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IN-VIVO CYTOTOXIC ACTIVITY OF *TERMINALIA ARJUNA* LEAF EXTRACTS USING BRINE SHRIMP (*ARTEMIA SALINA)* AND PHYTOCHEMICAL SCREENING

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ABSTRACT

The phytochemical study and cytotoxic potential of the leaves of *Terminalia arjuna* were investigated. The extraction was based on solvents of increasing polarities such as petroleum ether, chloroform, ethanol and aqueous. Preliminary phytochemical analysis indicates the presence of carbohydrates, saponins, steroids, tannins, proteins and phenolic compounds. The Brine Shrimp Lethality Assay (BSLA) of the extracts was carried out to detect possible cytotoxic effects of the plant leaves. *In vivo* cytotoxicity was evaluated in terms of LC_{50}. The extracts were potent against the brine shrimp with LC_{50} value of petroleum ether, chloroform, ethanol and aqueous extract were 11.22 µg/ml, 22.38 µg/ml, 30.19 µg/ml and 17.78 µg/ml respectively compared to vincristine sulphate, a reference drug, LC_{50} 0.52 µg/ml. The overall result indicates the presence of bioactive components in the all leaves extracts which could account for the reported pharmacological effects of the plant.

KEYWORDS

Terminalia arjuna leaves, Phytochemical investigation, Brine shrimp lethality assay, Cytotoxicity and LC_{50}.

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INTRODUCTON

Terminalia arjuna (Combretacae) is commonly known as Arjuna. The large evergreen tree is distributed throughout the greater part of Indian peninsula along rivers, streams, ravines and dry watercourses. It is basically a tropical species. It grows almost in all red lateritic soils. It grows in low land to hilly areas and it can tolerate half submergence for a few weeks. It is also planted for shade and decoration in avenues or parks. *Terminalia arjuna is* usually an evergreen tree

with new leaves appearing in the hot season (February to April) before leaf fall. Tree sometimes may be leaflets for a very short period before flowering. Fruit bearing begins 6-7 years after planting. For centuries, *Terminalia arjuna* have been used by folk healers throughout India in various Indigenous System of Medicine like Ayurveda, Siddha and Unani as astringent, cooling and diuretic in cirrhosis[1]. Various parts of these plants have claimed to be effective in a wide range of diseases[2]. The scientifically stated therapeutic properties for *Terminalia arjuna* have been reported to be used as anti-inflammatory, induction of apoptosis[3], cardio tonic, in fractures, antioxidant[4], ulcers, spermatorrhoea, leucorrhoea, diabetes, anti-fungal[5], cough, anti-allergenic[6], tumour, excessive perspiration, diarrhoea, asthma, diseases of heart, congestive heart failure[7], hypertension[8], anaemia, skin disorders, antineoplastic[9], gynaecological complaints, bacterial infections and urinary disorders. It is also believed to have the ability to cure hepatic, urogenital, venereal and viral diseases[10]. These observations prompted us to screen the cytotoxic activity by using the Brine Shrimp Lethality Assay (BSLA) method; it is based on the ability to kill laboratory-cultured *Artemia nauplii* brine shrimp. This BSLA is considered a useful tool for preliminary assessment of toxicity, developing antitumor agents and it has been used for the detection of fungal toxins and plant extract toxicity[11].

MATERIALS AND METHODS

Plant Material

The leaves were collected from the branches of *Terminalia arjuna* (Roxb) in Alangulam, Tirunelveli District, Tamilnadu, India, during February 2015. The plant material i.e., leaf of *Terminalia arjuna* (Roxb) was then identified and authenticated.

PREPARATION OF EXTRACTS

The extraction is the preliminary step involved in the phytochemical studies[12] and it is performed with solvents of increasing order of polarity. The dried, coarsely powdered *Terminalia arjuna* leaves (250gm) were first extracted with petroleum ether (50º-60ºC) in Soxhlet apparatus for 72 hrs, the petroleum ether extract was concentrated to a crude residue mass by using vacuum distillation. The marc obtained was dried in oven at 40ºC and extracted with chloroform (50º-60ºC) for 72 hours, followed by the extract was concentrated to get a dry crude residue mass by using vacuum distillation. Then the marc obtained was dried in oven at 40ºC and then extracted with ethanol (75º-80ºC) for 72 hours, followed by the extract was concentrated to a crude residue mass by using vacuum distillation. Finally the marc obtained was dried in oven at 40ºC and extracted with aqueous water for 72 hrs, followed by the extract was concentrated by vacuum distillation to get a crude residue mass.

