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Antimicrobial, antioxidant and anti-hemolytic effect of different fractions of ethanolic extracts of *Tamarindus indica* L. bark

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Abstract
This study has conducted an investigation on evaluation of antimicrobial, anti-hemolytic and antioxidant activity of different fractions ethanolic extract of *Tamarindus indica* L bark against pathogenic bacteria, fungi. Four different Species of Gram-positive and Gram-negative bacteria, four species of fungi, *Proteus* hemolysin are used in this study. *Tamarindus indica* L. is widely investigated but limited information is available on its bark (*Ti*-b). *Ti*-b was studied to combat against uropathogen *Proteus* for anti-hemolytic effect, isolated from UTI patient's catheter. Ethanol fractions of each specimen were tested for antimicrobial and antioxidant study. Active specimen was partitioned with TLC method to determine bioactive compound. Hemolysis caused by secreted hemolysins of *Proteus* was prominent (75%). Phenolic compound in *Ti*-b potentially inhibited that hemolysis. Phenolic compound(s) in *Ti*-b enabled to deactivate *Proteus* hemolysins and thereby strongly inhibited hemolysis. *Ti*-b show strong antioxidant property.

Keywords: Antimicrobial, antioxidant, anti-hemolytic, *Tamarindus indica* L. bark

Introduction
World is endowed with a rich heritage of medicinal plants and it has been used as remedies for disease [1]. Plants have the ability to synthesize a wide variety of chemical compounds that are used to perform important biological functions and to defend against attack from predators such as insects, fungi and herbivorous mammals [2].

Conventional systems of herbal medicine have been using from ancient times. Medicinal plants especially herbs have been the principal source of most of the drugs. Now-a-days about 70% of the world population is depending on medicinal herbs. Medicinal plants contain so many chemical compounds which are the major source of therapeutic agents to cure human diseases [3]. For a long period of time, medicinal plants have been the valuable source of natural products for maintaining human health, especially in the last decade, with more intensive studies for natural therapies. The use of phytochemicals for pharmaceutical purposes has been gradually increased.

According to World Health Organization the medicinal plants could be the best source to obtain a variety of drugs. Approximately 80% people in developed countries use traditional medicine, which has compounds derived from medicinal plants. Therefore, such plants should be investigated to better understand their properties, safety and efficiency [4]. The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments. In the last few years, a number of studies have been conducted in different countries to prove such efficiency [5-9]. Many plants have been used for their antimicrobial traits, which are due to compounds synthesized in the secondary metabolism of the plant. The antimicrobial properties of medicinal plants have been investigated by many investigators worldwide, especially in Indian region. Thirty one medicinal plant species have been reported by traditional healers as being used for UTIs, including leucorrhea, frequent or infrequent urination, cloudy urination, and burning sensations during urination in Bangladesh [10].

Production of cytotoxic hemolysins is common in both Gram-positive and -negative pathogenic bacteria including *Proteus*, but its extent in inter-species and intra-species varies greatly [11]. The hemolytic activity of *Proteus* bacteria is associated to hemolysins, HpmA and HlyA. Especially the predominant hemolysin HpmA is responsible for tissue damage which is activated when its N-terminal peptide is cleaved [12].

Materials and Methods

Bacterial strains

Eleven *Proteus* bacterial strains belonging four species i.e. *P. vulgaris* (hereafter termed as *Pv*), *P. mirabilis* (*Pm*), *P. hauseri* (*Ph*), and *P. penneri* (*Pp*) designated as 11(*Pv*), 66_1(*Pp*), 66_2(*Ph*), 66_3(*Pp*), 66_4(*Pp*), 66_5(*Pp*), 66_6(*Pp*), 66_7(*Pp*), 66_8(*Pp*), 91_1(*Pm*) and 91_2(*Pm*) isolated from municipal tap water of Rajshahi City, Bangladesh were used for anti-hemolytic test, four bacteria *Staphylococcus aureus, Bacillus cereus, Escherichia coli* and *Shigella dysenteriae* and four fungus *Aspergillus niger, Tichoderma viride, Tichoderma herzianum* and *Microphomina phaseolina are used for anti-microbial test* .

