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Phytochemical Screening, Antibacterial, Antioxidant and Cytotoxic Activity of the Bark Extract of *Terminalia Arjuna*

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Abstract

The soxhlet extracts of bark of *Terminalia arjuna* using different solvents were investigated for their phytochemical screening, antibacterial activity, antioxidant activity and cytotoxic activity. For phytochemical screening, some common and available standard tests were done. Phytochemical screening revealed the presence of phenols, flavonoids, tannin, saponin, alkaloids, glycosides, phytosterols and carbohydrate. The antimicrobial activity was performed by using the disc diffusion method where seven microorganisms were used as test organisms. Only methanol and ethanol extracts showed antimicrobial activity at higher concentrations. *In-vitro* antioxidant activities of the extracts were performed using DPPH free radical scavenging assay. Almost five extracts showed antioxidant activity, where methanol and ethanol extracts showed highest activity. All five

extracts were subjected to brine shrimp lethality bioassay for possible cytotoxicity. Concentration dependent increment in percent mortality of Brine Shrimp nauplii produced by the extracts indicated the presence of cytotoxic principles in these extractives.

Keywords: *Terminalia arjuna,* phytochemical screening, antibacterial activity, antioxidant activity, cytotoxic activity.

1. Introduction

The plants that possess therapeutic properties or exert beneficial pharmacological effects on the animal body are generally designated as medicinal plants. In most of the traditional systems of treatment, the use of medicinal plant include the fresh or dried part, whole, chopped, powdered or an advanced form of the plant usually made through extraction with different solvents play a major role and constitute the backbone of the traditional medicine (Mukherjee, 1986). Botanical medicines or phytomedicines refer to the use of seeds, berries, leaves, bark, root or flowers of any plant for medicinal purposes by significant number of people (Barret et al., 1999). Although there are no apparent morphological characteristics in the medicinal plants growing with them, yet they possess some special qualities or virtues that make them medicinally important. It has now been established that the plants which naturally synthesis and accumulate some secondary metabolites, like alkaloids, glycosides, tannins, volatiles oils and contain minerals and vitamins, possess medicinal properties (Ghani, 2003). Accordingly, the World Health Organization (WHO) consultative group on medicinal plants has formulated a definition of medicinal plants in the following way: "A medicinal plant is any plant which, in one or more of its organ, contains substance that can be used for therapeutic purpose or which is a precursor for synthesis of useful drugs" (Goldstein, 1974).

Medicinal plants constitute an important natural wealth of a country. They play a significant role in providing primary health care services to rural people. They serve as therapeutic agents as well as important raw materials for the manufacture of traditional and modern medicine. Substantial amount of foreign exchange can be earned by exporting medicinal plants to other countries. In this way indigenous medicinal plants play significant role of an economy of a country (Ghani, 2003).

Terminalia arjuna (Family - Combretaceae), a large tree, is found throughout the South Asian region. This tree is usually an evergreen tree with new leaves appearing in the hot season (February to April) before leaf fall. This tree is an exotic tree in Bangladesh. It is one of the most versatile medicinal plants having a wide spectrum of biological activity. The bark of *T. arjuna* is anti-dysentric, antipyretic, astringent, cardiotonic, lithotriptic and tonic while the powder of the bark acts as a diuretic in cirrhosis of liver and gives relief in symptomatic hypertension (Chatterjee, 1994). In studies in mice, its leaves have been shown to have analgesic and anti-inflammatory properties (Biswas et al., 2011). The purpose of the study was to find out the chemical nature of the extract and to check the biological activity of extract of the bark of *T. arjuna*.

2. Materials and Methods

2.1. Chemicals

All solvents n-hexane, petroleum ether, chloroform, ethanol and methanol were purchased from Merch, Germany. Mueller Hinton agar medium for antibacterial activity was purchased from HiMedia, India. All chemicals used in the study were of analytical grade.

2.2. Collection and Processing of Plant Samples

Plant sample of *T. arjuna* was collected from Savar, Dhaka in July 2011 and a plant sample was submitted to the Bangladesh National Herbarium for identification (Accession number 35904). Barks

were sun dried for seven days. The dried barks were then ground in coarse powder using high capacity grinding machine which was then stored in air-tight container with necessary markings for identification and kept in cool, dark and dry place for the investigation.