EVALUATION OF CYTOTOXIC ACTIVITY

Brine Shrimp Lethality Assay (BSLA)

This screening method used to detect and then monitor the fractionation of cytotoxic potency using brine shrimp rather than expensive *in-vitro* and *in-vivo* antitumor assays. The brine shrimp assay has advantages of being rapid (24 hours), inexpensive and simple (e.g., no aseptic techniques are required) [13]. The basis of this method is to evaluate cytotoxicity in terms of LC_{50} (lethality concentration). The test was carried out in three replicates of the concentration of the extracts and surviving brine shrimp were recorded after 24 hours[14]. About 1 gram of *Artemia salina* (Linnaeus) cysts were hatched in 1 litre capacity glass cylinder (jar) containing filtered artificial seawater prepared from commercial sea salt 40g/litre and supplemented with 6 mg/litre dried yeast. The air pump was fitted to the water to ensure complete aeration of the cysts. After 48 hours of incubation at room temperature (25-29°C) under continuous illumination of fluorescence lamp, newly hatched free-swimming pink-colored nauplii were harvested from the bottom outlet. As the cyst capsules floated on the surface, this collection method ensured pure harvest of nauplii. The freshly hatched free-swimming nauplii were used for the bioassay. The test samples (Pet. ether, chloroform, ethanol and aqueous extracts) were prepared by dissolving in dimethylsulphoxide (DMSO), (not more than 50µl in 5ml solution) and artificial sea water to make concentration 6.25µg/ml, 12.5µg/ml, 50µg/ml, 100µg/ml, 200µg/ml, and

400µg/ml. A vial containing 50µl DMSO diluted to 5ml was used as a control. Standard Vincristine sulphate was used as positive control. Then 10 matured alive shrimps were introduced into each of all experimental vials containing solution of concentrations 6.25µg/ml, 12.5µg/ml, 50µg/ml, 100µg/ml, 200µg/ml, 400µg/ml prepared from Pet. ether, Chloroform, Ethanol and Aqueous extracts of the leaves of plant *Terminalia arjuna.* Thus, there were a total of 30 shrimps per dilution. Then the volume was adjusted with artificial sea water up to 5ml per vial. The test tube was left uncovered under the lamp. After 24 hours, the vials were inspected using a magnifying glass and the number of dead nauplii in each vial was counted and recorded. The mortality end point of this bioassay was defined as the absence of control forward motion during 30s observation. The percentage mortality (%M) was calculated by dividing the number of dead nauplii by the total number, and then multiplied by 100%. This is to ensure that the death (mortality) of the nauplii is attributed to the bioactive compounds present in the plant extract. From this data the percent of the lethality of the brine shrimp nauplii for each concentration and control was calculated. An approximate linear correlation was observed when logarithm of concentration versus percentage of mortality was plotted on the graph paper and the values of LC_{50} were calculated using Microsoft Excel 2007[15].

RESULTS AND DISCUSSION

In the present investigation, the phytochemical screening has been done in various solvent extracts of *Terminalia arjuna* leaves extracts and the results are presented in Table No.1. The leaves powder with various chemical reagents showed the presence of carbohydrates, saponins, steroids, tannins, proteins and phenolic compounds. The petroleum ether extract showed the presence of steroids, saponins, glycosides, tannins, phenolic compounds and terpenoids. The chloroform extract showed the presence of carbohydrates, saponins, steroids, tannins, phenolic compounds and terpenoids. The ethyl alcohol extract showed the presence of carbohydrates, glycosides, saponins, steroids, tannins and phenolic compounds. The aqueous water extract showed the presence of carbohydrates, saponins, steroids, phenolic compounds and tannins. The four different extracts of *Terminalia arjuna* leaves were tested and exhibited good brine shrimp larvicidal activity. LC_{50} value of petroleum ether, chloroform, ethanol and aqueous extract were 11.22 µg/ml, 22.38 µg/ml, 30.19 µg/ml and 17.78 µg/ml respectively compared to vincristine sulphate, a reference drug, LC_{50} 0.52 µg/ml. Among all four extracts, the petroleum ether has more cytotoxic potential. Moreover, the chloroform and aqueous water extracts also endowed with considerable moderate activity. Among this ethanol extract only exhibited weak activity. The results are depicted in Table No.2-5 and Figure No.1-6.

