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# PHYTOCHEMICAL SCREENING AND ANTIBACTERIAL PROPERTIES OF *Punica granatum* EXTRACTS AGAINST GASTROINTESTINAL INFECTION AN *In-vitro* STUDY

**RAMSI VAKAYIL[1], NIVEDHITHA KABEERDASS[1], ABIRAMI KUPPUSAMY[1] AND MAGHIMAA MATHANMOHUN[1*]**

[1]Department of Microbiology, Muthayammal College of Arts and Science, Rasipuram, Namakkal DT, Tamilnadu, India.

**AUTHORS' CONTRIBUTIONS**

This work was carried out in collaboration with all authors. All authors read and approved the final manuscript.

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## ABSTRACT

**Background:** *Punica granatum* Linn is a bearing fruit shrub, belonging to the family Lythraceae. Every part of this plant is useful for humans such as seeds and fibers endow with vitamins-C, K and folate. Oil acids of punica, palmitic, stearic and oleic acid present in the seeds. Pomegranate is generally used as a conventional medication in India for the cure of enteric bacterial pathogens.
**Aim:** To study the phytochemicals and antibacterial activities of aqueous, alcoholic and chloroform crude extracts of *P. granatum* peel against some enteric pathogens.
**Materials and Methods:** The isolates were examined by Foldscope microscopy and biochemical tests. Phyto-chemical study analyzed the existence of alkaloids, flavonoids, phenols, saponins, terpenoids, steroids, tannins, reducing sugars, carbohydrates, and amino acids. Vancomycin, tetracycline, chloramphenicol, gentamycin, and co-trimoxazole are the antibiotic disc used in the disc diffusion assay.
**Results:** A multidrug-resistant pattern was observed in the isolated pathogens. As reported by natural medicine *P. granatum* is used for the treatment of enteric pathogens. Antimicrobial activity was performed by the standard procedure (Kirby-Bauer's – agar well diffusion method) using pomegranate extracts (aqueous, alcoholic and chloroform) against the enteric pathogens. Peel crude extracts of *P. granatum* were showed various degrees of antibacterial activities with the maximum mean inhibition zones reaches 36±1.0 mm in diameter for methicillin-resistant *Staphylococcus aureus* (MRSA) with ethanolic extract. The highest zone of clearance was found in ethanolic extract followed by aqueous extract, but only the lowest zone was in chloroform extract. Most significant inhibition was observed against *Staphylococcus aureus, Vibrio cholerae*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *E. coli*, *Salmonella typhi, Shigella dysentriae* followed by *Klebsiella sp.*
**Conclusion:** The end result attained that the peel extracts of *Punica granatum* have impressive pharmaceutical bioactive compounds and antibacterial property. It is used as natural medicine by itself to treat gastrointestinal disorders.

**Keywords:** Antimicrobial; enteric pathogens; multidrug resistance; phytochemicals.

**Corresponding author: Email: mmaghimaa@gmail.com;*

## 1. INTRODUCTION

Traditional herbal and medicinal plant products have been used by humans since the beginning of the human race. The green land of India has a prosperous flora of diverse plants and it is used in habitual therapeutics [1]. In recent times epitomize *P. granatum* as nature power fruit, usually named as Pomegranate, plentiful in India and in herbal remedies it is used to treat different kinds of diseases. The fruits are used for the preparation of juice, divergent value-addition of beverages, jam, and jelly [2]. Egyptians used the extract of this plant akin to fruits and barks for the treatment of numeral infections such as diarrhea and dysentery. It is used as a conventional medicine of Ayurveda for thousands of years [3]. Drug resistance is a foremost clinical problem to treat microbial infections of cholera and dysentery. Though some of the antibiotics are unexplored with antibacterial properties in the pharmacological approach and it supports their use in conventional anti-diarrhoeal remedy [4]. Peel extracts of this fruit have diverse pharmacological properties [5]. The antiviral property of these fruits was described against influenza, herpes, pox and HIV-1 virus [6,7]. The major composition of this plant is with hydrolyzable pigments of tannins and anthocyanins have been worthwhile property on the healthiness of mankind including antibacterial [8]. Punicalagin, caffeic, ellagic acid, and luteolin are the flavonoids of this plant, among them punicalagint only has high inhibitory effects on the influenza virus [9]. The mature pomegranate fruit consists of several fleshy appendages or covering of seeds detached with a white, membranous edible layer of pericarp [10].

