ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Antimicrobial activity and toxicity of methanol extract of *Cassia fistula* seeds

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ABSTRACT

The methanol extract of *Cassia fistula* seeds was investigated for potential antimicrobial activity against medically important bacterial, yeast and fungal strains. The antimicrobial activity was determined in methanol extract using the disk diffusion technique and the broth dilution method. The extract was effective on tested microorganism and the minimum inhibitory concentration (MIC) values were found in the range of 1.563- 50.00 mg/ml. Apart from that, the methanolic extract of *C. fistula* seeds was further tested for *in vivo* brine shrimp lethality test. The brine shrimp lethality test exhibited no significant toxicity (LC_{50} = 2.11mg/ml) against *Artemia salina*. The *C. fistula* seeds extract with high LC_{50} value signified that this plant is not toxic to human. Hence, this plant can be used to discover bioactive natural products that may leads in the development of new pharmaceuticals that address not fulfilled therapeutic needs.

Keywords: Antimicrobial activities, *Candida albicans*, *Cassia fistula* seeds, Toxicity study, *Artemia salina.*

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ISSN: 0975-8585

INTRODUCTION

Infectious disease caused by bacteria, viruses, fungi and parasites are still a major threat to public health, despite the tremendous progress in human medicine [1]. The past three decades have seen a dramatic increase in microbial resistance to antimicrobial agents [2]. Such situation stimulates the development of new anti-microbial agents in order to treat the infectious disease in an effective manner. So this matter continued to an era to identify the potential antimicrobial agent from the natural resources. The edible plants that used for traditional medicine contain a wide range of substance that can be used to treat abundant of infectious disease with reduced side effects [3]. In Asia, the use of medicinal plants to cure specific illness has been use for many years [4]. Furthermore, Malaysia is rich in various edible plants with diverse biological and pharmacological properties [5].

Basically, medicinal plants reflect the recognition of the validity of many traditional claims regarding the value of natural products in healthcare [6]. *Cassia fistula* L., (Leguminosae), a semi-wild Indian Labernum (also known as the Golden Shower), is distributed in various countries including Asia, South Africa, Mexico, China, West Indies, East Africa and Brazil. It is an ornamental tree with beautiful bunches of yellow flowers [7]. This plant is widely used by tribal people to treat various ailments including ringworm and other fungal skin infections [8]. *C. fistula* exhibited significant antimicrobial activity and showed properties that support folkloric use in the treatment of some diseases as broad-spectrum antimicrobial agents [9]. *C. fistula* plant parts are known to be an important source of secondary metabolites, notably phenolic compounds.

There is a still not sufficient study about *C. fistula* and there must be a research focused on achieving the definitive knowledge on this plant and utilization as antimicrobial agent. The present study was aimed to screen the methanol extract of *C. fistula* seeds against several pathogenic bacteria and fungi in order to detect new sources of antimicrobial agents and also to report the toxicity of the extract using *Brine Shrimp* assay. Toxicity studies also play an important role in identification and isolation of new compounds from crude extracts [10]. The toxicity activity of brine shrimp (*Artemia salina*) assay was developed by Michael et al. (1956) and adapted by others [12, 13]. It is a convenient preliminary toxicity test, since the brine shrimp is highly sensitive to a variety of chemical substances. The assay is considered a useful tool for preliminary toxicity assessment of plant extract [13, 14].

EXPERIMENTAL

Plant collection

The pods of *C. fistula* were collected from various areas in Universiti Sains Malaysia, Penang in November 2009 and authenticated by the botanist of the School of Biological Sciences at Universiti Sains Malaysia where the herbarium was deposited. The sun-dried pods break and the seeds are separated. Then the seeds were washed under running tap water and

dried in oven at 50°C. Then the dried seeds homogenized to fine powder and stored in airtight bottles.

Solvent extraction

One hundred fifty grams (150 g) of dried powder was extracted with 400 ml of 80% methanol (v/v) for one week. Then it was filtrated through filter paper and the entire extract of *C. fistula* seeds was evaporated under reduced pressure using rotary evaporator. The extract then evaporated at 50°C in oven to get a paste form. Then it was sealed in Petri plate and stored at RT for further studies. The filtrate was then re-dissolved again in 80% methanol (v/v).

Test Microorganism and growth media

The following Gram-positive and Gram-negative bacteria, yeasts and molds were used for antimicrobial activities studies: bacteria *included Staphylococcus aureus, Bacillus thuringiensis, Escherichia coli, Salmonella, Micrococcus sp.* and *Bacillus subtilis;* yeast included *Candida albicans;* molds included *Aspergillus niger*. The bacterial strains were grown in Nutrient Agar (NA) plates at 37°C, whereas the yeast and molds were grown in Sabouraud Dextrose Agar (SDA) media respectively, at 30°C. Then stock cultures were maintained at 4°C.

