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Augmentation of the growth of pathogenic bacteria by proteins of *Tridax procumbans L.*

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ABSTRACT

Human pathogens were treated with purified extract of calyx of *Tridax procumbans L.* and its effect on growth of the bacteria was observed. The extract had the ability to agglutinate the bacteria and no growth of the bacteria on the solid growth medium was seen when grown at appropriate condition, reflecting the properties to be of pharmaceutical importance.

Key words: Pathogenic bacteria, Proteins, *Tridax procumbans* L.

INTRODUCTION

Plant proteins are reported as the most widely used constituents in biomedical research. Pathogenic organisms often attach to human epithelial or erythrocytes cell surfaces via Plant protein-glycan interactions[1]. Bacterial species are important opportunistic pathogens having the ability to cause diarrhoea, dysentery and extra-intestinal infections[2]. Many pathogenic bacterial species can be distinguished by their carbohydrate rich surface antigen, which is referred to as glycoepitopes. These glycoepitopes are associated with capsular polysaccharides[3].

Plant proteins are used for direct clumping of microorganisms in microbiology. Clumping of Gram-negative *Diplococcus* by Wheat germ agglutinin (WGA) has been considered as positive test for *N. gonorrhea*. Similarly, *Bacillus anthracis* can be identified by clumping with *soybean* agglutinin[4]. Plant proteins are able to selectively clump *Pyogenic cocci*. Groups A, B, C, F and G streptococci can be readily distinguished by Plant proteins, however, *Salmonella genus* was unable to react with Con A.[5].

The interaction between most plant proteins and microbial surfaces are due to the glycoconjugates of microbial surfaces which is a potential site for clumping. Some plant proteins were able to selectively aggregate bacteria[6].

Medicinal plants are routinely exploited for the study of their conventional uses through the authentication of pharmacological effects and can be natural complex sources of new anti-infectious agents. The present study aimed at evaluating the agglutination activity of plant *Tridax procumbans calyx protein* against bacterial strains isolated from human infection following the agglutination assay of *Tridax procumbans calyx protein* with the pathogenic bacteria and to check the growth of clumped bacteria on the growth medium by evaluating the result with suitable statistical analysis.

For our convenience *Tridax procumbans* calyx protein has been designated as TPCP.

MATERIAL AND METHODS

Plant protein

Calyx of the plant *T procumbans L,* a wild medicinal plant of the family compositeae, was used as the source of plant protein[7].

Reagents and Glassware

Papain, BSA, guar–gum, D-glucose, D – galactose, sucrose, raffinose, N-Acetyl-D-glucosamine, D-glucosamine hydrochloride, α-pNPG, β-oNPG, Acrylamide, Bis-acrylamide, β – mercaptoethanol, coomassie brilliant blue (R 250), were obtained from Sigma chemicals, St Louis, M.O., U.S.A. Distilled water, glass wares like petri dishes, pipettes, glass rods, beakers, test tubes, etc. were sterilized in autoclaved at 121°C at 15 lbs pressure for 15 min. Other chemicals were of analytical grade.

Bacterial isolates

The bacterial isolates like *Enterococci faecalis, Escherichia coli, Klebsiella pneumonie, Morganella species, Proteius mirabilis, Pseudomonas aeruginosa, Providentia species, Salmonella typhi, Salmonella paratyphi- A and Salmonella paratyphi-B* were obtained from Department of Microbiology, Indira Gandhi Government Medical College, Nagpur and were grown on specific growth medium.

Purification of plant protein

Preparation of crude extract

Calyx of 45 days old plants (100 g) grown in the garden of University Department of Biochemistry, Nagpur University, Nagpur, India[7], were collected, washed four times under the tap water and two times with distilled water. After soaking between the folds of filter paper, they were homogenized in 1L of 1M NaCl and kept on shaker at 4°C for 2 hours, to ensure proper extraction of the Plant protein. The extract was passed through two layers of cheese cloth and the filtrate was centrifuged at 12000 rpm (9668g) at 4°C for 30 min. The supernatant obtained, designated as crude extract, was used for isolation and characterization of TPCP as described elsewhere[8].