Robust antioxidant activity is found in *P. granatum* juice and it is appreciably more than the fruit drinks of grapes and oranges on etc [11,12,13]. The antioxidant naturally found in the fruit will provide the non-aging of cells in mankind. And also it has brawny anti-inflammatory and anticancer properties towards numerous anthropoid malignancies [14]. The purpose of this study is to assess the phytochemicals and antimicrobial property of *P. granatum* peel extract against multidrug-resistant enteric pathogens in *invitro*.

## 2. MATERIALS AND METHODS

### 2.1 Collection of Plant Materials

The fresh, healthful, clean peel of *P. granatum* was gathered from the market of Rasipuram, Namakkal District, Tamilnadu, India during the starting of winter season. The taxonomic identities of the plant were confirmed.

### 2.2 Extract Preparation

The fruit peels were meticulously washed, shadow dried, pulverized and stored in air-tight bottles at 4°C. Fruit peel extract was prepared by soaking 50 gm of powder in 250 ml with three dissimilar solvents as aqueous, ethanol and chloroform for 8 hours using the soxhlet apparatus. The temperature varies sandwiched between extracts by way of 95-100°C for water, 65°C for ethanol and 60-62°C for chloroform. After 8 hours peel extract was collected, concentrated and stored at 4°C in airtight container for further use.

### 2.3 Phytochemical Analysis

The concentrated extract subjected to phytochemical analysis for screening antimicrobial properties. Solvents used in extraction procedures aqueous, ethanol and chloroform. Grade techniques were used for the scrutiny of alkaloids, flavonoids, phenols, saponins, terpenoids, tannins, reducing sugars, carbohydrates, steroids and amino acids in the extract [15,16,17,18].

#### 2.3.1 Test for alkaloids

Mayer's test: $Hgcl_2$ (1.36 gm), KI (5 gm) were disbanded in 60 ml and 10 ml of refined water respectively. The above-mentioned solvents were assorted were watery to l00 ml using refined water. Scant droplets of reagent were supplemented to one ml of peel extract and it creates the colorless precipitate indicates the existence of alkaloids.

Wagner's test: 1 ml of Wagner's test reagent was added in 3 ml of peel extract and advent precipitate of reddish-brown indicates the occurrence of alkaloids [19].

#### 2.3.2 Test for flavonoids

Few droplets of watery HCL and a small quantity of magnesium were supplemented to 0.5 ml of peel extract and simmered for a few minutes. The formation of deep red colour showed the existence of flavonoids.

#### 2.3.3 Test for saponins

In a test tube, 5 ml of refined water was supplemented with 0.2 g of peel extract and shaken vigorously the fusion was kept aside for 3 min. Formation of a honeycomb akin to froth indicates the existence of saponins [20].

#### 2.3.4 Test for phenols

In $Fecl_3$ test 1 ml of sample were supplemented with 2 ml of refined water subsequently scant drops of 10%

(aqueous) $Fecl_3$ solution. The existence of phenols is confirmed by a precipitate of blue or green.

### 2.3.5 Test for tannins

Lead acetate test: Five ml of peel extract was taken in a test tube and it was supplemented with scant droplets of lead acetate (1%). The creation of precipitate in yellow or red indicates the existence of tannins.

$FeCl_3$ test: Peel extract (2 ml) and $FeCI_3$ (2 ml) were assorted for the formation of blue or black precipitate specify the presence of tannins [21].

### 2.3.6 Test for steroids (Salkowski's test)

Salkowski's test: Five drops of conc. sulphuric acid was added in the blend of chloroform and filtered peel extract. Shaken tenderly and allowed to stand carefully. The presence of triterpenes (phytosterol) indicates the appearance of a golden yellow color.

Peel extract (1 mg) was added with chloroform (2 ml) and conc. $H_2SO_4$ (1 ml). The occurrence of steroids was noted by the reddish-brown colour.

### 2.3.7 Test for reducing sugars

Fehling's test: Each 1 ml of Fehling's A and B solutions was assorted and simmered. Peel extracts (1 ml) were added with assorted Fehling's solution and kept for ten minutes in a boiling water bath. Precipitation of yellow followed by brick red showed the existence of reducing sugars.

Benedict's test: In a test tube each 2 ml of peel extract and Benedict's solution were assorted and simmered for ten minutes in boiling water bath until the colour changes (yellow, green and red) confirmed the occurrence of reducing sugars.

