

EFFECT OF POLYPHENOL – TANNIC ACID ON ENTEROTOXIN PRODUCERS

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Abstract: Polyphenols are the micronutrients found in sufficient amount in our diet and evidence for their role in the prevention of degenerative diseases such as cancer and cardiovascular diseases. The health effect of polyphenols depends on the amount of consumed and their bioavailability. Tannic acid is a specific form of tannins, a type of polyphenol (chemical formula $C_{76}H_{52}O_{46}$) which corresponds with decagalloyl glucose, but in fact it is a mixture of polygalloyl glucose or polygalloyl quinic acid esters with numbers of galloyl moieties as per ranging from 2 – 12 depending on the plant source used to extract the tannic acid. Tannic acid is generally found in apricots, Asparagus, Black berries, Broccoli, Cabbage, Chocolate, Grapes, Spinach, Chestnut. In present study, the tannic acid was extracted from cocoa beans and tested qualitatively. Quantification was done by F-D method and free radical scavenging ability was checked by DPPH assay. Minimum inhibitory concentration of tannic acid for enterotoxin producing bacteria (*Escherichia coli*, *S.typhi*, *S. paratyphi A*, *S. paratyphi B*, *Vibrio*, *S.aureus*) by using laboratory strain. From presented study it was concluded that tannic acid has excellent free radical property and even the 10 mg/ml concentration of tannic acid found to be inhibitory for all tested strains. Tannic acid found to be effective to inhibit the activity of coagulase enzyme produced by *S.aureus*. The biofilm production of enterotoxin producing organisms was checked by using CRA method. And the effect of tannic acid on biofilm was checked by Crystal Violet Assay. Polyphenols stimulates the growth of probiotic organisms. The stimulatory effect of Tannic acid was also checked on probiotic organisms.

1. INTRODUCTION

Polyphenols comprise a heterogenous group of compounds characterized by hydroxylated phenyl moieties and generally classified into flavonoids and nonflavonoids. Depending on the oxidation state of the central pyran ring, flavonoids are divided into subclasses: flavonols, flavanols, flavones, flavanones, anthocyanidins and isoflavones and proanthocyanidins. Polyphenolic compounds in plant, including the catechins, exert anticarcinogenic, antimutagenic and cardioprotective effects linked to their free radical scavenging. The number and characteristics of these phenol structures underlie the unique physical, chemical, and biological (metabolic, toxic, therapeutic, etc.) properties of particular members of the class. Phenolic compounds, present in fruit and vegetables, wine, tea, extra virgin olive oil, chocolate, and other cocoa products, are important for the quality of plant based foods: they are responsible for the colour, substrates for enzymatic browning, and are also involved in flavour properties.

Tannic acid is found in the nutgalls. tannic acid was used along with activated charcoal and magnesium oxide in the “universal antidote,” formerly used for poisoning. These three ingredients in combination were believed to work better at absorbing poisons than any of the ingredients alone. Unfortunately, the activated charcoal soaked up the tannic acid, more or less inactivating it. This made the combination less effective. Tannic acid is also taken by mouth and applied directly for bleeding, chronic diarrhoea, dysentery, bloody urine, painful joints, persistent coughs, and cancer. Tannic acid is used for medicinal properties and Hence the effect of polyphenol - tannic acid on enterotoxin producers was studied.

2. MATERIALS AND METHODS

A. EXTRACTION OF TANNIC ACID FROM COCOA BEANS

Cocoa beans are used as plant source for extraction by using organic solvents viz. 80% methanol and acetone and efficiency of each of organic solvent for extraction was checked. 20 gm. of cocoa bean powder is mixed with 80%

Methanol and Acetone. Kept those flasks on rotary shaker for 48 hours at room temperature. Filter the supernatant and fraction was concentrated at 80°C. Cool the residue, weighed and stored at low temperature.

B. QUALITATIVE ANALYSIS

1. Ferric chloride test – Take extract in a tube and add 5% FeCl₃ and if the extract containing tannic acid it will show dark green to blue colour formation.
2. Lead acetate test – Take extract in a tube and 10% lead acetate. Allow it to stand and observe for thick precipitate.
3. Potassium dichromate test – Take extract in a tube and add 2% potassium dichromate solution and observe for dark colouration.