Polyacrylamide Gel Electrophoresis

The homogeneity of TPCP was tested on SDS – PAGE. Molecular weight of the purified plant protein was determined by the method of Weber and Osborn (1969). Acrylamide concentration for the running separator gel was 7.5% and that of the stacking gel was 2.5%, marker proteins such as Carbonic anhydrase 29kD, Trypsin 23kD, Myoglobin 17.2kD, Cytochrome C – 12.3kD, were used as standard proteins. After electrophoresis the gels were stained with 1% coomassie brilliant blue (R – 250) prepared in destaining solution containing 7% acetic acid. The gels were destained in 7% acetic acid[9, 10].

Protein Estimation

Proteins were estimated by the method of Lowry *et al.,* (1951). Bovine serum albumin was used as standard protein (Lowry *et. al.,* 1951)[11].

Growth of bacterial strains

The pathogenic bacteria were grown on appropriate medium on test tube slants (Table 1).

Preparation of the Bacterial growth medium

a) Blood Agar medium

Peptone	2%
Beef extract	1%
NaCl	0.5%
pH	7.4
Agar	3%
Autoclave upto	56^0
Add 10%	Human or Sheep Blood

b) **Macconkeys Agar medium**

Peptone	2%
Na Taurochlorate	0.5%
pH	7.4
Agar	3%
Red Indicator	0.1% aquas solution
Autoclave upto	56^0
Add	1% lactose

c) Shigella Agar (Ready to use Himedia)

d)Nutrient Agar medium

Yeast extract	5g
Peptone	10g
pH	6.8
Agar	15g
Distilled water	1L
Autoclave upto	56^0

Bacterial agglutination

Bacterial agglutination assay using purified TPCP was performed by the method of Zanette, *et. al.,* (2000)[14]. The bacterial agglutination titre was defined as the reciprocal of the highest dilution that was able to induce visible agglutination[12].

Qualitative determination of clumping of bacteria

This method is a quick screening method for qualitative determination of agglutination. A bacterium was carefully removed from the test tube slants and was suspended in PBS. One drop of 50µl of heavy bacterial suspension (OD=1) was mixed on a slide with fifty µl of TPCP (250 µg per ml), respectively[12]. Clumping of bacteria with TPCP is considered as positive test. One drop of 50µl added to 50µl of normal saline was considered as control.

Quantitative determination of the clumping of bacteria

The agglutination titre was determined using microtitre plate. The agglutination of bacteria by TPCP was determined by incubating fifty µl plant proteins (250 µg per ml) followed by serial dilution. Fifty µl of bacteria (OD=1) were added to each well separately. After gentle shaking of the reaction mixture (Plant protein + bacteria) was placed on a rotary shaker (50 rpm) for 10 min, the cells were allowed to settle for 45 minutes and the agglutination in the wells were monitored microscopically. Controls were run simultaneously[12].

The bacterial agglutination titer was defined as the reciprocal of the highest dilution of the *T. procumbans* plant protein extract that yielded visible agglutination activity. One Bacterial agglutination unit (BAU) was defined as the amount of plant protein extract, which causes complete agglutination under the experimental conditions[12].

Growth of clumped bacteria on solid medium

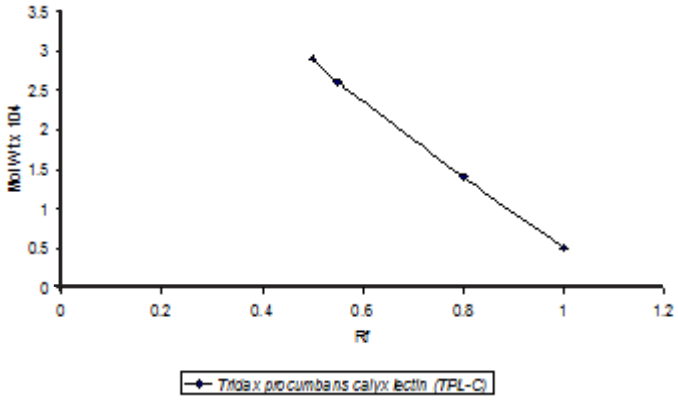
The appropriate agar plates were prepared with specific growth medium as all bacteria were pathogenic and they require specific growth medium. The agar plates were divided into four equal parts. The clumped bacteria by TPCP were determined by incubating fifty µl lectin (250 µg per ml) and fifty µl of bacteria (OD=1) were mixed separately. After gentle shaking, the reaction mixture (lectin + bacteria) was inoculated on agar medium for 24h at 37°C. The bacterial growth was observed on three successive days visually. Controls were run simultaneously[13].

