IN VITRO ANTHELMINTIC AND ANTIMICROBIAL POTENTIAL OF FLAVONOID RICH FRACTION FROM *Tamarindus Indica* SEED COAT

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Summary

Context: Microbial and helminthese infections are major cause of various types of illness in both human as well as livestock's. Under the chronic influence, object may suffer from variety of disorders like measles, malaria, trichinosis, cysticercosis, ascaridiasis. **Aim:** the objective of present study is to execute the ability of flavonoids rich fraction of *Tamarindus indica* seed coat extract (HTI) was tested for antimicrobial and anthelmintic bioassay using different species of microbes (Gram positive and Gram negative) as well as helminthese respectively. **Material and methods:** The total flavonoids contents of HTI were determined through spectrophotometrically. The agar well diffusion method was used to evaluate the antimicrobial ability of HTI through measuring the zone of inhibition (mm). In addition to microbicidal action, HTI was evaluated to determine the anthelmintic action by taking shortest paralysis and death time (min) for helminthese. **Results:** the results of *in-vitro* study showed that HTI (100 µg/mL), was much effective against both Gram positive as well as Gram negative strains as compared to standard drug tetracycline (10 µg/mL). Along with microbicidal action, HTI demonstrated significant (**p*< 0.05) anthelmintic action against earthworm (*Eisonia fatida*) and tapeworm (*Taenia solium*) as compared to standard drug piperazine citrate (10 µg/mL). **Conclusion:** The results showed that, HTI possess powerful microbicidal as well as anthelmintic action as compared to standard drug. Thus studies support the ethonomedicinal and folkloric claim about *T. Indica*.

Key words: Agar well diffusion**,** anthelmintic, flavonoids, gram negative, gram positive, *Tamarindus indica*

Introduction

The ability of a multi-cellular organism to protect itself against invasion by microbes depends on its ability to build up strong immune responses. [1] Infection due to microbes including bacteria, fungi, protozoa and gastrointestinal nematode are widespread in humans and livestock affecting enormous population of the world, particularly in developing countries.[2-4] Initially these

infections are not directly critical and may cause urinary tract inflammations (UTI), swelling and delay in wounds healings. Chronic infections in humans may lead to malaria, measles, pneumonia and paratyphoid fever retards the growth, lowered productivity and cognitive ability in children result in mortality.[5-8] During the past few decades, despite of several advances made in understanding the mode of transmission and the treatment of these parasites infection, but there are still no effective remedies to control certain helminthes and microbial invasion.[9] The random use of some drugs has generated several cases of resistance. In spite of the fact that, the development of resistance in microbes likes methicillin-resistant *Staphylococcus aureus* and extended-spectrum β-lactamase producing enterobacteriaceae isolates are continuing problems. Mortality rates have been shown to be higher among patients infected with antimicrobial-resistant pathogens compared with patients infected with susceptible pathogens.[10-12] Apart from chemotherapy, ethnopharmacological surveys revealed that the rationale for selection and scientific exploration of medicinal plants, since some of these native remedies are already used by significant numbers of people over extensive periods of time. Plants are impotent source of phytochemical as natural antimicrobial and anthelmintic agents commonly called 'biocides' is gaining popularity also minimize chances of resistance.[13, 14] *Tamarindus indica* L. (family-*Leguminosae*) is extensively used in traditional system of medicine in India and other countries for treating abdominal pain, diarrhea, wound healing, fever, constipation, blood disorders and acne. The plaster of leaves is applied for curing local inflammation.[15] Therefore in the present study, an attempt has been made to provoke the biocidal approach of *T. indica* through flavonoids rich fraction against microbes and helminthese.

Materials and methods

Plant material and extract preparation

Dried seeds of *Tamarindus indica* were collected from the tropical forest research institute (TFRI) of Jabalpur, Madhya Pradesh, India. The material was identified and authenticated by Prof. S.D. Upadhyaya, senior scientist and botanist, Department of Crop and Herbal Physiology, Jawaharlal Nehru Krishi Vishwavidyalaya (JNKVV), Jabalpur, M.P. and the voucher specimen number HD/CHPY/9581 was deposited at JNKVV. The seed coats were mechanically separated from the germ, grounded and classified using a 40-70 mesh and stored at 4°C prior to use. For the purpose of extraction and quantitative determination of flavonoids, the hydroethanolic seed coat extract of *T. indica* (HTI) was prepared by soaking the seeds coat powdered (100 g) in 95% of ethanol and water (1:1) for 72 hr. The solvent of the extract was removed under reduced pressure using a rotary vacuum-evaporator at 50°C and obtained content was subjected to phytochemical estimation for confirmed its efficiency.