Table No.1: Preliminary phytochemical screening of the dried powdered material and various extracts of the *Terminalia arjuna* leaves

S.No	Chemical Constituents	Powder	Petroleum Ether	Chloroform	Ethanol	Aqueous
1	Carbohydrates	+	-	+	+	+
2	Alkaloids	-	-	-	-	-
3	Steroids	+	+	+	+	+
4	Glycosides	-	+	-	+	-
5	Saponins	+	+	+	+	+
6	Flavonoids	-	-	-	-	+
7	Tannins	+	+	+	+	+
8	Phenolic Compounds	+	+	+	+	+
9	Proteins	+	-	-	-	-
10	Amino acids	-	-	-	-	-
11	Mucilages	-	-	+	-	-
12	Terpenoids	-	+	+	-	-

+ve indicates presence of respective constituents; -ve indicates absence of respective constituents

Table No.2: Brine Shrimp Lethality Assay for the petroleum ether extract of *Terminalia arjuna* leaves

S.No	*Terminalia arjuna* Extract	Concentration (µg/ml)	Log C	Number of surviving nauplii after 24 hours			Total number of survivors	% Mortality	LC_{50} Value
				T_1	T_2	T_3			
1	Pet. ether	6.25	0.7959	7	6	7	20	33.3	11.22
		12.5	1.0969	5	7	5	17	43.3	
		25	1.3979	3	2	3	8	73.3	
		50	1.699	1	2	1	4	86.6	
		100	2	0	0	0	0	100	
		200	2.301	0	0	0	0	100	
		400	2.6021	0	0	0	0	100	

Table No.3: Brine Shrimp Lethality Assay for the chloroform extract of *Terminalia arjuna* leaves

S.No	*Terminalia arjuna* extract	Concentration (µg/ml)	Log C	Number of surviving nauplii after 24 hours			Total number of survivors	% Mortality	LC_{50} Value
				T_1	T_2	T_3			
1	Chloroform	6.25	0.7959	7	8	8	23	23.3	22.38
		12.5	1.0969	6	5	7	18	40	
		25	1.3979	4	6	5	15	50	
		50	1.699	3	5	2	10	66.6	
		100	2	2	2	1	5	83.3	
		200	2.301	0	0	0	0	100	
		400	2.6021	0	0	0	0	100	

Table No.4: Brine Shrimp Lethality Assay for the ethanolic extract of *Terminalia arjuna* leaves

S.No	*Terminalia arjuna* extract	Concentration (µg/ml)	Log C	Number of surviving nauplii after 24 hours			Total number of survivors	% Mortality	LC_{50} Value
				T_1	T_2	T_3			
1	Ethanol	6.25	0.7959	7	9	8	24	20	30.19
		12.5	1.0969	5	7	8	20	33.33	
		25	1.3979	4	7	6	17	43.33	
		50	1.699	3	6	4	13	56.66	
		100	2	2	1	3	6	80	
		200	2.301	0	0	0	0	100	
		400	2.6021	0	0	0	0	100	

Table No.5: Brine Shrimp Lethality Assay for the aqueous water extract of *Terminalia arjuna* leaves

S.No	*Terminalia arjuna* extract	Concentration (µg/ml)	Log C	Number of surviving nauplii after 24 hours			Total number of survivors	% Mortality	LC_{50} Value
				T_1	T_2	T3			
1	Aqueous (Water)	6.25	0.7959	8	7	6	21	30	17.78
		12.5	1.0969	7	7	5	19	36.66	
		25	1.3979	5	4	2	11	63.33	
		50	1.699	4	4	1	9	70	
		100	2	2	1	1	4	86.6	
		200	2.301	0	0	0	0	100	
		400	2.6021	0	0	0	0	100	

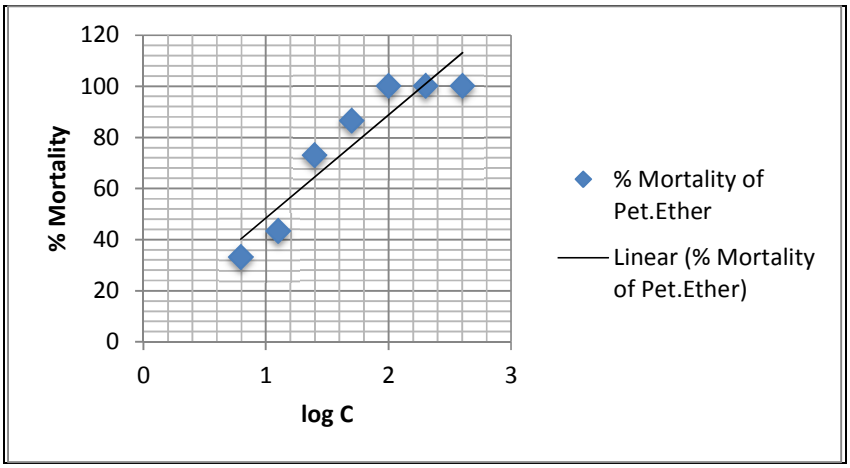

Figure No.1: Determination of LC_{50} value for the petroleum ether extract of *Terminalia arjuna* leaves, Linear correlation between Log Concentrations versus percentage mortality