Antimicrobial disk diffusion assay

The extract was tested for antibacterial and antifungal activities by the disk diffusion method according to the National Committee for Clinical laboratory standards (NCCLS, 2001). Nutient Agar (NA) and Sabouraud dextrose agar (SDA) sterilized in a flask and cooled to 45–50°C were distributed to sterilized Petri dishes with a diameter of 9 cm (15 ml). Then NA plates, containing an inoculums size of 10^6 colony-forming units (CFU)/ml of bacteria or 2 x 10^5 CFU/ml yeast cells or molds spores on SDA plates respectively, were spread on the solid plates with the inoculating loop. The filter paper discs (6 mm in diameter) were individually impregnated with 25µl of extract at concentration of 100 mg/ml was placed on the agar plates previously inoculated with test Microorganisms. Similarly, each plate carried a blank disc by adding methanol solvent alone in the center to serve as a negative control and antibiotic discs (6mm in diameter) of 30 µg/ml of chloramphenicol (for bacteria), and 30 µg/ml of Miconazole nitrate (for fungi) was used as a positive controls. All the plate was incubated at 37°C for 24 h for bacteria and 30°C for 48 h for fungi. The diameters of the inhibition zones were measured in millimeters. The sensitivity of the microorganisms to plant extract was determined by measuring the size of inhibitory zones on the agar surface around the discs. All the tests were performed in triplicate.

Determination of Minimum Inhibitory Concentration (MIC)

A 16 h culture was diluted in 10 ml of distilled water with reference to the 0.5 McFarland standards to achieve inocula of approximately 10^6 colony forming units. A serial

dilution was carried out to give final concentration between 0.10-100.00 mg crude extract per ml. The tubes were inoculated with 20µl of yeast suspension per ml of Sabouraud dextrose broth, homogenized and incubated at 30°C. Then after incubation 50 µl was withdrawn from each tube, inoculated on agar plates and incubated at 30°C for 24 h. The MIC value was determined at the lowest concentration of crude extract in the broth medium that inhibit the growth of the test microorganism.

Toxicity testing against the brine shrimp

Hatching shrimp

Brine shrimp eggs, *Artemia salina* were hatched in artificial seawater prepared by dissolving 38g of sea salt in 1L of distilled water. After 24-h incubation at room temperature (22°C - 29°C), the larvae were attracted to one side of the vessel with a light source and collected with pipette. Larvae were separated from eggs by aliquoting them three times in small beakers containing seawater.

Brine shrimp assay

Bioactivity of the extract was monitored by the brine shrimp lethality test [12]. Two milliliter of seawater was placed in all the bijoux bottles. A two-fold dilution of methanol extract carried out with the artificial seawater to obtain the concentration from 50 mg/ml to 0.098 mg/ml. The Potassium dichromate used as positive control and was prepared by dissolving in artificial seawater to obtain the concentration from 0.1 to 0.9 mg/ml [15]. The last bottle was filled with sea salt water only serving as a drug-free control or negative control. Hundred micro liters of suspension of larvae containing about 10 -15 larvae was added into each bottle and incubated for 24 h. The bottles were then examined and the number of dead shrimp in each bottle was counted. The total number of shrimp in each bottle was counted and recorded. The mean percentage mortality was plotted against the logarithm of concentrations. The concentration at which could kill 50% larvae (LC_{50}) was determined from the graph [16, 17].

Data analysis

The mean results of brine shrimp mortality against the logarithms of concentrations were plotted using the Microsoft Excel computer program, which also presents regression equations [18]. The regression equations were used to calculate the LC_{50} value. Extracts giving LC_{50} values greater than 1.0 mg/ml were considered to be nontoxic [19].

RESULTS AND DISCUSSION

The disk diffusion assay result of *C. fistula* seed extract with the inhibition zones formed by standard antibiotic disks, showed in Table 1. The extract had great *in vitro* potential of antimicrobial activities against all the tested bacterial strains. Maximum activity was observed

ISSN: 0975-8585

against *Micrococcus sp* (29 mm), followed by *Salmonella sp.* (25 mm), *Bacillus subtilis* (24mm), *Bacillus thuringienesis* (22 mm), *Staphylococcus aureus* (18 mm) and *Escherichia coli* exhibited weak inhibition zones (16 mm). The antimicrobial activity of this extract was also observed on the yeast *Candida albican* (21 mm) but it fails to inhibit the growth of filamentous fungi *Aspergillus niger*. In contrast, the inhibition zone of solvent control methanol (negative control) was zero so that it was not active against all of the tested microorganisms. However, the 2 antibiotics 30 µg/ml of chloramphenicols and Miconazole nitrate (positive control) were more effective than the seeds extract of *C. fistula* with the diameter of zone inhibition ranging between 28 and 31 mm.

