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Phytochemical analysis and *in vitro* antimicrobial activities of *Terminalia arjuna* leaf, bark and fruit extracts in different solvents

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Abstract

The soxhlet extracts of fruits, bark, and leaf of *Terminalia arjuna* were obtained using different solvent viz. water, methanol, ethanol, acetone, chloroform and petroleum ether and were analyzed for their phytochemical, antibacterial and antifungal activity. The phytochemical activity of leaf, bark and fruit extract of *T. arjuna* were performed using all six solvent. Results clearly indicate the presence of alkaloids, carbohydrates, cardiac glycosides, proteins, phytosterols, flavonoids, tannins, terpenoids, saponins, and phenols/ polyphenols. Moreover, proteins, flavonoids, tannins, and phenols were present in almost all leaf, bark and fruit extracts of *T. arjuna*. Antibacterial activity of the crude extract was studied against two each of gram-positive and gram-negative bacterial strains along with three fungal strains. All microbial strains (*Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Aspergillus niger, Aspergillus flavus* and *Candida albicans*) were procured from IMTech Chandigarh. Antimicrobial activity was performed using the disc diffusion method. The antibiogram of bark extract in methanol, acetone and petroleum ether showed significant ($p < 0.01$) antimicrobial activity. Similarly, significant antimicrobial activity ($p < 0.01$) was observed within the chloroform and aqueous extract of fruits. Maximum antibacterial and antifungal activity was found to be present in the aqueous extract of fruit indicating its probable significance in the reduction of infectious diseases within the feeding livestock population.

Keywords: *Terminalia arjuna,* Bark, Leaves, Fruit, Phytochemical Screening.

Introduction

Study of medicinal plants as natural products is widespread throughout the world [1]. From pre-historical period, medicinal plants have been used for traditional and conventional medicine formulations. People, in general, prefer these medicinal formulations due to their safe, effective and inexpensive mode. Henceforth, medicinal plants are the indispensable part of human healthcare system [2].

Terminalia arjuna tree has a cosmopolitan distribution and is found throughout the Indian subcontinent. It is present in the form of rows within the dry hill areas of several plants near water bodies - rivers, streams, and ravines. It is also planted for ornamental purposes. It thrives best on loose moist, fertile alluvial loams soil and shallow soil, often overlying more or less impervious rock. *T. arjuna* is an evergreen large deciduous tree reaching up to a height of 60-85 feet, bearing yellow flowers and conically shaped leaves [3]. Fruit is fibrous woody, 2.5-3.5 cm long, having five hard wings, striated with numerous curved veins. It has a buttressed trunk and a vast spreading crown from which the branches bent downwards. Flowering occurs between March to

June and fruiting between September to November [4].

Traditional Indian medicinal herb i.e., *T. arjuna* has many therapeutic applications in Ayurvedic, Unani and Homeopathic [4]. Its barks find applications in various medicinal practices as it is rich in calcium, magnesium salts, and glycosides which are prominently used in Ayurvedic medicines [5]. Juice of its leaves finds applications in the treatment of dysentery and headache [6, 7]. Due to the high antioxidant property of its fruits which is similar to Vitamin E, it helps in maintaining the cholesterol level [8]. It strengthens the heart muscle and also improves cardiovascular output [9]. *T. arjuna* is used to cure coronary artery disease, angina, heart failure, edema, and hypercholesterolemia [9]. Due to its diuretic, prostaglandin enhancing and coronary risk factor modulating properties, it is used in the treatment of asthma.

Active phytochemicals present in medicinal plants along with antimicrobial activity plays a key role in the prevention of various infectious diseases and could be a potential tool in combating antibiotic resistance among pathogenic microbes [10]. It has also been used traditionally as a milk decoction [11]. *T. arjuna* has been mentioned in Vagbhata and Ashtânga Hridayam as a medicine for the treatment of haemorrhage, wounds, and ulcers. Thus the study was conducted in order to investigate the presence of secondary metabolites in the leaf, bark, and stem (fig. 1) of *T. arjuna* plant and it's *in vivo* antimicrobial activity.