C. QUANTITATIVE ANALYSIS

The concentration of tannic acid in cocoa bean extract. was estimated by Follin-Denis method by keeping Gallic acid as standard stock with concentration 100 mcg/ml by using FC reagent (1:2 diluted).

D. DPPH ASSAY

Diphenyl picrylhydrazyl (DPPH) was used for detection of free radical scavenging property of extracted tannic acid. Pinch of DPPH was added in 50ml of methanol. In tube take 1ml of methanolic extract add 5ml of DPPH reagent to it. Observe for decolourisation of purple colour of DPPH and take OD at 530 nm.

E. CULTURES USED

The laboratory strains known for enterotoxin production such as *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi*, *Salmonella paratyphi A*, *Salmonella paratyphi B*, *Vibrio* were used for determination of MIC.

F. DETERMINATION OF MIC

Preparation of stock: Stock of various concentration was prepared by dissolving extracted tannic acid in Dimethyl Sulphoxide. Concentration taken 10 mg/ml with concentration range 0.5 mg/ml – 10 mg/ml

Procedure: Labelled a sterile petri plate for each concentration required also marked with grids. Add 1 ml of stock of each required concentration in 19 ml molten Muller Hinton agar butt, cool it slightly between palms and mix thoroughly. Pour it in sterile petri plate, allow it to solidify. Numbered the grids with respect to culture to be tested. Spot inoculated the culture on plates containing various concentration of tannic acid.

Interpretation: The cultures sensitive to the concentration of tannic acid contained on agar plate didn't grow at inoculum site, while those are not were grown.

G. DETECTION OF BIOFILM PRODUCTION (FREEMAN ET AL)

Detection of biofilm production on Congo red agar (CRA) supplemented with 5% sucrose is a simple qualitative method to detect biofilm production. The test organisms were streaked on (CRA) and incubated for 24 hours at 37°C. Black colonies with dry consistency indicated biofilm production.

H. EFFECT OF TANNIC ACID ON COAGULASE ACTIVITY OF *S.aureus*

In a tube 3ml of Citrated plasma, 0.1ml of *S.aureus* culture and 1ml of extracted tannic acid (10mg/ml) taken, labelled as test incubated at 37°C for 3 hours. And control kept without tannic acid.

I. EFFECT OF TANNIC ACID ON BIOFILM (CHRISTENSEN'S ET AL)

The effect of tannic acid on biofilms of test cultures was studied by crystal violet assay

J. EFFECT OF TANNIC ACID ON PROBIOTIC BACTERIA

Probiotic bacterial culture suspension prepared by using probiotic capsule "Vibact". Loopful from suspension was streaked on sterile Rogosa agar plate without tannic acid (control) and sterile Rogosa agar plate with 10 mg/ml tannic acid (test).

