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Effect of Polysaccharides of *Centella asiatica* and *Aegle marmalos* on the life span of *Artemia salina*

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Abstract

Centella asiatica (gotukola) and fruits of *Aegle marmalos* (Beli) are widely consumed in Sri Lanka as a vegetable, a fruit respectively. These two plants are used in ayurveda and indegenous medicinal system in Sri Lanka. Polysaccharides were isolated from the whole plants of *C.asiatica* and ripe fruits of *A. Marmalos.* The polysaccharides were purified using a polyamide column. The poysaccharides thus obtained were tested for the their bioactivity by the convenient and inexpensive brine shrimp method.

The polysaccharides showed the ability to enhance the life span of *A.salina,* independently of any nutritional effect that they may have.

Key Words : *C.asiatica, A.marmalos,* polysaccharide, bioactivity, brine shrimp, a.salina

1 Introduction

Many new natural compounds are isolated and characterized today without any information about their biological activity. There is a real need for reliable general convenient bioassays which can detect the pharmacological activities of higher plant constituents so that these methods could be employed by natural product chemists at low cost to guide phytochemical screening and fractionation. As most active plant principles are toxic at higher levels, a possible approach to develop an effective general bioassay might be simply to screen for substances that are toxic to zoological systems.

The method using *A.salina,* the brine shrimp, is a simple bioassay system that could be utilized by pharmacognostic and natural product chemists in the detection and isolation of higher plant constituents with varied pharmacological activities. The brine shrimp method could be applied to test for anaesthetics, insecticides and antibiotics.[1] A research group at Purdie University used *A.salina* to detect the activity of antileukemic principles from plant extracts[2]. Some others have used this method as a screening system for toxic fungi and to investigate their sensitivity to some known mycotoxins[3]. The chemical determination of aflatoxins and other mycotoxins have been confirmed by this bioassay.

A biological test to verify activity should be rapid and should not require expensive equipment or highly trained personnel. *A. salina* in a bioassay of this kind has several distinct advnatages. This method is relatively simple and could be carried out in most of the laboratories. The relative simplicity of the nervous system of the brine shrimp makes them particulary suitable for investigations on active compounds[4].

A.Salina is a primitive Crustacean found in lakes where saline content is almost high as saturation. In Sri lanka brine shrimps are normally found in lagoons. *A.salina* does not have any food competitors in high salinity where they normally live. They are bisexual. The unique feature which makes the brine shrimp valuable as a test organism is the viability of the eggs under adverse conditions. Some of the dried eggs remain viable on freezing conditions and some even survive in boiling water for a short time.

A. salina has another advantage of being readily available and inexpensive. The time scale is quite small (eggs hatch within 24 hours) and simple. The method requires neither special equipment nor special training. The environment of the larvae from hatch until the end of the experiment could be easily controlled[5]. The medicinal plants selected for the studies were *C.asiatica* (Gotukola) and *A.marmalos* (Beli) used by Sri Lankans as a vegetable and fruit respectively. These plants are also used in traditional and ayurvedic medicinal systems in Sri Lanka. The aqueous extracts of these plants were shown to be immunoactive in classial and alternative pathways of human complement[6,7].

The polysaccharides of *C.asiatica* and *A.marmalos* were shown to be immunoactive during earlied studies[7].

2. Materials and method

The plant materials, *C.asiatica* whole plant and *A.marmalos* ripe fruits were collected from Kottawa area. *C.asiatica* (whole plant, 100g.) And *A.marmalos* (ripe fruit,100g. Dry weight) were dried and grond using a cross beated mill and blender respectively.

Scheme 1.

Water soluble crude polysacharide were isolated from the methanol insoluble residue (Scheme 1)

Purification of polysaccharides

The water soluble lyophylized crude polysaccharide 1 g. was re-dissolved in of water (5 ml.) and fractionated using a polyamide column (38x2.8 ID cm)

The column was first eluted with water followed by 30% methanol in water (v/v), 60% methanol in water (v/v) 60% methanol in water (v/v) and ultimately 5% ammonia in methanol (v/v) Fractions of 15 ml. were collected. Purification of polysaccharides is given in scheme 2. Fractions collected were tested for polysaccharides and phenol using the concentrated sulphuric and acid and Ferric chloride reagent respectively.

Scheme II

Hatching the shrimp

Brine shrimp eggs were hatched in a large, partially illuminated petri dish filled with sea water. The eggs were sprinkled into darkened part of the the dish. After 48 hours the phototrophic naupli larvae were collected from the illuminated side, using a pipette. (Fig.1)

Bio-assay

Stock solutions were prepared by dissoving the following weights of polysaccharides in appropriate volume of sea water. Each petri dish contained 5 ml. of the prepared soution. Five replicates were made for each concentration.