Materials and Methods

Collection of Plant Material: *Terminalia arjuna* leaves, bark and fruits were collected from a location situated at the bank of Ajhari kund (Geographical location: 27°362 15.043 N; 77°352 00.863).

Bacterial Culture: *Bacillus subtilis* (MTCC 2057), *Escherichia coli* (MTCC 294), *Staphylococcus aureus* (MTCC 3160) and *Pseudomonas aeruginosa* (MTCC 2581) bacterial strains were employed in the antimicrobial analysis.

Fungal Cultures: *Aspergillus niger* (MTCC 282), *Aspergillus flavus* (MTCC 873) and *Candida albicans* (MTCC 227) fungal strains were used in the antimicrobial analysis.

Preparation of Extracts: 30 gm of each plant part (leaf, bark, and flower) was sequentially extracted with different solvents (200 ml each) on the basis of their decreasing polarity (Water> Methanol> Ethanol> Acetone> Chloroform> Petroleum ether) by using Soxhlet apparatus for 18 hours at a temperature equivalent to the boiling point of the solvent [12]. These extracts obtained were filtered by using Whatman No. 1 filter paper

Fig. 1. Plant parts of *T. arjuna* employed in the study. **(A)** Leaf and flowers. **(B)** Fruit. **(C)** Bark.

and concentrated by evaporating the solution at 40°C in a hot air oven for 24 hrs. Few drops of chloroform were added to the extract to prevent the growth of fungal contaminants and ultimately the dry extract obtained was stored at 4°C in sterilized sample bottles [13]. Percent extractive values of each extract were calculated by the following formula.

$$Percent\ Extract = \frac{Weight\ of\ dried\ extract}{Weight\ of\ dried\ plant\ material}\ x\ 100$$

Phytochemical screening of Extracts: The standard methods of Harbarne [14] were used to test for the presence of phytochemical in the different extracts of *T. arjuna* leaf, bark and fruit extracts.

Alkaloids [Mayer's Test]: 0.5-1 ml of the sample was taken in a test tube and few drops of Mayer's reagent were added to it. The solution was well shaken and allowed to stand for some time. The appearance of cream color ppt. indicates the presence of alkaloids.

Carbohydrates [Benedict's test]: 0.5-1 ml of the sample was taken in a test tube and few drops of Benedict's reagent (alkaline solution containing cupric citrate complex) were added to it. Upon boiling in a water bath, the appearance of a reddish-brown ppt indicates the presence of a reducing sugar.

Cardiac glycosides [Sodium nitroprusside test]: 0.5-1 ml of the sample was taken in a test tube and a pinch of sodium nitroprusside powder was added to it. 2-3 drops of 10% sodium hydroxide solution was added, mixed and allowed to stand for 2-3 min. The appearance of red color indicates the presence of cardiac glycosides.

Proteins [Ninhydrin test]: 0.2 % solution of Ninhydrin was added to 0.5-1 ml of the sample and subsequently heated. The appearance of a purple/violet color indicates the presence of protein.

Detection of phytosterols [Salkowski test]: 0.5-1 ml of sample was taken in a test tube, few drops of chloroform and conc. sulphuric acids were added. The solution was mixed well and kept undisturbed for some time. The appearance of a lower yellow layer indicates the presence of triterpenoids.

Flavonoids [Alkaline reagent test]: 0.5-1ml of sample was taken in a test tube; few drops of 10% sodium hydroxide solution were added. The appearance of an intense yellow color, which turns colorless on the addition of few drops of dil. Hydrochloric acid (HCl), indicates the presence of flavonoids.

Tannins and phenolic compounds [Ferric chloride test]: 0.5 ml of sample was taken in a test tube. Add few drops of ferric chloride. The appearance of blue- green color confirms the presence of tannins and phenols.