3. RESULTS

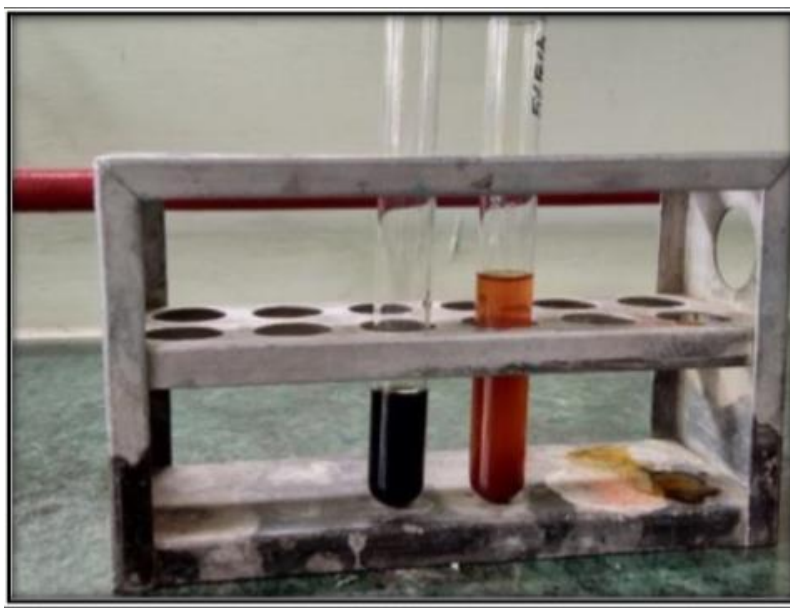
EXTRACTION OF TANNIC ACID

The extraction of tannic acid from cocoa beans by using organic solvent carried out successfully. Extraction was done by using Methanol and Acetone, to study the efficacy of organic solvent for tannic acid extraction. And it was found that Methanol was more efficient solvent for extraction of tannic acid extractions compare to Acetone. The tannic acid present in 20gms of Cocoa beans powder was found to be 0.335gms.

QUALITATIVE ANALYSIS OF TANNIC ACID

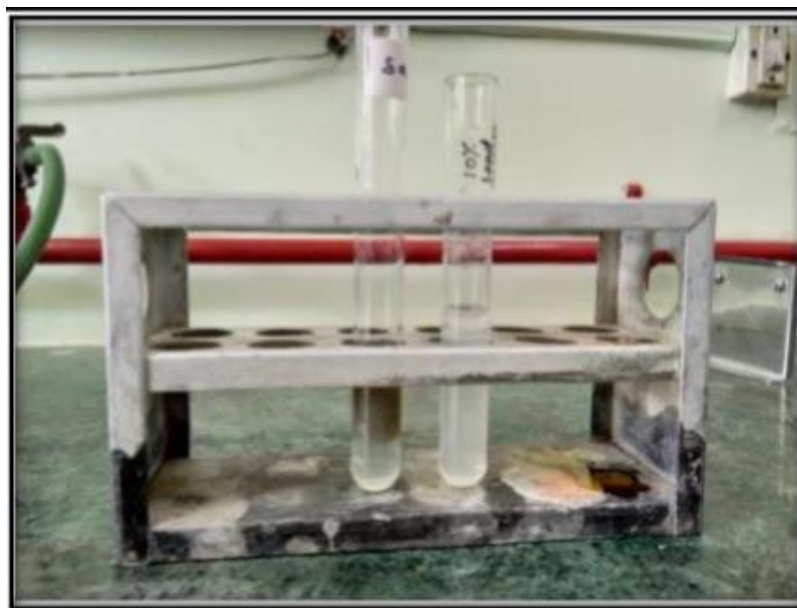
FERRIC CHLORIDE TEST:

In a tube containing cocoa bean extract on addition of 5% FeCl_3 , the solution turned into dark solution indicated the presence of tannic acid in extract.



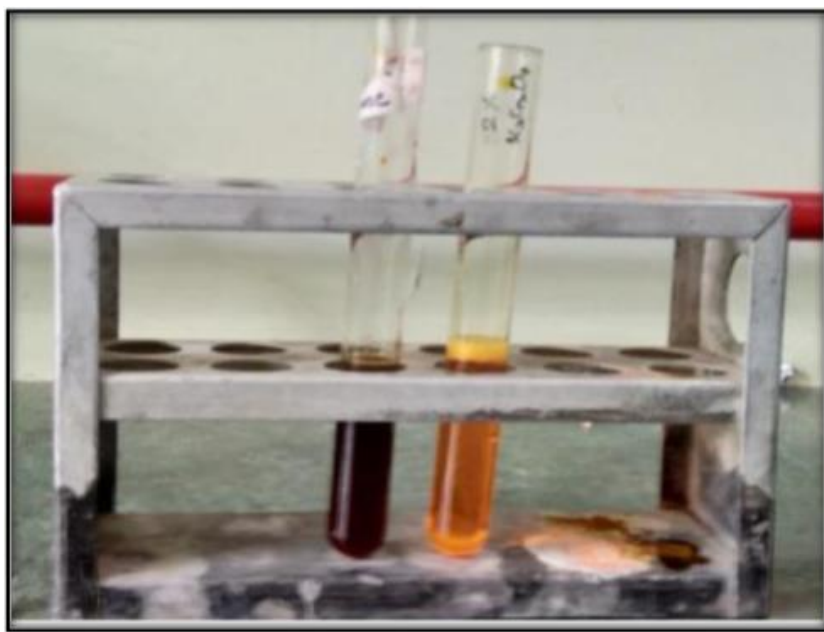
LEAD ACETATE TEST:

In a tube containing cocoa bean extract on addition of 10% Lead Acetate, thick precipitate observed in tube indicated the presence of tannic acid in the extract.