2.3. Extract Preparation

Measured amount of the powdered plant material was successively extracted in a soxhlet extractor at elevated temperature using n-hexane which was followed by petroleum ether, chloroform, ethanol and methanol. All extracts were filtered individually through filter paper and poured on petridishes to evaporate the liquid solvents from the extract to get dry extracts. After drying, crude extracts were stored in stock vials and kept in refrigerator for further use. Percent of Yield **(**Patil et al., 2010) was calculated as follows:

$$\text{Extract yield \%} = \frac{W_1}{W_2} \times 100$$

where, W_1= Net weight of powder in grams after extraction and W_2= Total weight of wood powder in grams taken for extraction.

2.4. Phytochemical Screening

Different extracts were screened for the presence of phenols, flavonoids, tannin, saponin, alkaloids, glycosides, phytosterols and carbohydrate by using standard protocols (Tiwari et al., 2011).

2.5. Preparation of Media

38 g of Mueller Hinton agar was taken in a conical flask and 1000 mL distilled water was added to it. The contents were heated in a water bath to make a clear solution and sterilized. Final pH (at 25°C) was 7.3 ± 0.2. Measured amount of this sterilized semisolid mueller hinton agar medium was poured in pre-sterilized glass petridishes under aseptic conditions in laminar flow.

2.6. Determination of Antibacterial Activity by Disc Diffusion Method

The antimicrobial activity for different extracts was determined by the disc diffusion method (Bauer et al., 1966). Both gram positive and gram-negative bacterial strains were used for the test. The bacterial strains used for the investigation are listed in Table 1. Solutions of known concentration (mg/mL) of the test samples were made by dissolving measured amount of the samples in calculated volume of solvents. Dried and sterilized filter paper discs were then impregnated with known amounts of the test substances using micropipette. Discs containing the test material were placed on nutrient agar medium uniformly seeded with the test microorganisms. Standard antibiotic discs (Kanamycin 30 µg/disc) and blank discs (impregnated with solvents) were used as a positive and negative control. These plates were then incubated at 37ºC for 24 h to allow maximum growth of the organisms. The test materials having antibacterial activity inhibited the growth of the microorganisms and a clear, distinct zone of inhibition was visualized surrounding the medium. The antimicrobial activity of the test agents was determined by measuring the diameter of zone of inhibition expressed in millimeter.

Table 1: Microorganisms used in antimicrobial assay

Gram positive bacteria	Gram negative bacteria
Staphylococcus aureus Bacillus subtilis Bacillus cereus	Salmonella typhi Escherichia coli Vibrio cholerae Klebsiella pneumoniae

2.7. Antioxidant Activity Determination by DPPH Free Radical Scavenging Assay

The free radical scavenging capacity of the extracts was determined using DPPH (Braca *et al.,* 2001). Freshly prepared 1, 1 diphenyl-2-picrylhydrazyl (DPPH) solution was taken in test tubes and extracts were added followed by serial dilutions (15.625 µg/ml to 250 µg/mL) to every test tube so that the final volume was 5 mL and after 30 min, the absorbance was read at 517 nm using a spectrophotometer. Ascorbic acid and butylated hydroxytoluene (BHT) was used as standard. Control sample was prepared containing the same volume without any extract and standard and the absorbance was read at 517 nm using a spectrophotometer. Methanol was served as blank.

2.8. Cytotoxicity Screening

Brine shrimp lethality bioassay is widely used in the bioassay for the bioactive compounds by the method of Meyer (Meyer et al., 1982). Here simple zoological organism (*Artemia salina*) was used as a convenient monitor for the screening. The eggs of the brine shrimp were collected and hatched in artificial seawater (3.8% NaCl solution) for 48 hr to mature shrimp called nauplii. The cytotoxicity assay was performed on brine shrimp nauplii using Meyer method. Measured amount of each of test samples were taken and dissolved in measured amount of pure dimethyl sulfoxide (DMSO) to get stock solutions. Then the solution was serial diluted to 400, 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.781 µg/mL with sea water. Then 2.5 ml of plant extract solution was added to 2.5 ml of sea water containing 10 nauplii. Positive control in a cytotoxicity study is a widely accepted cytotoxic agent and the result of the test agent is compared with the result obtained for the positive control. In the present study vincristine sulfate was used as positive control. Vincristine is a cytotoxic alkaloid and it was evaluated at very low concentration (40, 20, 10, 5, 2.5, 1.25, 0.625, 0.313, 0.156, 0.078 µg/mL). Measured amount of DMSO was added to each of three pre-marked test tubes containing 4.9 ml of simulated sea water and 10 shrimp nauplii to use as negative control groups.