Concentration (Mg.ml)	No.of replicates	Total requirements (mg)
2000	5	50000
1500	5	37500
1000	5	25000
500	5	12500

The bioassay was carried out in a petri dish. I mg. from the stock solutions of the extracts to be tested and 1 ml. of yeast solution (red brand dry yeast 3 mg.in 1 ml.of brine) were mixed in the petri dish and the volume was brought upto 4 ml.by addition of brine. Ten larvae were added using a pipette and the total volume was made upto 5 ml. with brine.Each petridish contained 10 larvae and a single experiment consisted of 15 replicates. The dishes were covered and allowed to stand at room temperature and survivals were counted every 24 hours for 10 days. A series of experiments were designed to test for the bioassay of *A.Salina.*

These are shown in table 3-6.

Nutrient Broth

This Nutrient broth make is DIFCO

3. Results and Discussion

According to Scheme 1 the polysaccharides were separated from the fruits *A.marmalos.* The aqueous fraction from Scheme II gave pure polysaccharide without any phenolics. The other fractions contained traces of polyphenolics. (Table I)

Same schemes were followed for *C.asiatica.* From 100g of dry plant material 0.93 gm of crude polysaccharide was obtained. (Scheme 1) Results of scheme 2 is shown in table II.

Our earlier workers have shown that 3 mg. of yeast is sufficient as a nutrient for *A.salina* to ensure its maximun life span. However, in this series of experiments, one experiment was designed to check the difference it would make by increasing the amount of added yeast. Results from this experiment (table III) shows that added yeast has not being effective to *increase the life span of A.Salina.* This experiment shows that 3 mg.of yeast would be sufficient to for its maximum life span.

The results in table IV show that the polysaccharide added to the media has undoubtedly extended the life span of *A.salina.* The results show that 2000 mg. of polysaccharide would be the optimum amount that ensures the maximum life span of *A.salina.*

The next set of experiments was designed by substituting yeast with a source of nitrogen in the form of a nutrient broth. The life span of *A.salina* was reduced which indicated that nutrient broth could not supply nutrition for *A.salina.* The polysaccharide enhances life span independently of any

nutrition effects they may have. The same experiments were repeated for *C.asiatica* polysaccharide and the results are shown in tables VII,VIII and XI. The results of *C.asiatica* show the same effect on brine shrimp as far as the bio activity is concerned. The *A.salina* bioassay described here could be a general screen for bioactive compounds.

Table I: Percentage composition of polysaccharides and phenolics in fractions obtained from *marmalos* by scheme 2.0

FRACTION	PURE POLYSACCHARIDES	POLYSACCHARIDES + PHENOLICS	PHENOLICS
F 1	72.5%	-	TRACES
F 2	-	21.1%	TRACES
F 3	-	3.5%	TRACES
F 4	-	-	1.2%

Table 2.0 Percentage composition of polysaccharides and phenolics in fractions obtained from *Centella asiatica* by scheme 2.

FRACTION	PURE POLYSACCHARIDES	POLYSACCHARIDES + PHENOLICS	PHENOLICS
F 1	72.5%	-	TRACES
F 2	-	21.1%	TRACES
F 3	-	3.5%	TRACES
F 4	-	-	1.2%

Table 3.0 - *Artemia salina* with yeast as a food source

Surveval period (hours)

Treatment	24	48	72	96	120	144	168	192	216	240
1	9	1	-	-	-	-	-	-	-	-
2	10	10	9	8	4	2	2	2	1	-
3	10	9	8	7	4	3	2	2	-	-
4	10	10	7	3	2	2	2	1	-	-
5	10	9	5	5	5	4	3	2	-	-
6	10	10	7	8	3	3	2	-	-	-

1 5ml of sea water + 10 larvae

2 5ml of sea water + 3 mg of yeast + 10 larvae

3 5ml of sea water + 6 mg of yeast + 10 larvae

4 5ml of sea water + 3 mg of yeast + 10 larvae + added extra 3 mg of yeast after one day

5 5ml of sea water + 3 mg of yeast + 10 larvae + added extra 3 mg of yeast after two days

6 5ml of sea water + 3 mg of yeast + 10 larvae + added extra 3 mg of yeast after three days

Table 4 - Survival in Aegle Marmalos Polysaccharide with yeast as a food source (3 mg of yeast in 1 ml suspension)

Surviving period (hours)

Treatment	24	48	72	96	120	144	168	192	216	240
1	10	10	9	7	4	2	-	-	-	-
2	10	10	8	7	5	5	4	4	4	3
3	10	10	9	8	5	4	4	4	4	4
4	10	10	9	9	8	8	7	7	7	6
5	10	10	10	10	9	9	7	7	7	7

1 5ml of sea water + 10 larvae

2 5ml of sea water + 10 larvae + 3mg of yeast + 500 ug polysaccharide + 10 larvae

3 5ml of sea water + 10 larvae + 3mg of yeast + 1000 ug polysaccharide + 10 larvae

4 5ml of sea water + 10 larvae + 3mg of yeast + 1500 ug polysaccharide + 10 larvae

5 5ml of sea water + 10 larvae + 3mg of yeast + 2000 ug polysaccharide + 10 larvae

6 5ml of sea water + 10 larvae + 3mg of yeast + 2500 ug polysaccharide + 10 larvae

Survival in A.marmalos polysaccharides only.