POTASSIUM DICHROMATE TEST:

In the tube containing cocoa bean extract on addition of 2% Potassium Dichromate, the solution turned into dark solution indicated the presence of tannic acid.



QUANTITATIVE ESTIMATION OF TANNIC ACID

Average Concentration of tannic acid in unknown sample was found to be = 1,081mcg/ml
 = 1.081mg/ml

DPPH ASSAY

Free Radical Scavenging activity of Tannic acid was Checked by DPPH assay.

Particulars	Absorbance at 530 nm
Test	
1] undiluted	0.07
2] diluted (1:2)	0.10
Control	0.39

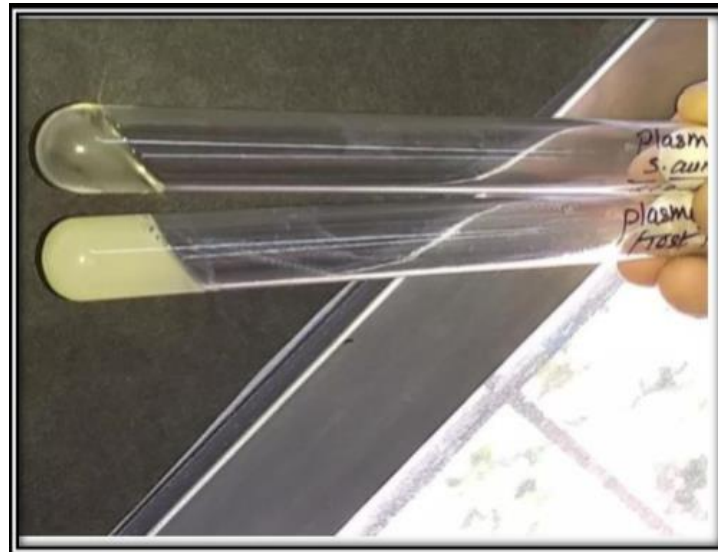
Calculation:

For Undiluted, % RSA = $[0.39 - 0.07/0.39] \times 100 = 82.05 \%$

For Diluted (1:2), % RSA = $[0.30 - 0.10/0.39] \times 100 = 74.35 \%$

EFFECT OF TANNIC ACID ON COAGULASE ENZYME

In control tube, clot was observed indicated that positive coagulase activity shown by *S.aureus*. In contrast in test tube, No clot was observed indicated that tannic acid had inactivated the coagulase enzyme secreted by *S.aureus*. From this, it was concluded that tannic acid present in food found to effective on *S.aureus* which is one of the known enteric pathogen for *Staphylococcal* food poisoning.



DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION:

cultures Conc. Of Tannic Acid (mg/ml)	cultures					
	<i>S.aureus</i>	<i>E.coli</i>	<i>S.typhi</i>	<i>S.paratyphi A</i>	<i>S.paratyphi B</i>	<i>Vibrio</i>
0.5	+	+	+	+	+	-
1.0	+	-	-	+	+	-
1.5	+	-	-	+	+	-
2.5	+	-	-	+	+	-
3.0	+	-	-	+	+	-
3.5	+	-	-	+	+	-
4.0	+	-	-	+	+	-
4.5	+	-	-	+	+	-
5.0	+	-	-	+	+	-
5.5	+	-	-	+	+	-
6.0	+	-	-	+	+	-
6.5	+	-	-	+	+	-
7.0	+	-	-	-	-	-
7.5	+	-	-	-	-	-
8.0	+	-	-	-	-	-
8.5	+	-	-	-	-	-
9.0	+	-	-	-	-	-
9.5	+	-	-	-	-	-
10.0	-	-	-	-	-	-