Test for Terpenoids: 2 ml of sample was taken in a test tube, 2 ml of chloroform and conc. sulphuric acids were carefully forming a layer of each. The appearance of reddish brown color indicates the presence of terpenoids.

Test for Saponins: 2 ml of sample was dissolved in 2 ml of Benedict's reagent. The appearance of a blue-black color precipitate indicates the presence of saponins.

Phenols/polyphenols: A small amount of sample was dissolved in distilled water and 0.5 ml Folin-ciocalteu reagent was added to it. 2 ml of 20% sodium carbonate was added to the solution. The appearance of a bluish color indicated the presence of phenols.

Screening for in vitro antimicrobial activity: The antimicrobial susceptibility tests were carried out using disc diffusion assay [15]. Sterile filter paper discs (Whatman no. 1, diameter 5 mm) were impregnated with 40 ml of the extract (10 mg/ ml) and left to dry in vacuum so as to remove residual solvent. The bacterial and fungal pathogens were initially grown on Mueller-Hinton broth and Czapekdox broth medium respectively. Bacterial and fungal suspensions were prepared by obtaining the inoculum size 1×10^7 CFU/ml in a sterilized medium [12]. Under aseptic condition

Mueller-Hinton agar media and Czapekdox Agar medium agar medium were poured in sterilized petri dishes for growth of different pathogenic bacterial and fungal strains [16]**.** Using a sterile cotton swab, 500 µl of the suspensions were spread over the Mueller-Hinton and Czapekdox agar plates for obtaining uniform microbial growth on test plates. Different extract discs were then placed in triplet on the Mueller-Hinton and Czapekdox agar plates at concentration of 10 mg/ml. Results were compared with that of the standard Himedia antibiotic disc i.e., Gentamicin (10 mcg/disc) and Ketoconazole (10 mcg/disc) as standard for bacterial and fungal strains [17]. The plates were then incubated at 37°C for bacterial strains (24 hrs) and at 27°C for fungal strains (48 hrs**)** [18]. Each experiment was repeated thrice and the average inhibition zones for leaf bark and fruit extracts were recorded and compared with the standard reference antibiotics [19].

Statistical Analysis: Statistical analysis of the data (Zone of inhibition) obtained was carried out using one way analysis of variance (ANOVA) using SPSS ver. 20.0 software and Duncan's multiple range test (DMRT) at $p < 0.05$ and $p < 0.01$ to determine the significant difference in mean values among the treated and the control. All values were expressed as mean ± S.E.M (standard error of the mean).

Results and Discussion

Phytochemical screening: Phytochemical analysis of methanol, ethanol, chloroform, acetone, petroleum ether and aqueous (water) extracts of leaves, bark, and fruits of *T. arjuna* revealed the presence of alkaloids, carbohydrates, cardiac glycosides, proteins, phytosterols, flavonoids, tannins, terpenoids, saponins and phenols/ polyphenols. Similar results have also been obtained by other researchers globally [20, 21]. The presence/ absence of various phytochemicals in different plant part (leaf, bark, and fruit) extracts are shown in table 1. The plant leaf extract revealed the presence of flavonoids, proteins, phenols/ polyphenols and carbohydrates ("+" in all extract) while the bark extract displayed the presence of all the active phytochemical ingredients except cardiac glycosides and phytosterols [22].

The fruit extract displayed the high content of proteins, flavonoids, tannins and phenol/ polyphenol ("+" in all extracts) indicating its potential to act as a source for fruit protein concentrate (FPC) along with leaf protein concentrate (LPC) for the feeding livestock's [23]. Almost similar results with slight variations have also been reported in other studies [20, 21, 22, 23].