Table 5: Survival in *Aegle Marmalos* Polysaccharide extract only

Surviving Period (houes)

Treatment	24	48	72	96	120	144	168	192	216	240
1	10	10	9	7	-	2	2	-	-	-
2	10	8	6	2	-	-	-	-	-	-
3	10	1	5	1	-	-	-	-	-	-
4	10	7	5	1	-	-	-	-	-	-
5	10	7	4	1	-	-	-	-	-	-
6	10	7	4	2	-	-	-	-	-	-

1 5 ml of sea water + 10 larvae

2 5 ml of sea water + 500 ug Polysaccharide + 10 larvae

3 5 ml of sea water + 1000 ug Polysaccharide + 10 larvae

4 5 ml of sea water + 1500 ug Polysaccharide + 10 larvae

5 5 ml of sea water + 2000 ug Polysaccharide + 10 larvae

6 5 ml of sea water + 2500 ug Polysaccharide + 10 larvae

Table VI. Survival in *Aegle Maralos* polysaccharide with 50 nutrient broth

Surviving Period (hours)

Treatment	24	48	72	92	120	144	168	192	216	240
1	10	10	9	8	4	2	2	1	-	-
2	10	9	8	3	-	-	-	-	-	-
3	10	8	7	2	-	-	-	-	-	-
4	10	8	6	1	-	-	-	-	-	-
5	10	8	6	2	-	-	-	-	-	-
6	10	8	6	2	-	-	-	-	-	-

1 5ml of sea water + 10 larvae

2 5ml of sea water + 50 of nutrient broth + 500 ug Polysaccharide + 10 larvae

3 5ml of sea water + 50 of nutrient broth + 1000 ug Polysaccharide + 10 larvae

4 5ml of sea water + 50 of nutrient broth + 1500 ug polysaccharide + 10 larvae

5 5ml of aea water + 50 of nutrient broth + 2000 ug polysaccharide + 10 larvae

6 5ml of ses water + 50 of nutrient broth + 2500 uh polysaccharide + 10 larvae

Table VII. Survivals in Centella Asiatica polysaccharide with food surce yeast (3 mg of YEAST IN 5 mg suspension)

Surviving Perod (hours)

Treatment	24	48	72	96	120	144	168	192	216	240
1	10	10	9	7	3	2	2	-	-	-
2	10	10	9	8	6	5	5	5	5	5
3	10	10	9	8	5	5	5	5	5	5
4	10	10	9	9	9	8	7	7	7	7
5	10	10	10	10	10	9	7	7	7	7
6	10	10	10	10	9	9	9	8	8	7

1 5 ml of aea water + 10 larvae

2 3 ml of yeast + 5 ml of sea water + 500 ug Polysaccharide + 10 larvae

3 3 mg of yeast + 5 ml of sea water + 1000 ug Polysaccharide + 10 larvae

4 3 mg of yeast + 5 ml of sea water + 1500 ug Polysaccharide + 10 larvae

5 3 mg of yeast + 5 ml of sea water + 2000 ug Polysaccharide + 10 larvae

6 3 mg of yeast + 5 ml of sea water + 2500 ug Polysaccharide + 10 larvae

Table VIII. Suevival in Centells Asiatica polysaccharide (extract only)

Surviving Period (hours)

Treatment	24	48	72	96	120	144	168	192	216	240
1	10	10	9	8	4	2	-	-	-	-
2	10	9	7	3	-	-	-	-	-	-
3	10	8	6	1	-	-	-	-	-	-
4	10	8	5	-	-	-	-	-	-	-
5	10	8	5	-	-	-	-	-	-	-
6	10	8	5	-	-	-	-	-	-	-