RESULTS AND DISCUSSION

The TPCP could be purified in good yield on cross-linked guar gum by affinity chromatography[8]. The purified protein exhibited little low molecular weight of 23kD on SDS – PAGE (Fig. 1 and 2)[13]. The results show that *T. procumbans* calyx protein was able to agglutinate pathogenic bacteria. The bacterial agglutination titre was least with *Vibrio cholera and Serratia species* as compared to other pathogenic bacteria (Table 3).

Table 1: Growth medium for pathogenic bacteria

No	Name of microorganism	Required Growth medium
1	*Enterococci faecalis*	Macconkeys Agar
2	*Escherichia coli*	Macconkeys Agar
3	*Klebsiella pneumonie*	Macconkeys Agar
4	*Morganella species*	Macconkeys Agar
5	*Proteius mirabilis*	Macconkeys Agar
6	*Pseudomonasaeruginosa*	Macconkeys Agar
7	*Providentia*	Macconkeys Agar
8	*Salmonella typhi*	Macconkeys Agar
9	*Salmonella paratyphi- A*	Macconkeys Agar
10	*Salmonella paratyphi-B*	Macconkeys Agar
11	*Salmonella entritidis*	Macconkeys Agar
12	*Serratia species*	Macconkeys Agar
13	*Shigella dysenteria*	Shigella Agar
14	*Staphylococcus aureus*	Blood Agar
15	*Vibrio cholera*	Macconkeys Agar



(a) carbonic anhydrase,
(b) - Trypsin and *T. procumbans* calyx protein (TPCP).
(c) Myoglobin,
(d) Cytochrome C
(from top to bottom)

Fig. 1: Electrophoretic mobilities of standard proteinsand *T. procumbans* calyx protein(TPCP). Acrylamideconcentration. 7.5%, standard proteins

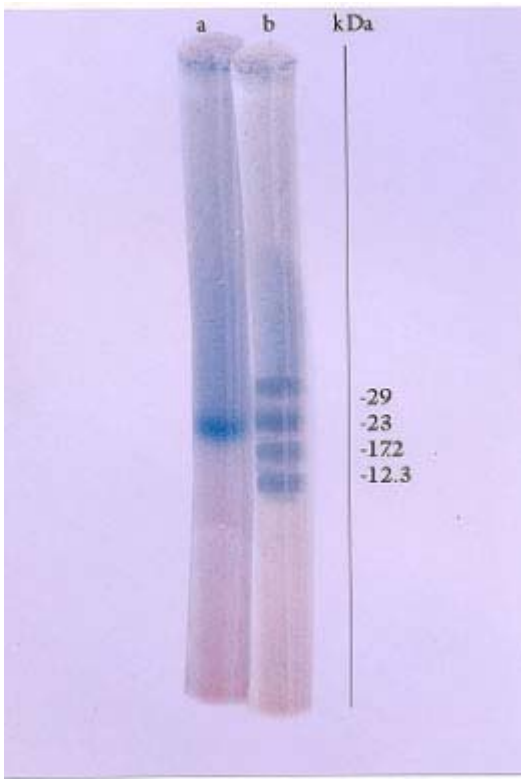


Fig 2. SDS-PAGE: (a). *T. procumbans* calyx protein (TPCP), (b). Standard proteins

The characteristics of bacterial strains that specifically were agglutinated by TPCP are shown in Table 2 and 3; Fig. 3. TPCP proved to be effective agglutinating agent for *Staphylococcus aureus, Enterococci faecalis, Escherichia coli, Pseudomonas* species, *Shigella species, Proteius mirabilis, Morganella* species and *Providentia species.* Whereas, *Salmonella* species and *Klebsiella* species were unable to agglutinate with TPCP. The bacterial strains gave positive agglutination after 4th serial dilution. The strains of *Serratia* and *Vibrio cholera* required lowest titre for agglutination as compared to the other bacteria (Table 3; Fig. 3).