Table1: Antimicrobial activity (zone of inhibition and MIC) of Cassia fistula seed extract compared with commercial antibiotics

Microorganisms	zone of inhibition (mm)[a]			MIC (mg/ml) (mm)[b] of the extract
	Extract	Chloramphenicol	Micanozole nitrate	Extract
Bacteria				
Staphylococcus aureus	18	28	ND	12.500
Bacillus thuringienesis	22	31	ND	12.500
Escherichia coli	16	30	ND	50.000
Salmonella sp.	25	29	ND	3.125
Micrococcus sp.	29	32	ND	1.563
Bacillus subtilis	24	30	ND	3.125
yeast				
Candida albican	21	ND	22	6.250
Fungi				
Aspergillus niger	-	ND	21	-

[a] The values (average of triplicate) are diameter of zone of inhibition at 100mg/ml crude extract and 30µg/ml of miconazole nitrate and chloramphenicol

[b]Broth dilution method, mean value n= 3.

ISSN: 0975-8585

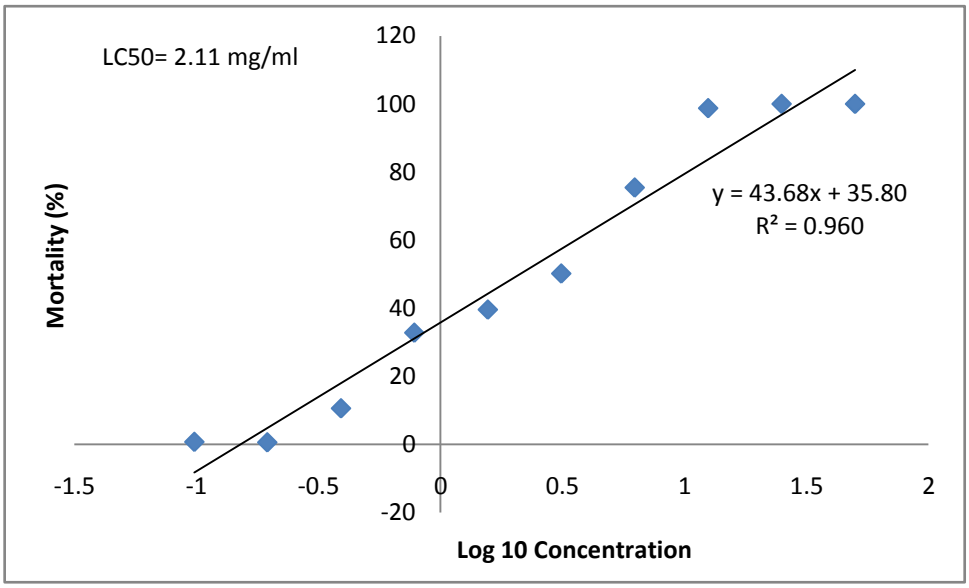

Figure 1: The toxicity effects of the *Cassia fistula* seed extract using brine shrimp lethality assay after 24 hours

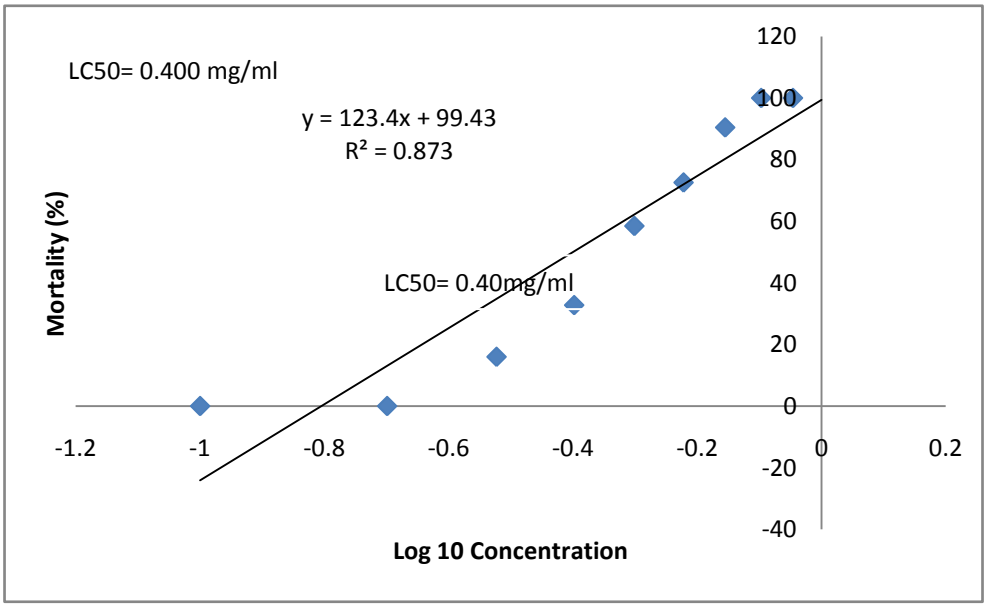

Figure 2: The toxicity effects of the potassium dichromate using brine shrimp lethality assay after 24 hours