1 5 ml of sea water + 10 larvae

2 5 ml of sea water + 500 ug of Polysaccharide + 10 larvae

3 5 ml of sea water + 1000 ug of Polysaccharide + 10 larvae

4 5 ml of sea water + 1500 ug of Polysaccharide + 10 larvae

5 5 ml of sea water + 2000 ug of Polysaccharide + 10 larvae

6 5 ml of sea water + 2500 ug of Polysaccharide + 10 larvae

Table XI. Survival in Centella Asiatica polysaccharide with nutrient broth

	Surviving Period (hrs)									
Treatment	**24**	**48**	**72**	**96**	**120**	**144**	**168**	**192**	**216**	**240**
1	10	10	9	8	4	2	2	1	1	-
2	10	9	8	2	-	-	-	-	-	-
3	10	8	7	2	-	-	-	-	-	-
4	10	8	6	1	-	-	-	-	-	-
5	10	8	6	1	-	-	-	-	-	-
6	10	8	5	1	-	-	-	-	-	-

1 5 ml of sea water + 10 larvae

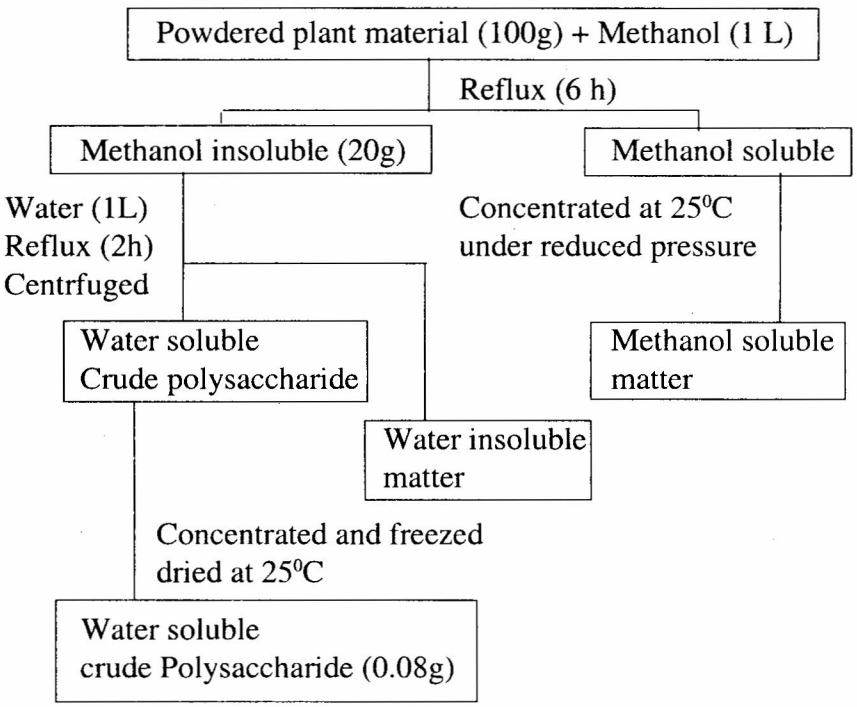
2 5 ml of sea water + 50 of nutrient broth + 500 ug of Polysaccharide + 10 larvae

3 5 ml of sea water + 50 of nutrient broth + 1000 ug of Polysaccharide + 10 larvae

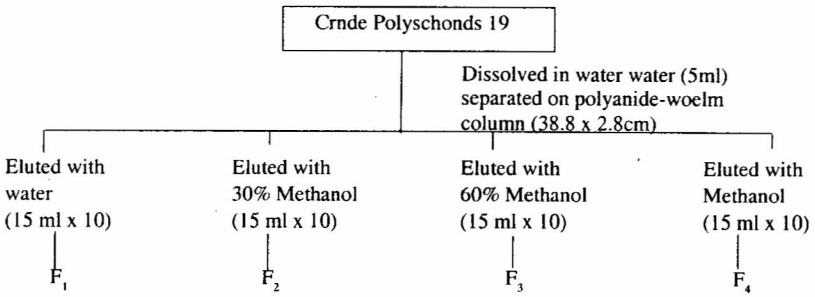
4 5 ml of sea water + 50 of nutrient broth + 1500 ug of Polysaccharide + 10 larvae

5 5 ml of sea water + 50 of nutrient broth + 2000 ug of Polysaccharide + 10 larvae

6 5 ml of sea water + 50 of nutrient broth + 2000 ug of Polysaccharide + 10 larvae



Scheme 1-Isolation of crude polysaccharide from *C. asiatica*



Scheme II : Purification of Crude Polysaccharide

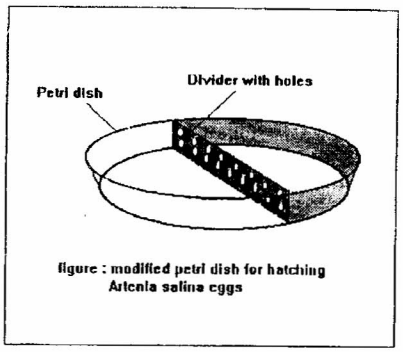


Fig 1. Masited petridish for liatliag of *A salina* eggs

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