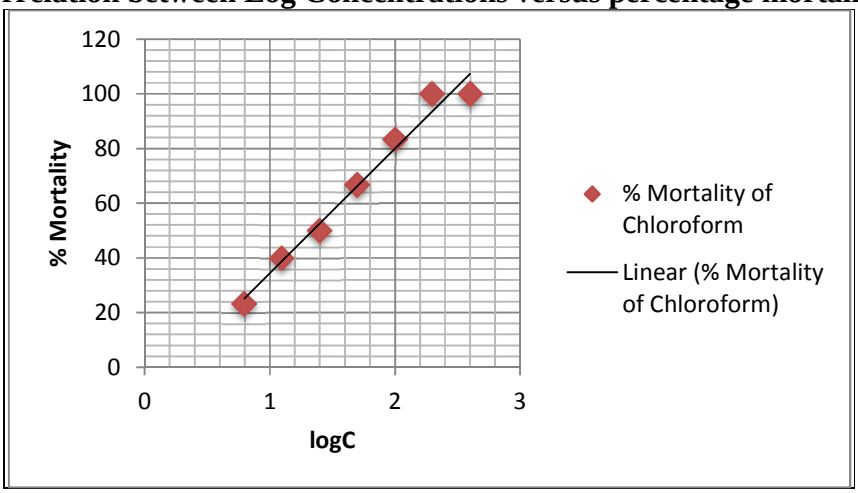

Figure No.2: Determination of LC_{50} value for the chloroform extract of *Terminalia arjuna* leaves, Linear correlation between Log Concentrations versus percentage mortality

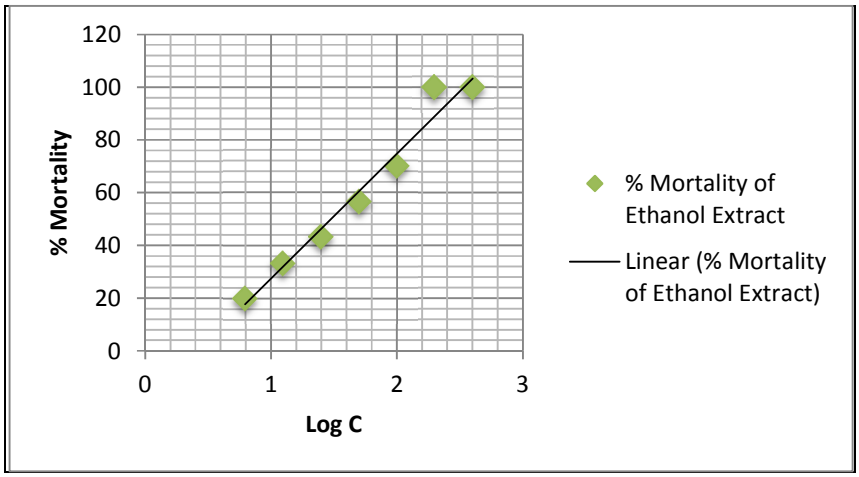

Figure No.3: Determination of LC_{50} value for the ethanolic extract of *Terminalia arjuna* leaves, Linear correlation between Log Concentrations versus percentage mortality

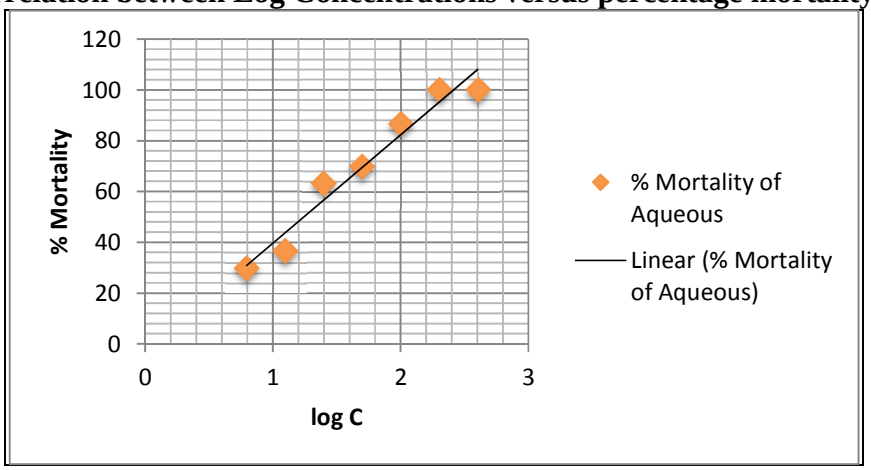

Figure No.4: Determination of LC_{50} value for the aqueous extract of *Terminalia arjuna* leaves, Linear correlation between Log Concentrations versus percentage mortality