Plant material

Plant parts were collected from the medicinal plant garden of University of Rajshahi and around Rajshahi City area, Bangladesh on November to December 2013, and duly identified by a plant taxonomist, Department of Botany, University of Rajshahi, Bangladesh where a specimen voucher (75/05.07.2008, 32/10.05.2007) was recorded in the department herbarium for future reference. Barks were air-dried under shade. Once dried, the plant material was ground, extracted by maceration for more than 72 hrs with ethanol, filtered (Paper Whatman No. 3) and the solvent was vacuum evaporated in a Soxhlet apparatus (Rotary Evaporator, RE 300, Bibby Sterilin Ltd, UK). Then solutions were evaporated to dryness and further dilutions were made in the same solvent to obtain the required extract concentrations for the different assays. The concentrated ethanolic extract of *Tamarindus indica* bark was fractionated by modified Kupchan method into *n*-Hexane, petroleum ether, ethyl acetate and acetone.

Hemolysis assay

Hemolytic Proteus isolates

Proteus strains isolated in our previous study were screened for their hemolytic activities where human erythrocyte cells were exposed to bacterial toxins, hemolysin, and the hemolysis was quantified by OD_{530nm}. All test isolates were found to be hemolytic with variable potentials. Isolate 11(*Pv*) was strongly hemolytic followed by isolates 66_5(*Pp*), 66_7(*Pp*), 66_4(*Pp*), 66_8(*Pp*), 66_3(*Pp*), 66_1(*Pp*), 66_6(*Pp*). Isolates 66_2(*Ph*), 91_1(*Pm*) and 91_2(*Pm*) were weakly hemolytic compared to others. It is thought that *Pm* and *Pv* are more pathogenic than other *Proteus* species. However, the results obtained here showed that *Pp* and *Pv* are equally pathogenic in respect of hemolysis. Koronakis *et al.* [13] showed three types of hemolytic activities (intracellular, cell associated, cell free) in *Pv* and two types (intracellular, cell associated) in *Pm*. Here we found that *Pp* is more hemolytic than *Pm* that might be the results of three types of hemolytic activities of *Pp*. Quantization of three hemolytic activities in *Pv* were measured where hemolysis by cell free toxins was prominent (75%) compared to that of positive control (Triton X-100). However, the hemolysis by cell associated and intracellular toxin were 72 and 5.9%, respectively.

Anti-hemolytic activity test

0.3 ml of blood, 0.5 ml of supernatant toxin, extract of a concentration of 1 mg per ml and sufficient physiological buffer was added to a falcon tube to make final reaction volume of 10 ml. Then it incubated for 4 hours. Dextran and titran x 100 is used as control instead of extract. After incubation it was centrifuged at 4000 rpm for 10 minutes. Then absorbance of each solution was noted at 530 nm at UV spectrophotometer.

Antimicrobial susceptibility tests

The antimicrobial susceptibility tests were determined using the standard disc diffusion method [14]. Standardized inoculums of the overnight grown LB broth cultures were spread on Mueller-Hinton agar plates using sterile swabs. The plates were dried at room temperature for 2 hrs before placing the antibiotic disks at equidistance. The plates were incubated at 37 ºC and the diameters of zone of inhibition were measured after 18 hr. Four pathogenic bacteria were selected for the antibacterial activity test, two of which were gram positive and two were gram negative. The pure cultures were collected from the microbiological research laboratory of Department of Pharmacy, Rajshahi University. Antibacterial activity of leaf extracts were determined against two Gram positive (Staphylococcus aureus, Bacillus cereus,) and two Gram negative bacteria (Escherichia coli, Shigelladysenteriae). Antifungal activity was determined against four fungi (Aspergillus niger, *Tichoderma viride, Tichoderma herzianum, Microphomina phaseolina*). Test organisms were the laboratory stocks of Microbiology Lab, Pharmacy Department, Rajshahi University.