Phytochemical analysis and total phenolic content

The HTI was subjected to preliminary qualitative test and total phenolic contents to identify various phytoconstituents present in the seeds coat.[16] It was observed that HTI contains tannins, saponins, steroids, carbohydrates and well rich with flavonoid, isoflavonoid and polyphenolic compounds [Table 1]. The total phenolic concentrations of extracts were determined using the Folin-Ciocalteu method described previously [Table 2].[17] In brief, 200 µL of diluted extract was added to a test tube and then mixed with 1000 µL of Folin-Ciocalteu reagent (1:10). Thirty seconds later and just prior to 8 min, 800 µL of Na_2CO_3 (7.5%) was added. The reaction mixture was incubated at 24°C for 1 hr and absorbance (at 765 nm) of mixtures was traced against blank.

The standard curve was prepared using 1, 10, 100 and 200 mg/L solutions of Gallic acid in ethanol: water (1:1). Total phenol values are expressed in terms of gallic acid equivalent (mg/g of dry mass), which is a common reference compound [Figure 1].

Determination of Total Flavonoid

The concentrated HTI was again exhaustively defatted by refluxing with n-hexane and benzene (15 hr twice). HTI (0.5 mL of 1:10 g/ mL) in ethanol were separately mixed with 1.5 mL of ethanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate and 2.8 mL of distilled water. It remained at room temperature for 30 min. A yellow color indicated the presence of flavonoids.[18]

Antibacterial assay

The agar well diffusion method was conducted to evaluate the inhibitory spectrum of extracts against test microorganisms. The method allows determination of minimum inhibitory concentration (MIC) against various microbial strains.[19]

Microbial Strains

In the present study, all strains including *Staphylococcus aureus* (MTTC-3160) and *Bacillus subtilis* (MTCC-1790) which were Gram-Positive in addition to *Escherichia coli* (MTCC-2960), *Pseudomonas auruginosssa* (MTTC-4676), *Klebsiella pneumonia* (MTTC-3030) which were Gram-Negative bacterial strains with one yeast *Candida albicans* (MTCC-183) used. These strains were obtained from microbial type culture and collection (MTCC) IMTECH, Chandigarh, India.

Preparation of Inoculums

The method required the preparation of inoculums in which the peptone water medium was sterilized by autoclaving at 15 lbs/sq/inch for 15 min. Loop full organisms were transferred from a laboratory maintained culture in to a conical flask containing sterilized peptone water medium. The flask was incubated for 24 hr at 37°C. All the mentioned bacterial strains were incubated at 37 ± 0.1°C, for 24 hr in Nutrient broth and fungal strain of micro-organism inoculated in Sabouraud's dextrose broth and incubated at 28 ± 0.1°C for 48 hr.[20, 21]

Antimicrobial Screening

The antimicrobial activity was evaluated by employing 24 hr cultures of microorganism using different cultured media. The sterilized 96-well microtitre plates were filled with a cultured media with uniform thickness and microbial strains were transferred aseptically. The plates were left at room temperature and allowed for solidification. In each plate three holes of the height 10 cm and 6 mm diameter were made using aluminum sterile borer. The HTI and the standard drug were dissolved in di-methyl sulphoxide (DMSO) and the 0.1 mL of was introduced into the cylindrical hole by means of micropipette. In each hole, the HTI (100 µg/mL), tetracycline (10 µg/mL) and amphotericin B (10 µg/ml) as standard were added under aseptic conditions and labeled accordingly. The plates were kept in the refrigerator for 2 hr for diffusion and incubated at 37 ± 1°C for 24 hr for bacterial strains and 28 ± 0.1°C for 48 hr for fungal strain and zone of inhibition (mm) was evaluated [Table 3].[19]

Anthelmintic Assay

The tapeworm *Taenia solium* (Cestoda), round worm *Trichinella spiralis, Ascaridia galli* (Nematoda) and the earthworm *Pheretima posthuma* (Annelida, Megascolecidae) were used for evaluating the anthelmintic activity [Table 4]. Formulated suspension (10 mL) containing different concentrations of HTI (25, 50, and 100 mg/ mL in distilled water) were prepared and six worms of about the same size per petridish for different species were used and cultured on nematode growth (NG) agar seeded. The paralysis and death time were recorded at room temperature. The time for paralysis was noted when no movement of any sort were observed except when the worms were shaken vigorously. Time for death of worms were recorded after ascertaining that worms neither moved when shaken vigorously nor when dipped in warm water (50°C). The warm environment stimulates & induces movement in the worms, if alive. Piperazine citrate (10 mg/mL) was used as standard drug and distilled water served as the control [Table 5]. [22]

Statistical Analysis

Data are given as means ± SEM, of at least triplicate experiments. Significant differences between groups were determined by analysis of variance (ANOVA) complemented with Dunnett´s post hoc multiple range tests where the *P* value ≤0.05 was considered as significant.