The clumped bacteria by the extract of *Tridax procumbans L.* did not show growth on solid medium (Fig. 4).

The agglutination of bacteria with TPCP indicates that these bacteria carry the receptor sites. This could be recognized by TPCP and bind with the bacteria thereby forming clumps.

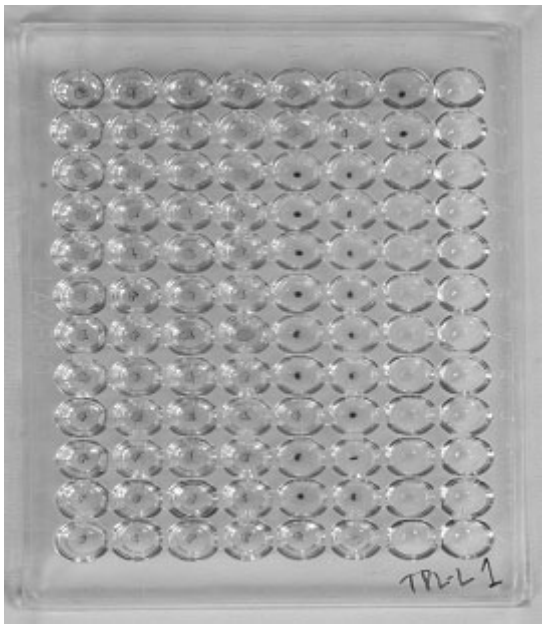
The binding properties and specificities of TPCP may have practical benefit; and it can be used to classify the bacteria, which can serve as an

additional test for identification of several pathogenic bacteria. For instance, these studies could be used as a screening test for *Salmonella species and Klebsiella* that do not have the property to agglutinate with TPCP. In addition, the studies may serve as the basis of a novel therapeutic approach[14]. Plant proteins are able to selectively clump *Pyogenic cocci*, similarly, groups A, B, C, F and G streptococci can be readily distinguished. *Salmonella genus* was unable to react with Con A. The *Shigellae* are simpler from classification viewpoint because they do not possess flagellar H-antigens and consists of four species. Of the four main *Shigella* species tested, only *Shigella flexneri* was readily clumped by Con A. *Shigella boydii* and *Shigella dysenteriae* reacted only slowly with the lectin Con A suggesting that it is possible that lectins could classify *Enterobacteriaceae*, *Salmonella* genus and *Shigella species* [8].

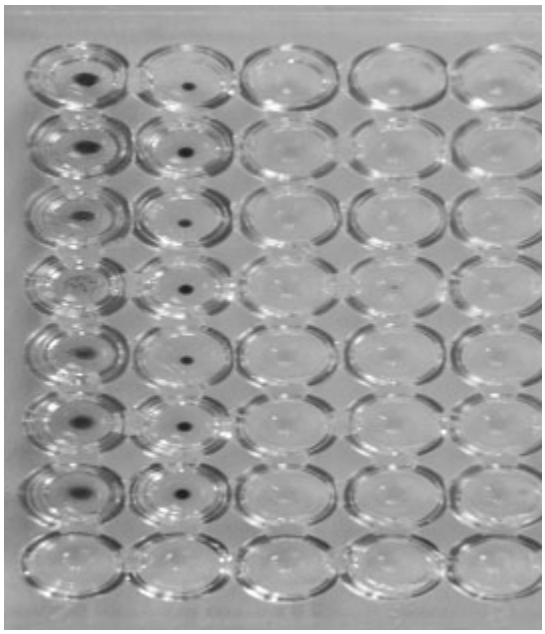
Table 2: Characteristics of pathogenic bacteria used for agglutination with TPCP[a]