3. Results

3.1. Percent of Yield Determination

The obtained yield of the plant extracts has been presented in Table 2.

Table 2: Yield of extraction of bark of *Terminalia arjuna* in different solvents

Plant Part	Solvent	Yield (%)
Bark	n-Hexane	0.35
	Petroleum ether	0.70
	Chloroform	0.80
	Ethanol	35.70
	Methanol	31.70

3.2. Phytochemical Screening

Phytochemical screening revealed the presence of phenols, flavonoids, tannin, saponin, alkaloids, glycosides, phytosterols and carbohydrate (Table 3).

Table 3: Result of chemical group test of various extracts of bark of *Terminalia arjuna.*

TESTS	EXTRACT					
	Methanol	Ethanol	Pet-ether	n-hexane	Chloroform	Water
Phenols	+++	++	+	-	+	++
Flavonoids	+++	+++	+	-	+	++

Table 3: Result of chemical group test of various extracts of bark of *Terminalia arjuna.* - Continued

Tannin	++	+++	-	-	-	++
Saponin	+	++	-	-	-	++
Alkaloids	+	+++	+	+	+	+
Glycosides	+++	+	-	-	-	+
Phytosterols	+	+	-	++	-	-
Carbohydrate	++	+++	-	-	-	+++

+++: Present in high concentration, ++: Present in moderate concentration, +: Present in low concentration and -: Absent

3.3. Antimicrobial Screening

The result of antimicrobial screening of different extracts of *T. arjuna* has been presented in Table 4.

Table 4: Antimicrobial screening of different extracts of *Terminalia arjuna*

Test microorganisms	**Diameter of the zone of inhibition (mm)**						
	Sample	**30 µg/disc**	**100 µg/disc**	**300 µg/disc**	**500 µg/disc**	**1000 µg/disc**	**1500 µg/disc**
Gram positive bacteria							
Staphylococcus aureus	ME	-	-	-	-	9	13
	EE	-	-	-	-	11	14
	PE	-	-	-	-	-	-
	HE	-	-	-	-	-	-
	CE	-	-	-	-	-	-
	K	24	27	25	25	27	27
Bacillus subtilis	ME	-	-	-	-	11	8
	EE	-	-	-	-	10	10
	PE	-	-	-	-	-	-
	HE	-	-	-	-	-	-
	CE	-	-	-	-	-	-
	K	20	20	20	22	22	20
Bacillus cereus	ME	-	-	-	-	9	12
	EE	-	-	-	-	9	11
	PE	-	-	-	-	-	-
	HE	-	-	-	-	-	-
	CE	-	-	-	-	-	-
	K	25	25	25	25	25	25
Gram negative bacteria							
Salmonella typhi	ME	-	-	-	-	13	14
	EE	-	-	-	-	11	18
	PE	-	-	-	-	-	-
	HE	-	-	-	-	-	-
	CE	-	-	-	-	-	-
	K	28	28	29	29	29	28
Escherichia coli	ME	-	-	-	-	11	11
	EE	-	-	-	-	10	12
	PE	-	-	-	-	-	-
	HE	-	-	-	-	-	-
	CE	-	-	-	-	-	-
	K	21	21	25	25	25	21
Vibrio cholerae	ME	-	-	-	-	14	12
	EE	-	-	-	-	12	12
	PE	-	-	-	-	-	-
	HE	-	-	-	-	-	-
	CE	-	-	-	-	-	-
	K	32	32	31	32	32	31

Table 4: Antimicrobial screening of different extracts of *Terminalia arjuna* - continued

Klebsiella pneumoniae	ME	-	-	-	-	9	11
	EE	-	-	-	-	9	15
	PE	-	-	-	-	-	-
	HE	-	-	-	-	-	-
	CE	-	-	-	-	-	-
	K	26	24	26	26	24	26