Results

From perusal observation (Table: 1 and 2) preliminary phytochemical analysis of HTI showed the presence of saponins, tannins and well rich fraction of polyphenolic like flavonoids and phenols. The HTI showed broad spectrum of a strong antimicrobial activity against all tested antimicrobial strains, the susceptibility of the test microorganisms was found to be significant ($p<0.05$) in the cases of Gram-Positive followed by Gram-Negative strains and fungus *candida albicans* as compared to standard antibiotics, respectively (Table 3). The diameters of growth of inhibition zones (clear zones around wells) ranged from 11-24 mm (including the diameter of the disk, 10.2 mm) with the highest inhibition zone values observed against the pathogens *Staphylococcus aureus* (24 ± 1.35 mm) and *Bacillus subtilis* (22 ± 0.95 mm).

The results of study indicates that, HTI was comparatively more significant ($p<0.05$) than the piperazine citrate against tapeworms (*Taenia solium*) and earthworms (*Eisonia fatida*), but not showed prominent lethal action against round worms (*Ascaridia galli*) and earthworm (*Pheretima posthuma*) than that of piperazine citrate (Table 5). Among all tested concentration, the HTI at 100 mg/mL showed significant ($p<0.05$) anthelmintic action (paralysis and death) as compared to piperazine citrate. The mean paralysis and death time of HTI against *Taenia solium* (9.1 ± 0.39 and 13.9 ± 0.53 min, respectively), *Eisonia fatida* (5.13 ± 0.35 and 15.61 ± 0.25 min, respectively) were much shortest whereas the time for piperazine citrate was found to be circuitous against *Taenia solium and Eisonia fatida* (16.4 ± 0.39; 10.2 ± 0.28 and 6.3 ± 0.15; 17.5 ± 0.29) respectively.

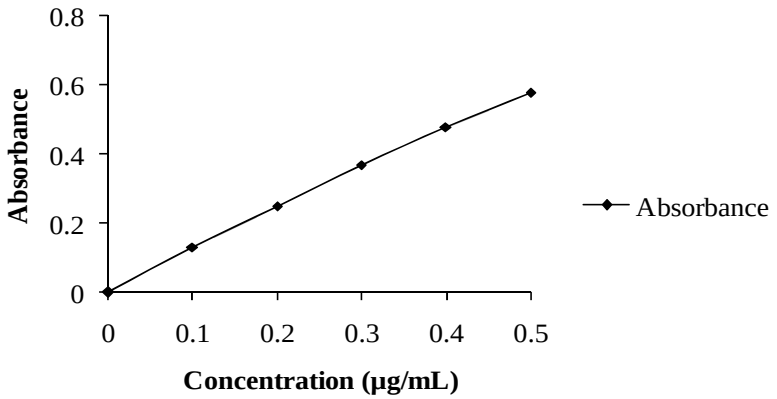

Fig 1: Determination of total phenolic compounds from HTI

Table 1: Preliminary phytochemical screening of HTI

Phytochemicals	Specific tests	Results	Observation
Flavonoids	Shinoda test	+	Dark red color solution obtained
Tannins	Phlonata nins test	+	Brick red precipitate was formed
Alkaloids	Wagner's reagent	+	Light red color solution obtained
Saponins	Frothing test	+	Light red solution with bulky foam obtained
Glycosides	Salkowskii test	-	-

Table 2: Flavonoids and Phenol contents in HTI

Hydroethanolic seed coat extract of *T. indica* (HTI)	Flavonoids (mg/g)	Phenol (mg/g)
	26.12 ± 0.54	65± 0.21

Each value was obtained by calculating the average of three experiments ± standard deviation

Table 3: effect of HTI on diameter zone of inhibition in cultured discs

Diameter zone of inhibition (mm)			
Microorganism	HTI (100 µg/mL)	Amphotericin B (10 µg/mL)	Tetracycline (10 µg/mL)
Staphylococcus aureus	24 ± 1.35*	-	21 ± 1.56
Bacillus subtilis	22 ± 0.95*	-	10 ± 1.42
Escherichia coli	18 ± 1.10	-	17 ± 0.89
Klebsiella pneumonia	11 ± 0.20*	-	9 ± 0.68
Pseudomonas aurigenosa	20 ± 1.27*		16 ± 1.15
Candida albicans	13 ± 1.02	17 ± 1.25	-

Effect of HTI on microbial growth and all data are given as means ± SEM of at least triplicate experiments, where the *P** value ≤0.05 was considered as significant as compared to standard drug.