Determination of DPPH• scavenging activity

Phenolic compounds, flavonoids, flavonols and proanthocyanidins function as antioxidants and scavengers of free radicals by rapid donation of a hydrogen atom to radicals. DPPH• is a free radical compound and it has been widely used to test the free radical scavenging ability of antioxidants. Phenolic, flavonoids, flavonols and proanthocyanidins have ability to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH•) free radicals. DPPH free radical is reduced to the corresponding hydrazine when it reacts with hydrogen donors. DPPH can generate stable free radicals in aqueous or methanol solution. With this method, it is possible to determine the anti-radical power of an antioxidant by measuring the decrease in the absorbance of DPPH at 517 nm. In the radical form, this molecule has an absorbance at 517 nm which disappears after acceptance of an electron or hydrogen radical from an antioxidant compound to become a stable diamagnetic molecule.

Free radical scavenging activity of different fractions of ethanolic extract of *Tamarindus indica* bark was determined by DPPH radical scavenging assay as described by Choi et al, 2000 [15]

A solution of 0.004% DPPH in methanol was prepared and 3ml of this solution was mixed with 1ml of 6.25µg/ml, 12.50µg/ml, 25µg/ml, 50µg/ml, 100µg/ml and 200µg/ml of different fractions of ethanolic extract of *Tamarindus indica* bark in methanol. The reaction mixture was vortex thoroughly and left in the dark at RT for 30 minutes. The absorbance of the mixture was measured spectrophotometrically at 517 nm. Ascorbic acid was used as reference standard. Percentage DPPH radical scavenging activity was expressed as $S\% = [(A_0 - A_1)/A_0)] \times 100$, where A_0 is the absorbance of the control without sample, and A_1 is the absorbance of the extractives/standard. Then percentage DPPH radical scavenging activity was plotted against concentration, and from the graph IC_{50} was calculated.

TLC and chemical nature of bioactive compound

For compound separation using thin layer chromatography (TLC), 100 µl of extract was spotted onto the heat activated TLC plate made in the laboratory. Different solvent systems were used as mobile phase and finally ethylacetate: n-hexane (2:1) was selected and used on the basis of best separation obtained. After separation, TLC plate was undertaken to various phytochemical tests using spray technique and the presence of polyphenolic compound in one bioactive fraction was confirmed by treating the fraction with $FeCl_3$ which turned the color of compounds to black [16-17].

Detection of compounds

The following detection methods were applied for the detection of compounds:

(i) Visual detection: The developed chromatogram was examined visually to detect the presence of colored compounds.

(ii) UV light: The developed and dried plates were observed under UV light (Short range: 254 and long range: 366 nm) to locate UV absorbing/quenching compounds.

(iii) Iodine vapor: The developed chromatogram was placed in a closed jar containing crystals of iodine and kept for few minutes. The compounds, which appeared as brown spots, are marked. Unsaturated compounds absorb iodine. Bound iodine is removed from the plate by air blowing.

TLC Spray Reagents [Stahl and Egon, 1969]

(a) Vanillin-Sulfuric Acid	:	For higher alcohol's, phenols, steroids and essential oil.
Spray reagent	:	1 g vanillin is dissolved in 100 ml sulfuric acid.
Treatment	:	The chromatogram was sprayed with 1% vanillin-sulfuric acid reagent and heating at 120 °C is carried out until the spots attain maximum intensity.
(b) Ferric chloride - Ethanol	:	For some of phenolic compounds
Spray reagent	:	5% ferric chloride in absolute ethanol
Treatment	:	The plates are sprayed with the reagent to detect the Phenolic compounds. Phenolic compound show blue spot with the reagent.

Results and Discussion

DPPH free radical scavenging activity

The antioxidant activity of the crude ethanol extract of *Ti-b* was evaluated by DPPH radical scavenging assay. The results of DPPH free radical scavenging assay are given in [figure 1]. All the extractives showed DPPH free radical scavenging activity. The IC_{50} values of *Ti-b* and catechin for DPPH scavenging were shown below. The results demonstrated that *Ti-b* exhibited strong scavenging activity which was found to be very similar to the reference standard catechin and ascorbic acid.