"-" indicates "no activity", Methanol Extract = ME, Ethanol Extract = EE, Pet-ether Extract = PE, n-Hexane Extract = HE, Chloroform Extract = CE, Kanamycin = K

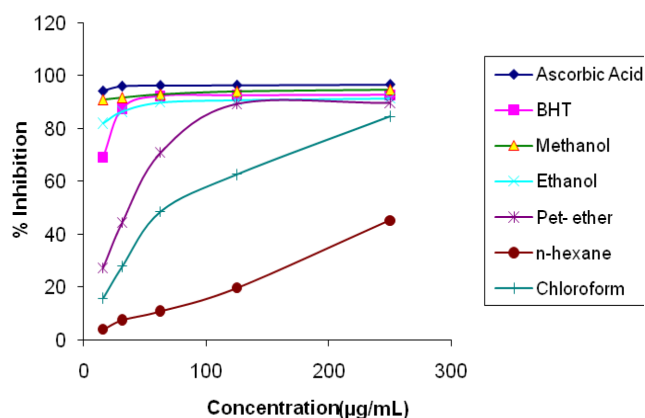
3.4. DPPH Free Radical Scavenging Assay

The free radical scavenging activity of different extracts of *T. arjuna* was studied by its ability to reduce the DPPH, a stable free radical and any molecule that can donate an electron or hydrogen to DPPH, can react with it and thereby bleach the DPPH absorption. DPPH is a purple colored dye having absorption maximal of 517 nm and upon reaction with a hydrogen donor its purple color fades or disappears due to conversion of it to 2, 2-diphenyl-1-picryl hydrazine resulting in decrease in absorbance. The methanol and ethanol extracts showed maximum activity of 94.72% and 91.48% respectively at 250 µg/mL, where as ascorbic acid and BHT at the same concentration exhibited 96.66% and 92.59 % inhibition respectively. Five extracts exhibited considerable DPPH free radical scavenging activity as indicated by their IC_{50} values shown in Table 5 and Figure 1. IC_{50} indicates the potency of scavenging activity. Standard ascorbic acid and BHT were found to have an IC_{50} of 5.698 µg/mL and 8.816 µg/mL. In comparison to standard ascorbic acid and BHT, methanol, ethanol, petroleum ether and chloroform extract of *T. arjuna* bark showed IC_{50} of 6.34, 7.76, 25.63 and 61.89 respectively. n-hexane fraction is seen to have the least free radical scavenging activity.

Table 5: IC_{50} values of different extracts of *Terminalia arjuna* in DPPH scavenging assay.

Extracts/standard	**IC_{50} (µg/ml)**
Methanol	6.34
Ethanol	7.76
Pet-ether	25.63
n-hexane	6828.229
Chloroform	61.890
Ascorbic acid	5.698
Butylated hydroxytoluene (BHT)	8.816

Figure 1: Comparative DPPH radical scavenging activity of *Terminalia arjuna* bark extracts, Ascorbic acid and Butylated hydroxytoluene (BHT)