DETECTION OF BIOFILM PRODUCTION

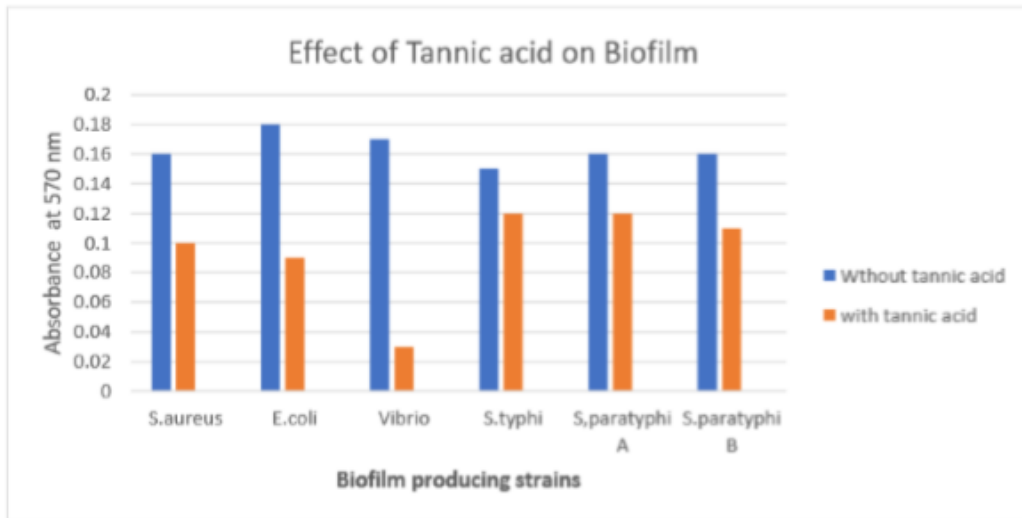
Six cultures were streaked on Congo red agar and out of those, five cultures had produced black colour colonies with dry consistency were *Vibrio*, *E.coli*, *S.aureus*, *S.typhi*, *S. paratyphi A* were biofilm producers, while *S.paratyphi B* has not produced black colour colonies with dry consistency indicated it is not biofilm producer.



EFFECT OF TANNIC ACID ON BIOFILM PRODUCED BY ENTEROTOXINS PRODUCERS BY CRYSTAL VIOLET ASSAY:

The effect of tannic acid on Biofilm produced by enterotoxin producers was checked by Crystal Violet Assay. Results are recorded in table given below.

Cultures used	Absorbance at 570 nm	
	Without tannic acid	In presence of tannic acid
<i>S.aureus</i>	0.16	0.10
<i>E.coli</i>	0.18	0.09
<i>Vibrio</i>	0.17	0.03
<i>S.typhi</i>	0.15	0.12
<i>S.paratyphi A</i>	0.16	0.12
<i>S.paratyphi B</i>	0.16	0.11

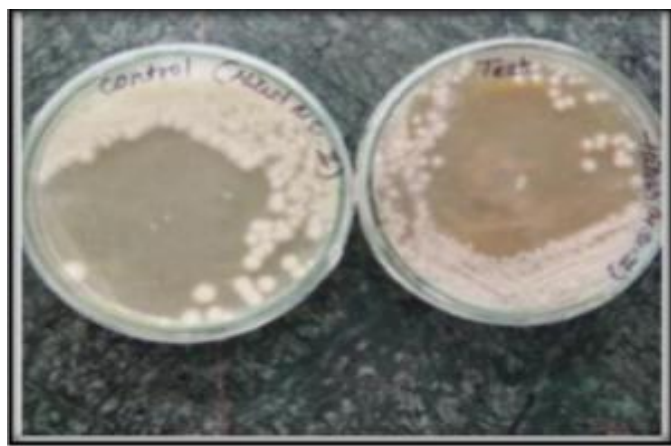


The tannic acid was found to be effective on the biofilm production of tested strain and the cytotoxicity was calculated as follow.

Cultures used	% Cytotoxicity
<i>S.aureus</i>	37.5%
<i>E.coli</i>	50.0%
<i>Vibrio</i>	82.35%
<i>S.typhi</i>	20.0%
<i>S.paratyphi A</i>	25.0%
<i>S.paratyphi B</i>	31.25%

THE EFFECT OF TANNIC ACID ON PROBIOTIC ORGANISMS

Polyphenols stimulates the growth of gut microbiota but inhibits the growth of pathogenic bacteria, hence the effect of tannic acid extracted from cocoa beans was checked on probiotic organisms. It is found that probiotic organisms are growing in the presence of tannic acid (10mg/ml), where *S.aureus* didn't grow at this concentration. The comparative study of growth pattern on control and test plates was carried out, and it was observed that on test plate growth rate was as same as it was on control but the colony size was reduced. It might be due to the high concentration of tannic acid which increasing acidic condition. The concentration of tannic acid can be optimised to get a normal size colony on Rogosa Agar plate.