A graphical presentation on free radical scavenging activity of the Catechin and ascorbic acid (standard) and ethanolic extract of *Ti-b* at different concentrations may be:

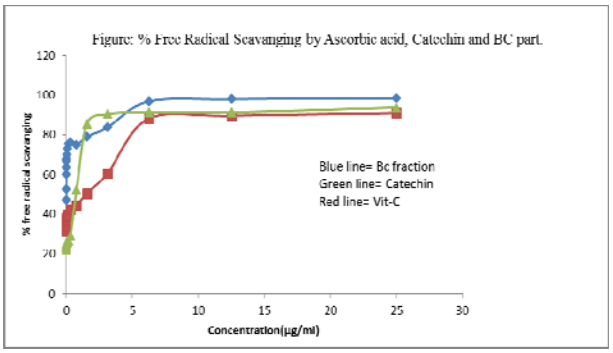

Fig 1: Determination of DPPH free radical scavenging activity of the catechin and ascorbic acid (standard) and ethanolic extract of *Ti-b* at different concentrations.

The results of DPPH radical scavenging assay of extracts and standard catechin are shown in Figure. The IC_{50} values of *Ti-b*, catechin and ascorbic acid for DPPH scavenging were 0.0012µg/ml, 0.75 µg/ml, 1.56 µg/ml, respectively. Among the extracts, the highest scavenging activity were found in *Ti-b* having IC_{50} value of 0.0012µg/ml, which is strong than that of catechin and ascorbic acid. The activity of different extracts exhibited the following order:

Ti-b > Catecin>Ascorbic acid

Antimicrobial activity

Different fraction of ethanolic extracts shows mild antibacterial and antifungal activity. In antibacterial screening at a concentrations of 250 µg/disc, the produced zone of inhibition against *Staphylococcus aureus, Bacillus cereus, Escherichia coli* and *Shigella dysenteriae* for NHF were 14mm, 10mm, 12mm and 9mm respectively, and that for PEF were 15mm, 16mm, 15mm and 12mm respectively in comparison with Kanamycin-30µg/disc the produced zone of inhibition against the same bacteria were 30mm, 27mm, 28 mm and 28mm, respectively [Table 1].

In antifungal screening at a concentrations of 250 µg/disc, the produced zone of inhibition against *Aspergillus niger, Tichoderma viride, Tichoderma herzianum* and *Microphomina phaseolina* for NHF were 12mm, 8mm, 8mm and 10mm respectively, and that for PEF were 15mm, 10mm, 15mm and 12mm respectively in comparison with Kanamycin-30 µg/disc the produced zone of inhibition against the same fungi were 30mm, 30mm, 30mm and 32mm, respectively [Table 2].

Table 1: In vitro antibacterial activity of different fractions of ethanolic extract of *Ti-b*

Bacteria	Zone of inhibition (mm)				
	NHF -250 µg/disc	PEF- 250 µg/disc	EAF -250 µg/disc	ACF -250 µg/disc	Kanamycin-30 µg/disc
S. aureus	14± 0.2	15± 0.3	12± 0.2	8± 0.2	30± 1.2
B. cereus	10± 0.3	16± 0.6	10± 0.3	12± 0.3	27± 0.9
E. coli	12± 0.4	15± 0.2	14± 0.4	10± 0.4	28± 0.8
S. dysenteriae	9± 0.2	12± 0.3	8± 0.2	10± 0.2	28± 0.9

(-) sign indicates no activity. Values were expressed as mean ± SD (n = 3).

Table 2: In vitro antifungal activity of different fractions of ethanolic extract of *Ti-b* and Kanamycin

Fungi	Zone of inhibition(mm)				
	NHF -250 µg/disc	PEF- 250 µg/disc	EAF -250 µg/disc	ACF -250 µg/disc	Kanamycin-30 µg/disc
Aspergillus niger	12± 0.3	15± 0.6	16± 0.2	14± 0.2	30± 0.8
Tichoderma viride	8± 0.3	10± 0.3	12± 0.3	12± 0.3	30± 1.2
Tichoderma herzianum	8± 0.2	15± 0.4	8± 0.4	8± 0.4	30± 0.8
Microphomina phaseolina	10± 0.2	12± 0.2	9± 0.2	10± 0.2	32± 1.3

(-) sign indicates no activity. Values were expressed as mean ± SD (n = 3).