3.5. Cytotoxicity Screening

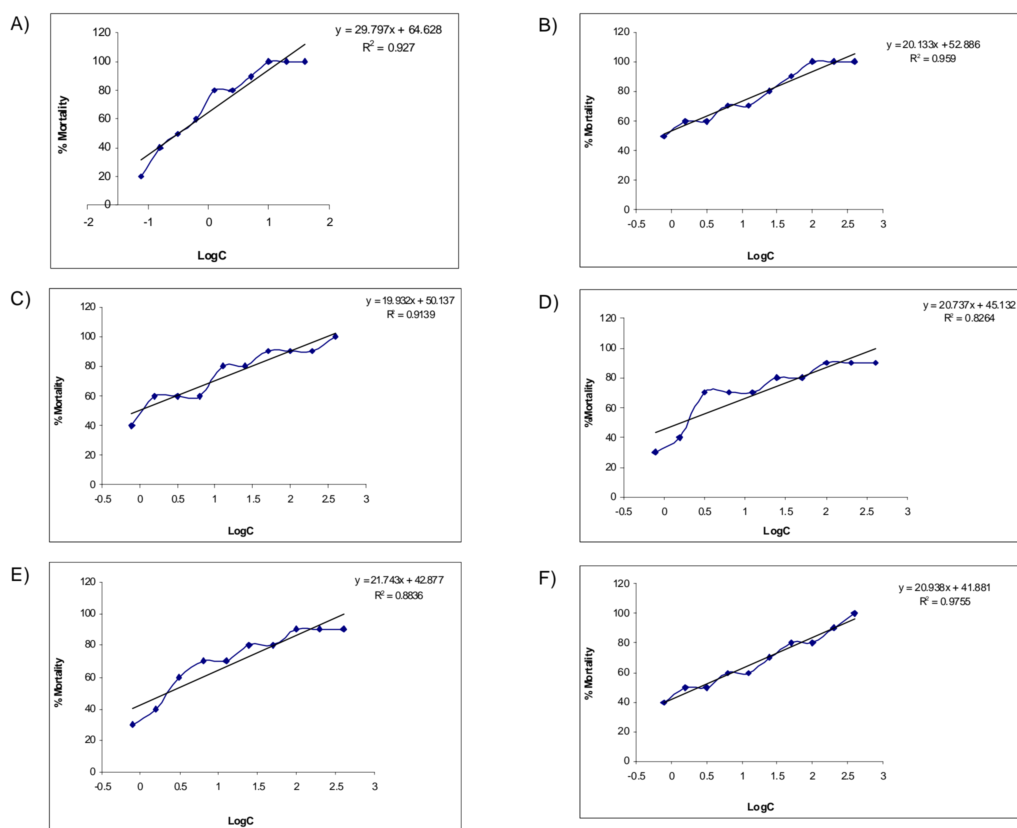
In the present bioactivity study the five crude extracts and pure compounds showed positive results indicating that the test samples are biologically active. The methanol, ethanol, petroleum ether, chloroform and n-hexane extract of the dried bark of *T. arjuna* were subjected to brine shrimp lethality bioassay following the procedure which has been utilized by Meyer et al., 1982. The results of the different extracts of *T. arjuna* (% mortality at different concentrations and LC_{50} values) were shown in Table 6 and Figure 2.

Vincristine sulphate (VS) was used as positive control and the LC_{50} was found as 0.323 µg/mL. Compared with the negative control, VS (positive control) gave significant mortality and the LC_{50} values of the different extractives were compared with negative control.The LC_{50} values of methanol, ethanol, petroleum ether, chloroform and n-hexane were found to be 0.719 µg/mL, 0.984 µg/mL, 1.717 µg/mL, 2.127 µg/mL and 2.442 µg/mL respectively (Table 6).

Table 6: LC_{50} values of the five extracts of *Terminalia arjuna* and standard

Test Samples	Regression line	R^2	LC_{50} (µg/ ml)
Vincristine	y = 29.79x + 64.62	R^2 = 0.927	0.323
Methanol	y = 20.13x + 52.88	R^2 = 0.959	0.719
Ethanol	y = 19.93x + 50.13	R^2 = 0.913	0.984
Pet-ether	y = 20.73x + 45.13	R^2 = 0.826	1.717
Chloroform	y = 21.74x + 42.87	R^2 = 0.883	2.127
n-hexane	y = 20.93x + 41.88	R^2 = 0.975	2.442

Figure 2: Determination of LC_{50} of A) vincristine sulphate, B) methanol extract, C) Ethanol extract, D) petroleum ether extract, E) chloroform extract and F) n-hexane extract of *Terminalia arjuna* against brine shrimp nauplii.



Varying degree of lethality of *Artemia salina* was observed with exposure to different dose levels top the test samples ranging from 0.781-400 µg/mL. The degree of lethality shown by the extractives was found to be directly proportional to the concentration of the extractives ranging from the lowest concentration (0.781 µg/mL) to the highest concentration (400 µg/mL). This concentration dependent increment in percent mortality of Brine Shrimp nauplii produced by the *T. arjuna* extracts indicates the presence of cytotoxic principles in these extractives.

4. Discussions

4.1. Percent of Yield Determination

Among the extracts ethanol extracted highest yield (35.70 %) whereas n-hexane extract yield was found to be low (0.35 %).