Antimicrobial activity: Antimicrobial activity of the leaf, bark and fruit extracts were tested against selected microorganisms and inhibition zone was recorded (table 2). Plant extracts of leaf, bark, and fruit of *T. arjuna* showed significant ($p < 0.01$) antimicrobial potential against *B. subtilis*, *S. aureus*, *E. coli*, *P. aeruginosa*, *A. niger*, *A. flavus* and *C. albicans* (fig. 2). Among all the extracts, aqueous extract of fruit displayed maximum

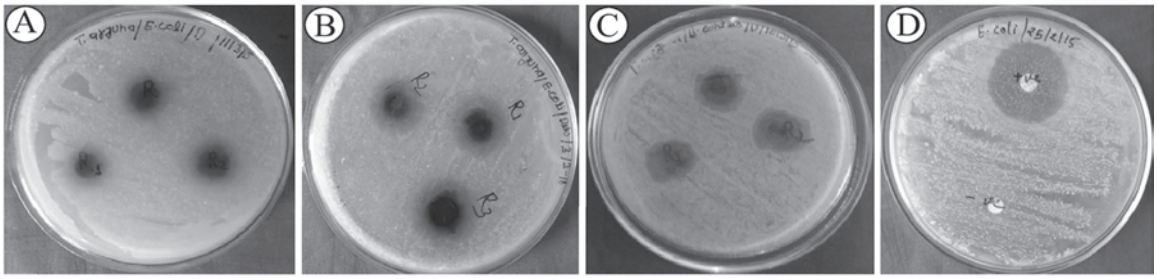

Fig. 2. Antimicrobial activity of *T. arjuna* methanolic extracts. **(A-C)** Antimicrobial activity of leaf **(A)**, bark **(B)** and fruit **(C)** extract against *E. coli* culture. **(D)** Positive control (G: 10 mcg) and negative controls (disk rinsed in methanol) employed during the study.

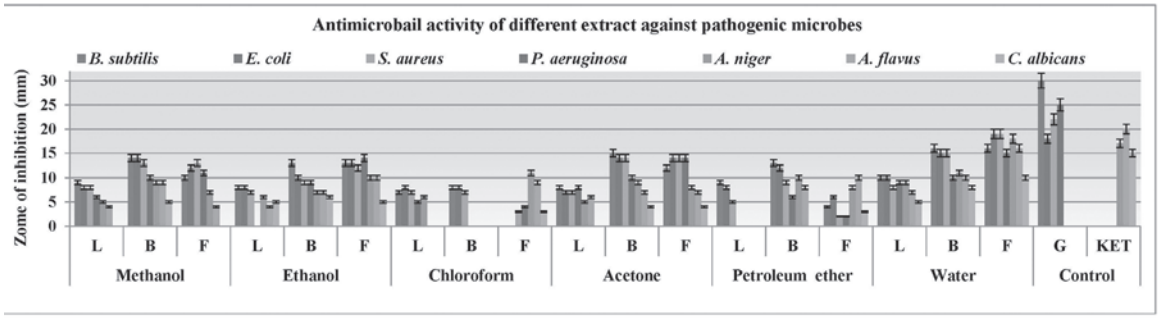

Fig. 3. A comparative antimicrobial activity of different extracts of leaf, bark and fruits of *T. arjuna* in different solvents employed. Considerable high antimicrobial activity was found to be associated with the aqueous extract of the fruits as compared with the activity of other extracts. The antimicrobial activity was compared with that of control.

antimicrobial activity against both bacterial and fungal strains (fig. 3). Most susceptible bacterial pathogens were found to be *E. coli* and *S. aureus* against which, the extracts displayed maximum inhibition zone (7 to 19 mm in leaf, bark and fruit extracts) similar results have also been obtained by other researchers [23, 24]. Among fungal strains genus, *Aspergillus* (*A. niger* and *A. flavus*) displayed maximum inhibition zone (4 to 18 mm in leaf, bark and fruit extract) while other researchers have reported the antifungal activity of leaf and bark extract against *C. albikans* [24]. The antimicrobial activity accessed was compared with that of standard antibiotics (Gentamycin and Ketoconazole) (table 3) (fig. 3).

The values represent the mean SEM of experiments performed in triplet sets. Statistical analysis was performed using one way ANOVA followed by DMRT revealed the results to be significant ($p < 0.01$).