Antihemolytic activity of plant extracts

Isolate 11(*Pv*) chosen on the basis of its strong hemolytic activity was undertaken to a test of hemolysis in the presence of ethanolic extracts (500 µg/ml). *Ti*-b showed promising anti-hemolytic activities compared to that of control where *Ti*-b was stronger. The higher OD_{530nm} values of other plant specimens might be the consequences of partial hemolysis by the extracts. Although the antibacterial activity of *Ti*-b was weak (data not shown), its anti-hemolytic activity was too strong. Therefore, it is noteworthy that *Ti*-b has the phytochemical(s) which enables to neutralize hemolysins, HlyA and/or HpmA, produced by the isolate. Thus *Ti*-b here offered a strong protection against hemolysis occurred by *Proteus* hemolysins [Figure 2].

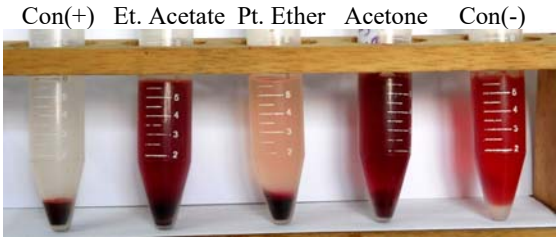

Fig 2: Anti-hemolytic activity of plant extracts. Hemolytic reaction of isolate was conducted in the presence of plant extracts at 500 µg/ml concentration. Con (−) left: reaction mixture in the absence of strain and extract, Con (−) right: reaction mixture in the absence of extract. Petroleum ether fraction of *Ti*-b had the best anti-hemolytic activity.

Identification of chemical nature of bioactive compound in Ti-b

Partial separation of *Ti*-b extract was conducted by TLC using various organic solvent systems whereas ethyl acetate: n-hexane (2:1) system separated seven fractions namely F1 to F7 (Figure 3). Each fraction including baseline components (Bc) were analyzed for their anti-hemolytic activities. Interestingly Bc had a strong anti-hemolytic activity against hemolysis caused by *Proteus* hemolysin [Table 3]. To know the chemical nature of Bc, the visualized bands were solvent extracted and sprayed with various test reagents. The black color of Bc appeared after spray of 5% $FeCl_3$ solution indicated that it is polyphenolic compound. This result manifested that polyphenol(s) in *Ti*-b was a hemolysis inhibitor. The molecular mechanism of polyphenol mediated inhibition of hemolysis caused by *Proteus* toxin is not clear. Nonetheless the obtained result will open up the opportunity to develop drugs that may minimize the bacterial toxin mediated sufferings of the infected patients.

Table 3: Fractionated Petroleum ether part by PTLC was further tested for anit-hemolytic activity.

Con	Bc	F_1	F_2	F_3	F_4	F_5	F_6	F_7
3.41	0.82	2.96	2.90	2.96	2.96	1.60	2.90	2.87

Fig 3: A representative image of preparative thin layer chromatography (PTLC).

Phenolic test

For some of phenolic compounds Spray reagent	:	5% ferric chloride in absolute ethanol.
Treatment	:	The plates are sprayed with the reagent to detect the Phenolic compounds. Phenolic compound show blue spot with the reagent.

The baseline components show positive results.

Table 4: Phytochemical tests to identify the nature of bioactive compound

	Steroid	Terpenoid	Flavanoid	Phenol	Tannin	Glycoside
Bc	-	-	-	+	-	-

Conclusion

Tamarindus indica bark shows potent antioxidant activity and mild anti microbial activity which may open the door of using alternative medicine. The hemolysis of human blood cell caused by secreted *Proteus* bacterial toxins was robustly inhibited by ethanol extract of *Tamarindus indica* bark. Phenolic compound in extract played the role in anti-hemolytic activity by deactivating HlyA and/or HmpA. The result has opened up the possibility of the use of phenolic compound(s) in drug development to relieve the sufferings of patients having UTI and wound infections. Further investigation is needed for the identification of the target compound(s).

Abbreviation

Ti-b = *Tamarindus indica* bark, UTI= Urinary tract infection, TLC= Thin layer chromatography, NHF= N-haxane fraction, PEF= Pet ether fraction, EAF= Ethyl acetate fraction, ACF= Acetone fraction, BC= Baseline component

Data analysis

For data processing, the software Microsoft Excel 2007 was used. Results of triplicate experiments were averaged, and means ± standard deviations were calculated.