Table 4: Organism used for Anthelmintic potential

Scientific name	Group	Common name	Host	Disease
Trichinella spiralis	Nematoda	Round worm, pork worm, trichina worm	Rats, humans bears, Pigs	Trichinosis
Taenia solium	Cestoda	Pork, tapeworm	Pig and humans	Taeniasis (adult) and cysticercosis (larval)
Pheretima posthuma	Annelida	Earthworm	----------	---------
Ascaridia galli	nematoda	Roundworm	Birds	Ascaridiasis

Discussion

Phytochemical estimation of hydroethanolic seed coat extract of *Tamarindus indica* (HTI) reveals presence of tannins, saponins, steroids, carbohydrates and flavonoids. Out of these phytoconstituents polyphenols and flavonoids have been well known to exhibit antibacterial activities with distinguished characteristics in their reactivity with proteins related polyamides polymers.[23] Another probable mechanism for growth inhibition of microorganisms by phenolic compounds may be due to iron deprivation or hydrogen bonding with vital proteins such as microbial enzymes.[24] The findings suggested that ability of HTI to penetrate the cell walls with hydrophobic surroundings (Gram negative) and hydrophilic environment (Gram positive) of bacteria responsible for the bactericidal action which can be isolated and identified by some analytical techniques.[25] Helminthic infections of the gastrointestinal tract (GIT) of humans and animals have been recognized to adversely affect the health status of large populations with a consequent lowering of resistance to other diseases. It has been confirmed that, previously reported all anthelmintics are toxic to earthworms therefore substance which is toxic to earthworms is worthy for investigation as an anthelmintic.[26] In parasitic studies, functional proteins such as acetylcholinesterase (AChE) that might elicit protective host immunity against infection have been identified. AChE is a well established enzyme of the invertebrates nervous system and muscles. Various AChE inhibitors are being used as anthelmintic drugs.[27] Some anthelmintics seem to act through the deleterious generation of reactive oxygen and nitrogen species to which helminthes have no or relatively low antioxidant defenses when compared with aerobic organisms.[28] The predominant effect of piperazine citrate on worm is to cause a flabby paralysis which results in exclusion of the worm by peristalsis. Consequently piperazine citrate produces hyper polarization by increasing chloride ion conductance of worm muscle membrane and reduced excitability that leads to muscle relaxation and flaccid paralysis.[29] The findings of the present study are promising, particularly in veterinary medicine for the ethnoveterinary management of nematode and cestode parasitic diseases in pigs, sheep and goats, thus justifying its use in traditional medicine system.

Table: 5 Anthelmintic activity of hydroethanolic seed coat extract of *Tamarindus indica* (HTI)

Groups	Concentration (mg/mL)	Time (min) for paralysis (P) and/or death (D) of different worms (mean values)							
		Eisonia fatida Earthworm		*Pheretima posthuma* Earthworm		*Ascaridia galli* Roundworm		*Taenia solium Tapeworm*	
		P	D	P	D	P	D	P	D
Control		-	-	-	-	-	-	-	-
HTI	25	9.1±0.21	23.24±0.53	13.2±0.42	29.1±0.33	8.4±0.36	21.3±0.37	18.0±0.53	40.3±0.21
	50	8.34±0.27	20.62±0.46	11.4±0.31	26.3±0.54	7.54±0.29	18.28±0.43	14.35±0.47	29.2±0.36
	100	5.13±0.35*	15.61±0.25*	7.3±0.11	21.8±0.48	5.14±0.23	15.59±0.45	9.1±0.39*	13.9±0.53*
Piperazine citrate	10	6.3±0.15	17.5±0.29	4.7±0.22	7.1±0.49	2.9±0.57	5.51±0.46	10.2±0.28	16.4±0.39

All Values represent Mean± SD; n=6 in each group. Significant (*P** value ≤0.05) differences between test groups and standard treated groups were determined by analysis of variance (ANOVA) complemented with post hoc Dennett's multiple range tests. Formulation containing 25, 50, 100 mg/ml of HTI produced dose dependent paralysis ranging from loss of motility to loss of response to external stimuli.

Conclusion

The present study focused the potential of seed coat extract of *T. indica* against gram positive and gram negative bacterial strains as well as explore its ability to induce paralysis and death of helminthese (anthelmintic action) as compared to standard drugs. These findings were in support with folk claims about the use of plants. In future, there will be a wide scope to isolate the active chemical moiety of *T. indica* which will ensure its prospective.

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