Leaf extract in methanol, ethanol, chloroform, acetone, petroleum ether and water displayed significant ($p < 0.01$) antibacterial activity among all bacterial strains except that of ethanol and petroleum ether against *P. aeruginosa*. Leaf extracts in different solvent exhibited antifungal activity against genus *Aspergillus* except that of petroleum ether (table 2). Similar work on leaf extract carried out by different researchers has produced synonymous findings [24, 25]. Bark extract in almost all (except chloroform extract against *P. aeruginosa* and all fungal strains) displayed significant ($p < 0.01$) antibacterial antifungal activity (fig. 3) which was in accordance with the findings of other research workers [23, 24, 25]. The fruit extract demonstrated maximum antimicrobial among all the plant part extracts. It displayed antimicrobial activity against all pathogenic bacterial and fungal strains except for *B. subtilis* and *E. coli* in chloroform extract and for *C. albicans* in the methanolic extract. Susceptibility differences between Gram-positive and Gram-negative bacteria may be due to cell wall structural differences between these classes. Work carried out independently by different research groups have reported similar findings with the fruit aqueous extract displaying the maximum antimicrobial activity [21, 27]. The maximum antimicrobial activity of fruit extract could be attributed to the presence of flavonoids, tannins and phenol/ polyphenols. This signifies its importance towards the development of novel chemotherapeutic agents.

Conclusion

The study evidenced the presence of active phytochemical compounds within the leaf, bark and fruit extracts of *T. arjuna*. The phytoconstituents present in these plant extracts were responsible for the variation in antimicrobial activity (zone of inhibition) against the pathogenic

Table 1: Phytochemical screening of *T. arjuna* (leaf, bark, and fruit).

S. No.	Solvents	Plant Part	Methanol Ether	Ethanol	Chloroform	Acetone	Petroleum	Aqueous
1.	Alkaloids	L	+	-	-	+	+	-
		B	+	+	+	+	+	+
		F	-	-	-	-	-	-
2.	Carbohydrates	L	+	+	+	+	-	-
		B	+	+	+	+	+	+
		F	-	-	-	-	-	-
3.	Cardiac Glycosides	L	-	-	-	+	+	-
		B	-	-	-	-	-	-
		F	-	-	-	-	-	-
4.	Proteins	L	+	+	+	+	+	+
		B	+	+	+	+	+	+
		F	+	+	+	+	+	+
5.	Phytosterols	L	-	-	-	-	+	-
		B	-	-	-	-	+	+
		F	+	-	-	+	+	+
6.	Flavonoids	L	+	+	+	+	+	+
		B	+	+	+	+	+	+
		F	+	+	+	+	+	+
7.	Tannins	L	+	+	+	-	-	-
		B	+	+	+	+	+	+
		F	+	+	+	+	+	+
8.	Terpenoids	L	+	-	+	-	+	-
		B	+	-	+	-	+	+
		F	-	-	-	-	-	-
9.	Saponins	L	-	-	+	+	-	-
		B	+	+	+	+	+	+
		F	-	-	-	-	-	-
10.	Phenols/ polyphenols	L	+	+	+	+	+	+
		B	+	+	+	+	+	+
		F	+	+	+	+	+	+

Where, “+”: Presence; “-”: Absence, “L”: Leaf, “B”: Bark and “F”: Fruit.

Table 2: Antimicrobial activity of different extracts of *T. arjuna* against pathogenic microbes.