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Conflict of Interest
Authors' declare no conflicts of interest.

References
1. Animesh Biswas, Bari MA, Mohasweta Roy, Bhadra SK. Inherited folk pharmaceutical knowledge of tribal people in the Chittagong Hill tracts, Bangladesh. Indian journal of traditional knowledge. 2010; 9(1):77-89.
2. Lai PK, Roy JJ. Antimicrobial and chemopreventive properties of herbs and spices. Curr Med Chem. 2004; 11(11):1451-60.
3. Maurya U, Srivastava S. Traditional Indian herbal medicine used as antipyretic, antiulcer, anti-diabetic and anticancer: A review. Int J Res Pharm Chem. 2011; 1:1152-9.
4. Ellof JN. Which extractant should be used for the screening and isolation of antimicrobial components from plants? J Ethnopharmacol. 1998; 60:1-6.
5. Ayoola GA, Coker HA, Adesegun SA, Adepoju-Bello AA, Obaweya K, Ezennia EC *et al*. Phytochemical screening and antioxidant activities of some selected medicinal plants used for malaria therapy in Southwestern Nigeria. Trop J Pharm Res. 2008; 7:1019-4.
6. Li XM, Brown L. Efficacy and mechanisms of action of traditional Chinese medicines for treating asthma and allergy. J Allergy Clin Immunol. 2009; 123:297-6.
7. Sharan K, Siddiqui JA, Swarnkar G, Maurya R, Chattopadhyay N. Role of phytochemicals in the prevention of menopausal bone loss: evidence from *in vitro* and *in vivo*, human interventional and pharmacokinetic studies. Curr Med Chem. 2009; 16:1138-7.
8. Russo M, Spagnuolo C, Tedesco I, Russo GL. Phytochemicals in cancer prevention and therapy: truth or dare? Toxins. 2010; 2:517-1.
9. Howes MJR, Perry E. The role of phytochemicals in the treatment and prevention of dementia. Drugs & Aging. 2011; 28:439-8.
10. Hossan MS, Hanif A, Agarwala B, Sarwar MS, Karim M, Rahman MTU *et al.* Traditional use of medicinal plants in Bangladesh to treat urinary tract infections and sexually transmitted diseases. Ethnobotany Res Appl. 2010; 8:61-4.
11. Hacker J, Hughes C. Genetic analysis of bacterial hemolysin production. Bull Inst Pasteur. 1985; 83:149-5.
12. Cestari SE, Ludovico MS, Martins FH, Dejato-da-Rocha SP, Elias WP, Pelayo JS. Molecular detection of HpmA and HlyA hemolysin of uropathogenic *Proteus mirabilis*. Curr Microbiol. 2013; 67:703-7.
13. Koronakis V, Cross M, Senior B, Koronakis E, Hughes C. The secreted hemolysins of *Proteus mirabilis*, *Proteus vulgaris*, and *Morganella morganii* are genetically related to each other and to the alpha-hemolysin of *Escherichia coli*. J Bacteriol. 1987; 169:1509-5.
14. Bauer AW, Kirby WMM, Sherris JC *et al.* Antibiotic susceptibility testing by a standardized single disk method. Amer J Clin Pathol. 1966; 45:493-496.
15. Chaity FR, Khatun M, Rahman MS. In vitro membrane stabilizing, thrombolytic and antioxidant potentials of Drynaria quercifolia L., a remedial plant of the Garo tribal people of Bangladesh. BMC Complement Altern Med. 2016; 16:184.
16. Jaiswal Y, Tatke P, Gabhe S, Vaidya A. Rapid high performance thin layer chromatographic method for quantitation of catechin from extracts of Cashew leaves – a short report. Pol J Food Nutr Sci. 2013; 63:49-4.
17. Mandloi S, Mishra R, Verma R, Mugal S, Rajshree S. Phytochemical analysis of the leaf extract of *Terminalia catappa* L. Indian J Applied & Pure Bio. 2013; 28:65-0.