In addition, the stem

bark, leaf and seeds of *A. squamosa* were also tested and its stem bark was used as positive control (Hopp et al., 1998).

Materials and Methods

Plant materials

The plant materials, stem bark and leaf, were collected at Naresuan University, Phitsanulok, Thailand. Except for the stem bark, leaf and seeds of *A. squamosa* were collected in Kampangpech, Thailand. All of the samples were collected in June 2002. The plant specimens were identified by comparison with the herbarium specimens at Department of Pharmaceutical Chemistry and Pharmacognosy, Faculty of Pharmaceutical Sciences, Naresuan University, Phitsanulok. The voucher specimen of these plants have been deposited at Medicinal Plants Information Center, Faculty of Pharmaceutical Sciences, Naresuan University, Phitsanulok.

Extraction

The 200 grams of fresh plant materials were cut or milled into small pieces and macerated in methanol for three days on the shaker and filtered. The marc was macerated in methanol repeatedly for three days on the shaker. The filtrate were then pooled and concentrated under reduced pressure. The small amounts of crude extracts were kept as references and for BSLT. The remaining portion of the active extracts were partitioned with water and dichloromethane (1:1). The aqueous layer were dried by using vacuum freeze dryer. The organic layer were further partitioned with hexane and 90% methanol. Both layers were evaporated under reduced pressure until dryness. All extracts were kept in the freezer until testing.

Hatching the brine shrimp

Brine shrimp eggs (*Artemia salina*, Sanders™ Great Salt Lake, Brine Shrimp Company L.C., U.S.A.) were hatched in artificial sea water prepared from commercial sea salt (Aqua Marine, Thailand) 40 g/1 and supplemented with 6 mg/1 dried yeast. The two unequal compartments plastic chamber with several holes on the divider was used for hatching. The eggs were sprinkled into the larger compartment which was darken, while the smaller compartment was illuminated. After 48 hours incubation at room temperature (25-29°C), nauplii (larvae) were collected by pipette from the lighted side whereas their shells were left in another side.

Bioassay

The procedure for BSLT was modified from the assay described by Solis et al. (1993). Ten milligrams of the extracts were made up to 2 mg/ml in artificial sea water except for water insoluble compounds which were dissolved in DMSO 50 μl prior to adding sea water.

Serial dilutions were made in the wells of 96-well microplates (Nunc, Denmark) in triplicate in 120 μl sea water. Control wells with DMSO were included in each experiment. A suspension of nauplii containing 10-15 organisms (100 μl) was added to each well. The plates were covered and incubated at room temperature (25-29°C) for 24 hours. Plates were then examined under the binocular steromicroscope and the numbers of dead (non-motile) nauplii in each well were counted. One hundred microlitrs of methanol were then added to each well to immobilize the nauplii and after 15 minutes the total numbers of brine shrimp in each well were counted. Analysis of the data was performed by probit analysis on a Finney computer program to determine the lethal concentration to half of the test organisms (LC_{50}).

Results and Discussion

Brine shrimp lethality activity of the plant extracts of Meliaceae family members and *A. squamosa* were shown in Tables 1 and 2, respectively. Crude extracts resulting in LC_{50} values of less than 250 μg/ml were considered significantly active and had the potential for futher investigation (Rieser et al., 1996). They were the extracts of stem bark of *Azedirachta indica, Azedirachta indica* var. *siamensis, Melia azedarach* and *Sandoricum indicum.* These extracts were further fractionated by partition method.

Table 1 Brine shrimp bioassay results of plant extracts of Meliaceae

Plant	Part use	Fraction	LC_{50}(μg/ml)
Azedirachta indica	Stem bark	Crude extract	158.18
		Hexane fraction	>1000
		90% methanol fraction	181.54
		Water fraction	>1000
	Leaf	Crude extract	>1000
Azedirachta indica var. *siamensis*	Stem bark	Crude extract	133.28
		Hexane fraction	741.74
		90% methanol fraction	29.99
		Water fraction	729.16
	Leaf	Crude extract	>1000
Melia azedarach	Stem bark	Crude extract	8.63
		Hexane fraction	11.99
		90% methanol fraction	3.27
		Water fraction	61.73
	Leaf	Crude extract	>1000
Sandoricum indicum	Stem bark	Crude extract	234.08
		Hexane fraction	48.89
		90% methanol fraction	56.03
		Water fraction	796.99
	Leaf	Crude extract	724.28
Swietenia macrophylla	Stem bark	Crude extract	>1000
		Hexane fraction	nt
		90% methanol fraction	nt
		Water fraction	nt
	Leaf	Crude extract	704.83

Note: nt = not tested

Table 2 Brine shrimp bioassay results of *A. squamosa* extracts compared with previously reported data[a]

Plant	Part use	Fraction	LC_{50} (μg/ml)
A. squamosa	Stem bark	Crude extract	6.53
	Leaf	Crude extract	1.49
		Hexane fraction	65.63
		90% methanol fraction	0.63
		Water fraction	>1000
	Seed	Crude extract	0.15
		Hexane fraction	4.09
		90% methanol fraction	0.10
		Water fraction	3.71
A. squamosa[a]	Stem bark	Crude extract	1.55

Note: a data obtained from Hopp et al. (1998).

The results revealed that the active principles were mainly distributed in 90% methanol fraction, except for *S. indicum* which the hexane portion was the most active fraction. This result indicated that partition method was an advantage procedure to eliminate a large amount of inactive fractions which, in turn, reduced cost and time to find out the active compounds (Table 3).

Table 3 The percentage yield of fractions obtained from partitioning

Plant	Part use	Partitioning solvent	%
Azedirachta indica	Stem bark	Hexane	3.6
		90% methanol	17.6
		Water	78.8
Azedirachta indica var. *siamensis*	Stem bark	Hexane	11.4
		90% methanol	27.0
		Water	61.6
Melia azedarach	Stem bark	Hexane	13.4
		90% methanol	21.1
		Water	65.5
Sandoricum indicum	Stem bark	Hexane	11.7
		90% methanol	17.0
		Water	71.3
Annona squamosa	Leaf	Hexane	40.5
		90% methanol	28.5
		Water	31.0
	Seed	Hexane	10.2
		90% methanol	43.4
		Water	46.4

Among five Meliaceae plants, the most active extract was the 90% methanol fraction of stem bark of *Melia azedarach*. This fraction has a potential to be a candidate for the investigation of cytotoxic compounds even though such activity against lymphocytic leukemia P388 cell lines have already been confirmed from the root bark of this plant (Itokawa et al., 1995; Takeya et al., 1996). Although several potent cytotoxic compounds have been isolated from the stem bark of *Annona squamosa* (Li et al., 1990; Hopp et al., 1996; Hopp et al., 1997), its leaf and seed have never been tested. This study found that these parts exhibited even higher potency than the stem bark. Therefore, further isolation of the highly active fractions (the leaf and seed of *A. squamosa*, and the stem bark of *M. azedarach*) may lead to the discovery of new cytotoxic compounds. Besides cytotoxic activity, these fractions should also be evaluated for the pesticide activity.

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