4. CONCLUSION

Polyphenols present in natural compounds present in natural product has health benefits. Tannic acid is weak acidic compound from tannins family are present in nuts, tea, cocoa beans etc. Tannic acid shows antimicrobial, antioxidant properties which enhances the shelf life of product and prevent the growth of some infection causing bacteria. In 20gms of cocoa beans powder tannic acid content found to be 0.335gms which has free radical scavenging property checked by DPPH assay. The minimum inhibitory concentration of tannic acid determined for enterotoxin producing organisms. Where all tested strains showed inhibition at concentration 10mg/ml. and *Vibrio* didn't grow in presence of tannic acid indicated that all tested cultures of enterotoxin producing bacteria were sensitive to tannic acid.

Tannic acid was inhibited the activity of coagulase enzyme produced by *S.aureus*. Even the tannic acid showed the reduction in biofilm of enteric pathogens, which indicated that tannic acid inhibited the adherence of these pathogenic organisms on intestinal lining. The extracted tannic acid had supported the growth of probiotic organisms in presence of tannic acid (10mg/ml) on Rogosa agar medium which indicated that polyphenols stimulate the growth of normal gut microbiota and probiotic organisms. Hence the intake of natural product containing polyphenolic compounds is must for maintenance of good health.

5. DISCUSSION

Tannic acid is a specific form of tannins, a type of polyphenol. It's weak acidity is due too the numerous phenol group. Tannic acid will hydrolyse into glucose and gallic acid or ellagic acid units. As polyphenols has numerous health benefits and boost the host immunity. Tannic acid has anti-bacterial and anti enzymatic and astringent properties.

The presented study carried out to check the effect of tannic acid on enterotoxins producers. The tannic acid was extracted by using organic solvent i.e Methanol from cocoa beans and even the free radical scavenging property shown by tannic acid which shown that by tannic acid has antioxidant ability which prevent the deterioration of food. The effect of tannic acid on tannic acid was checked on enterotoxin producing bacteria viz, *S.aureus*, *Vibrio*, *E.coli*, *S.typhi*, *S.paratyphi A*, *S.paratyphi B* and it was found that these all organisms were sensitive for tannic acid concentration 10mg/ml. while *Vibrio* was found to be highly sensitive for tannic acid concentration 0.5mg/ml.

The tannic acid inhibited the activity of coagulase enzyme produced by *S.aureus*. Coagulase is enzyme convert the inactive prothrombin in active fibrinogen and formed clot., it helps to hide the bacteria from host immune system. Tannic acid inactivated the enzyme as clot had not formed in presence of tannic acid. The biofilm production of tested cultures was detected by streaking a cultures on Congo red agar medium, and found that except *S.paratyphi B* , all were biofilm producing organisms. The effect of tannic acid on biofilm of all test cultures. It is highly effective on biofilm of *vibrio* by showing 82.35% of cytotoxicity which was calculated by carrying out Crystal Violet Assay/ Cytotoxicity assay.

Polyphenols are known to enhance the growth of gut microbiota. The concentration (10mg/ml) at which at which the growth of *S.aureus* was inhibited, in presence of the same concentration probiotic organisms grown on Rogosa agar medium. Hence the tannic acid extracted from cocoa beans was found to be effective on enterotoxin producers by inhibiting their growth and it stimulated the growth of probiotic organisms. This study showed that the regular intake of polyphenols from natural products will be beneficiary for consumers. Tannic acid present in food also found to be effective against the enteric pathogenic bacteria. It can be used for treatment of diarrhoea and other gastric infections. Even tannic acid will help to stimulate of probiotic organisms which will help in digestion of fibrous food and probiotic organisms will help to remove the pathogenic organisms from intestinal linings.

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