S. No.	Extract (mg/disc)	Solvents	Plant Parts	Inhibition zone (in mm) against pathogenic microbes after 24 hrs incubation						
				B. subtilis	*E. coli*	*S. aureus*	*P. aeruginosa*	*A. niger*	*A. flavus*	*C. albicans*
1.	(10 mg/ disc)	Methanol	Leaf	9 ± 0.21	8 ± 0.1	8 ± 0.2	6 ± 0.2	5 ± 0.2	4 ± 0.1	Nil
			Bark	14 ± 0.1	14 ± 0.1	13 ± 0.1	10 ± 0.2	9 ± 0.2	9 ± 0.2	5 ± 0.2
			Fruit	10 ± 0.1	12 ± 0.2	13 ± 0.1	11 ± 0.2	7 ± 0.1	4 ± 0.2	Nil
2.		Ethanol	Leaf	8 ± 0.2	8 ± 0.1	7 ± 0.2	Nil	6 ± 0.2	4 ± 0.2	5 ± 0.1
			Bark	13 ± 0.1	10 ± 0.1	9 ± 0.2	9 ± 0.1	7 ± 0.2	7 ± 0.1	6 ± 0.2
			Fruit	13 ± 0.2	13 ± 0.1	12 ± 0.2	14 ± 0.2	10 ± 0.2	10 ± 0.2	5 ± 0.1
3.		Chloroform	Leaf	7 ± 0.2	8 ± 0.1	7 ± 0.2	5 ± 0.2	6 ± 0.2	Nil	Nil
			Bark	8 ± 0.1	8 ± 0.2	7 ± 0.1	Nil	Nil	Nil	Nil
			Fruit	Nil	Nil	3 ± 0.2	4 ± 0.2	11 ± 0.2	9 ± 0.2	3 ± 0.1
4.		Acetone	Leaf	8 ± 0.1	7 ± 0.1	7 ± 0.1	8 ± 0.2	5 ± 0.1	6 ± 0.1	Nil
			Bark	15 ± 0.1	14 ± 0.1	14 ± 0.2	10 ± 0.1	9 ± 0.1	7 ± 0.1	4
			Fruit	12 ± 0.2	14 ± 0.2	14 ± 0.2	14 ± 0.1	8 ± 0.1	7 ± 0.1	4
5.		Petroleum ether	Leaf	9 ± 0.2	8 ± 0.2	5 ± 0.1	Nil	Nil	Nil	Nil
			Bark	13 ± 0.1	12 ± 0.1	9 ± 0.2	6 ± 0.1	10 ± 0.1	8 ± 0.1	Nil
			Fruit	4 ± 0.2	6 ± 0.1	2 ± 0.1	2 ± 0.1	8 ± 0.2	10 ± 0.2	3 ± 0.2
6.		Aqueous	Leaf	10 ± 0.1	10 ± 0.2	8 ± 0.2	9 ± 0.2	9 ± 0.1	7 ± 0.1	5 ± 0.1
			Bark	16 ± 0.2	15 ± 0.1	15 ± 0.2	10 ± 0.2	11 ± 0.1	10 ± 0.2	8 ± 0.1
			Fruit	16 ± 0.1	19 ± 0.2	19 ± 0.2	15 ± 0.1	18 ± 0.2	16 ± 0.1	10 ± 0.2

Table 3: Antimicrobial potential of extracts against standard antibiotics.

Antibiotic	Dose (mcg)	Zone of inhibition (mm) against pathogenic agents						
		B. subtilis	*S. aureus*	*E. coli*	*P. aeruginosa*	*A. niger*	*A. flavus*	*C. albicans*
Gentamicin	10	30 ± 0.15	18 ± 0.21	22 ± 0.1	25 ± 0.18	-	-	-
Ketoconazole		-	-	-	-	17 ± 0.22	20 ± 0.21	15 ± 0.22

microbes. Among the different extract of leaf, bark and fruit the antibiogram assay of aqueous and methanolic extract displayed significant ($p < 0.01$) antibacterial potential against test microbes. The present investigation has revealed the broad spectrum antibacterial and antifungal activity of polar constituents of *T. arjuna* plant parts against bacterial strains. The maximum antimicrobial activity of the aqueous extract indicated its potential use as a feed for livestock's with the dual benefit of protection from infection and strengthening the immune system. Further studies are required to isolate and characterize the bioactive principles for developing novel antimicrobial drugs in near future.

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Conflict of interest

The authors declare that there is no conflict of interest.

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