4.2. Phytochemical Screening

Methanol and ethanol extracts of bark of *T. arjuna* contain phenols, flavonoids, tannin, saponin, alkaloids, glycosides, phytosterols and carbohydrate. Pet-ether extract of bark of *T. arjuna* contains alkaloids and n-hexane extract contains alkaloids and phytosterols, chloroform extract contains flavonoids and alkaloids. Water extract of bark of *T. arjuna* contains phenols, flavonoids, tannin, saponin, alkaloids, glycosides and carbohydrate. Alkaloids present in all six extracts of bark of *T. arjuna.*

4.3. Antimicrobial Screening

Antimicrobial screening of different extracts of *T. arjuna* did not show any antimicrobial activity at 30 µg/mL, 100 µg/mL, 300 µg/mL, 500 µg/mL, 1000 µg/mL and 1500 µg/mL concentrations. But methanol and ethanol extracts showed antimicrobial activity at 1000 µg/mL and 1500 µg/mL concentrations against all test microorganisms.

4.4. DPPH Free Radical Scavenging Assay

The DPPH test showed the ability of the test compound to act as a free radical scavenger. DPPH assay method is based on the ability of 1, 1-diphenyl-2-picrylhydrazyl (DPPH), a stable free radical, to decolorize in the presence of antioxidants (Kumarasamy et al., 2007). DPPH, a protonated radical, has characteristic absorbance maximal at 517 nm, which decreases with the scavenging of the proton radical. This property has been widely used to evaluate the free radical scavenging effect of natural antioxidants (Jao et al., 2002). The stable free radical DPPH has been widely used to test the free radical-scavenging ability of various dietary antioxidants (Brand-Williams et al., 1995). Because of its odd electron, DPPH gives a strong absorption band at 517nm in visible spectroscopy. As this electron becomes paired off in the presence of a free radical scavenger, the absorption vanishes, and the resulting decolourization is stoichiometric with respect to the number of electrons taken up. The bark extracts showed considerable radical scavenging activity in a concentration-dependent manner. Methanol (6.34), ethanol (7.76), petroleum ether (25.63) and chloroform (61.89) extract of the dried bark exhibited a good potential to act as a free radical scavenger with IC_{50} for DPPH inhibition comparable to that of Ascorbic acid (5.698 µg/mL) and BHT(8.816 µg/mL) which are known free radical scavengers. Figure 1 is exhibiting the comparative % inhibition among *T. arjuna* bark extracts and standard compounds (Ascorbic acid & Butylated Hydroxytoluene). The highest scavenging effect was showed by Methanol extract with a value of 6.34 µg/mL followed by ethanol and petroleum ether having value of 7.76 and 25.63 respectively as opposed to that of the scavenging effects of ascorbic acid and BHT of 5.698 and 8.816 respectively. These results indicated that extract has a noticeable effect on scavenging the free radicals. In fact, IC_{50} value of methanol and ethanol extract appeared to

have slightly better than standard BHT at 50% inhibition. However, a maximum inhibition was achieved at a higher concentration of 250 µg/mL compared to 250 µg/mL for both of Ascorbic acid and BHT respectively.

4.5. Cytotoxicity Screening

The brine shrimp lethality assay (BSLA) has been used routinely in the primary screening of the extracts as well as isolated compounds to assess the toxicity towards brine shrimp, which could also provide an indication of possible cytotoxic properties of the test materials. Brine shrimp nauplii have been previously utilized in various bioassay systems. Among these applications have been the analyses of pesticidal residues, mycotoxins, stream pollutants, anesthetics, dinoflagelate toxins, morphine-like compounds, carcinogenicity of phorbol esters and toxicants in marine environment. A number of novel antitumor and pesticidal natural products have been isolated using this bioassay (McLaughlin et al., 1991; Meyer et al., 1982). The variation in BSLA results (Table 6) may be due to the difference in the amount and kind of cytotoxic substances (e.g. tannins, flavonoids or triterpenoids) present in the extracts.

5. Conclusion

Based on the results of the present study, it can be suggested that the extracts of *Terminalia arjuna* possess antimicrobial, antioxidant and cytotoxic effects. The phytochemical screening revealed chemical constituents that form the foundation of their pharmacological activity. Methanol and ethanol extracts of *T. arjuna* has good efficacy against gram positive and gram negative bacteria in higher concentration. Almost all extracts exhibited potential antioxidant activity. The high toxicity exerted by the extracts of *T. arjuna* in brine shrimp lethality bioassay suggests bioactive principles in the plant.

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