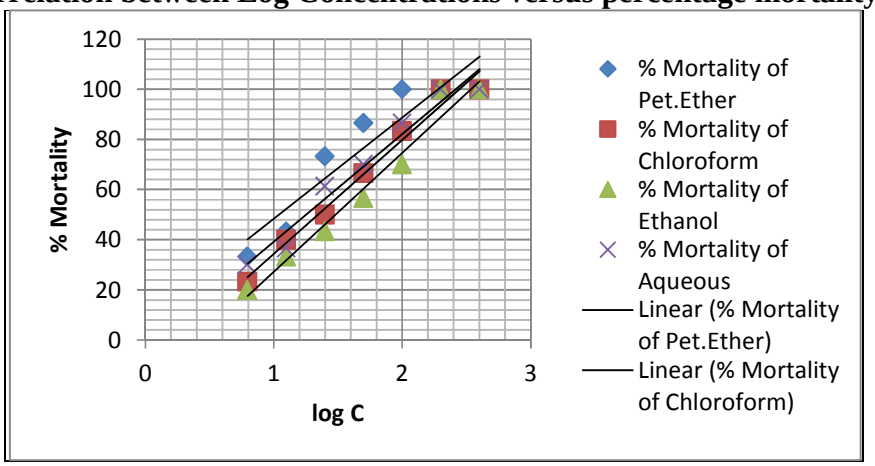

Figure No.5: Determination of LC_{50} value for the petroleum ether, chloroform, ethanolic and aqueous water extracts of *Terminalia arjuna* leaves, Linear correlation between Log Concentrations versus percentage mortality

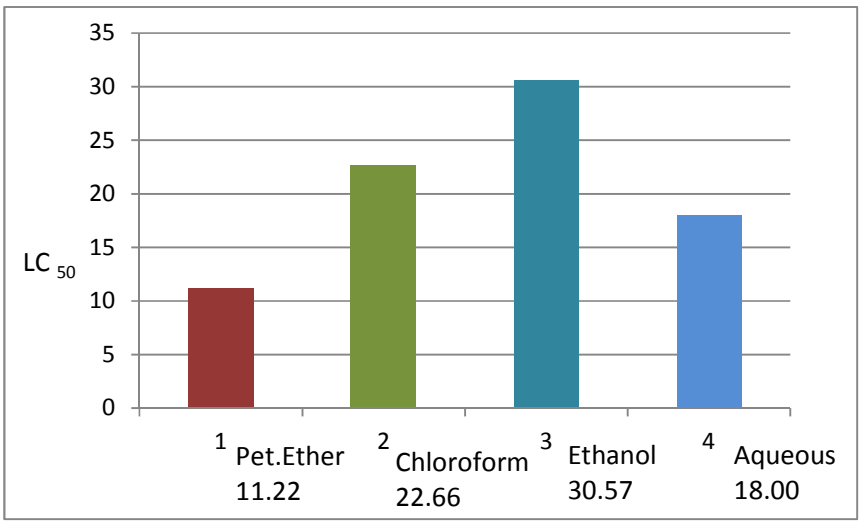

Figure No.6: Comparison of LC_{50} values of petroleum ether, chloroform, ethanolic and aqueous water extracts of the *Terminalia arjuna* leaves

CONCLUSION

The phytochemical screening of the *Terminalia arjuna* leaves are exhibited the presence of various phytochemical constituents such as, saponins, steroids, tannins and phenolic compounds. The leaves extracts of *Terminalia arjuna* exhibit cytotoxic activity against the brine shrimp. The cytotoxic activity against the brine shrimp are considered as containing the active or potent components because of their LC_{50} values are less than 100µg/ml. Various extracts of the leaves of the plant possess LC_{50} value less than 100µg/ml and therefore they are present to have curative properties against several pathogens. Although, BSLA is inadequate in determining the mechanism of action of the bioactive substances in the plant, it is very useful by providing a preliminary screen that can be supported by a more specific bioassay once the active compounds has been isolated. Thus, some useful antitumor or anti-proliferative drugs of therapeutic importance may develop out of the research work.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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