ISSN: 0975-8585

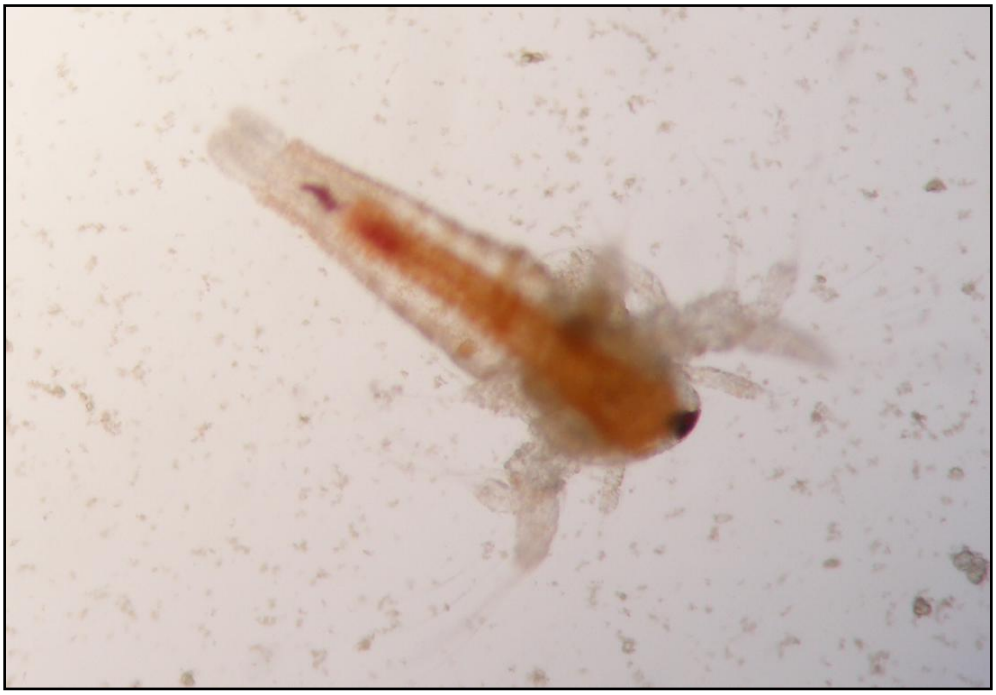

Figure 3: Light microscope micrograph of the *Cassia fistula* seeds extract treated *Artemia salina*

Based on the initial antimicrobial screening assay the strains which show positive results against seed extract of cassia fistula were selected for further studies to determine the MIC value and the MIC value are as shown in Table 1. The MIC values against all the tested bacterial and fungal strains are ranged from 1.563- 50.000 mg/ml (Table 1) and found to be active against bacteria such as *Salmonella sp., Bacillus subtilis* and yeast such as *Candida albicans* with MIC values of 3.130 mg/ml to 6.250 mg/ml and *Micrococcus sp*. has lowest MIC value 1.563 mg/ml. Meanwhile, the MIC value ranged from 12.500 mg/ml- 50.000 mg/ml for bacterial stains such as *Staphylococcus aureus, Bacillus thuringenesis and Escherichia coli.* Basically, the MIC values are indicates the potential of the extract to inhibit the microbial growth at lowest concentration.

Results of the toxicity evaluation against brine shrimp of the *C. fistula* seeds extracts are shown in Figures 1. The *C. fistula* seeds extract exhibited no significant activity toxicity against brine shrimp with an LC_{50} value of 2.11 mg/ml (24 h). This signified that *C. fistula* seeds might not be toxic to human. The Figure 3 shows a light microscope micrograph of the death of *Artemia salina* after treatment with the methanol seeds extract of *C. fistula.* The potassium dichromate which used as positive control was exhibit significant toxicity (LC_{50} value <1.0 mg/ml) against the brine shrimp. Since *C. fistula* seeds extract was not toxic against brine shrimp therefore it can be used as an antimicrobial agent in known dosage.

ISSN: 0975-8585

ACKNOWLEDGEMENT

S. Jo Thy Lachumy was supported by Universiti Sains Malaysia fellowship from Institute for Postgraduate Studies, Universiti Sains Malaysia.

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