S. No.	Name of microorganism	Agglutination	Gram-ve/+ve	Cell wall infection	Resulting composition
1	*Enterococci faecalis*	+	-	Mannose Specific	Bacillary dysentery
2	*Escherichia coli*	+	-	Mannose Specific require Ca+ +	Urinary tract meningitis in children Pneumonia, Urinary tract.
3	*Klebsiella pneumonie*	-	-	Mannose Very large capsule	Pneumonia
4	*Morganella species*	+	-		Urinary tract infection Wound infection
5	*Proteius mirabilis*	+	-		Urinary tract infection Wound infection
6	*Pseudomonas aeruginosa*	+	-	Galactose	Urinary tract, wounds, Burns, lung infection.
7	*Providentia species*	+	-		Diarrhea Urinary tract infection Wound infection
8	*Salmonella typhi*	-	-	Mannose	Typhoid fever
9	*Salmonella paratyphi- A*	-	-	Mannose	Paratyphoid fever
10	*Salmonella paratyphi-B*	-	-	Mannose	Paratyphoid fever
11	*Salmonella entritidis*	-	-	Mannose	Typhoid fever
12	*Serratia species*	+	-		Meningitis Pneumonia,
13	*Shigella dysenteria*	+	-	Mannose	Bacillary dysentery
14	*Staphylococcus aureus*	+	+		Boils carbuncles food poisoning
15	*Vibrio cholera*	+	-		Cholera

a. TPCP – *Tridax procumbans calyx protein.*



a) *Tridax procumbans* calyx protein (TPCP)



b) Positive control (Plant protein + O positive group erythrocytes) and Negative control (Plant proteins only)

Fig. 1: Agglutination of pathogenic bacteria with TPCP (microtiter plate showing carpet and button)

a) Agar Slants

b) Agar plates

Fig. 2: Determination of the growth on solid medium: The clumped bacteria by the extract of TPCP did not show growth on the agar medium. Positive control (Plant protein + Bacteria) and Negative control (Plant proteins only)

Table 3: Agglutination of pathogenic bacteria with TPCP[a]

No	Name of organism and Agglutination with TPCP	No.of Samples	Average titre			Bacterial Agglutination Units		
			Crude	ASF[b]	ACASF[c]	Crude	ASF	ACASF
1	*Enterococci faecalis* +	5	1:2	1:4	1:8	512**	256**	128*
2	*Escherichia coli* +	5	1:2	1:4	1:8	512**	256**	128*
3	*Klebsiella pneumonie* -	5	-	-	-	-	-	-
4	*Morganella species* +	5	1:2	1:4	1:8	512**	256**	128*
5	*Proteius mirabilis* +	5	1:2	1:4	1:8	512**	256**	128*
6	*Pseudomonas aeruginosa* +	5	1:2	1:4	1:8	512**	256**	128*
7	*Providentia species* +	5	1:2	1:4	1:8	512**	256**	128*
8	*Salmonella typhi* -	5	-	-	-	-	-	-
9	*Salmonella paratyphi- A* -	5	-	-	-	-	-	-
10	*Salmonella paratyphi-B* -	5	-	-	-	-	-	-
11	*Salmonella entritidis* -	5	-	-	-	-	-	-
12	*Serratia species* +	5	1:4	1;8	1:16	256**	128*	64**
13	*Shigella dysenteria* +	5	1:2	1:4	1:8	512**	256	128*
14	*Staphylococcus aureus* +	5	1:2	1:4	1:8	512**	256	128*
15	*Vibrio cholera* +	5	1:4	1:8	1:16	256**	128*	64**

Control

1. Normal saline

2. Plant protein only.

*p <= 0.001, **p <=0.02, ***p<=0.05.

a. TPCP – *Tridax procumbans* calyx protein.

b. ASF – Ammonium sulphate fraction.

c. AC-ASF – Affinity column Ammonium sulphate fraction.

Experiments were run in triplicates.

The results presented here indicate that the probable specific receptor for TPCP that agglutinated pathogenic bacteria and obstruct their growth by blocking the surface receptors. Thus this extract can be used for the treatment of bacterial dysentery or for preparation of creams for topical application on wounds to prevent bacterial invasion of tissues.

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Precautions

Safe working and the prevention of infection was strictly followed according to Health Services Advisory Committee (2003).

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