### 2.3.8 Test for carbohydrates

In Molisch's test, dispersed and percolated plant extract is supplemented with 5 ml of refined water, 2 drops of alcoholic ά- naphthol solution and by a dropper cautiously pour alongside the test tube drop by drop the conc $H_2SO_4$ (inclined tubes). A creation of violet colour at the union of two liquids shows the existence of carbohydrates [22].

### 2.3.9 Test for Aminoacids

In a test tube, peel extract (3 ml) is supplemented with three drops of 5% lead acetate solution, boiled on a water bath for 10 mins. The existence of amino acids as in the ninhydrin test is indicated by purple or blue colour change [23].

## 2.4 Clinical Sample Collection and Culture Preparation

The inclusion of peel extract as complementary or alternative medicine is endorsed by WHO to evaluate *in vitro* antibacterial effects on a cohort of Multi-drug resistant enteropathogenic bacteria isolated from fourteen diarrhoeal stool samples collected in sterile containers from hospitals at Namakkal and transported to the laboratory. Samples inoculated in Macconkey broth and after 24 hours pure culture obtained in the McConkey agar plate. The pure cultures were maintained on nutrient agar slants.

## 2.5 Physiological and Biochemical Analysis

Isolates were recognized according to their biochemical and Physiological Foldscope profilings alike (Gram reaction, shape, motility, endospore formation, utilization of citrate, indole, catalase, oxidase, carbohydrate fermentation, methyl red, Voges Proskauer, TSI and urease tests) is carried out [24].

## 2.6 Antimicrobial Sensitivity Test

An entirety of 10 isolates were analyzed for antibiotic sensitivity test which was carried out via disc diffusion process in opposition to five-grade antibiotic disks (Tetracycline, Gentamycin Chloramphenicol, Co-trimoxazole, and vancomycin) incubated for 24 h [25]. By means of a spotlessly clean swab, the chosen colonies were spread on the muller hinton agar plate and permitted to arid for 2–5 min. Later then, by a disc dispenser, the antibiotic discs were applied to the plates and incubated at 25-30°C for 18–24 h. The zone of inhibition via plate ruler was deliberated and expounded the isolates as Resistant (R), Intermediate (I) or Susceptible (S) [26,27].

## 2.7 Determination of Antibacterial Activity

The peel extracts were set (aqueous, ethanolic and chloroform) to acquire a concentration in opposition to check organisms [28] and then this inoculum was swabbed homogeneously onto the Muller-Hinton Agar plates and punched outwells of diameter 6mm in all plates for antibacterial activity via well diffusion technique. Diverse concentrations (25 mcg, 50 mcg, and 75 mcg) of 50 µl of each were transferred into these wells and the plates were incubated at 37°C overnight [6,7]. The enteric pathogen sensitivity pattern or outline (aqueous, alcoholic and chloroform)

of the peel extracts was deliberated and measured the (diameter in millimeter) of zone of inhibition (ZOI). Latterly, a clear zone of inhibition of the bacteria after (24 hr and 48 hrs) readings were observed and for each mishmash of extract and the bacterial strain, experiments were performed in triplicates.

## 3. RESULTS

### 3.1 Screening of Phytochemical Analysis

The presence of alkaloids, flavonoids, saponins, tannins, terpenoids, phenols, reducing sugars, carbohydrates and amino acids in the peel extract of *Punica granatum* and some of the extracts (aqueous, ethanolic and chloroform) is via phytochemical study as shown in Table 1. The presence of (alkaloids, saponins, terpenoids, phenols, reducing sugars, carbohydrates and amino acids) in the aqueous and ethanolic extract the existence of alkaloids, flavonoids, saponins, tannins, and amino acids is showed only in the chloroform extract. For alkaloids in the extract shows a higher concentration of water and ethanol, anti-diuretic activity and used as anti-bacterial agents for enteric pathogens which implies the presence of these phytochemicals.

### 3.2 Foldscope Microscopy and Biochemical Characterization

The enteropathogenic bacteria were isolated from hospitalized patient's diarrhoeal stool samples (14 nos.) and examined for Foldscope microscopy and biochemical characterization (Tables 2 and 3). The colony morphology of these isolates was circular, small, convex, smooth colony, large, opaque, shiny, mucoid, dome-shaped, tiny, swarming growth and translucent colonies. Under Foldscope microscopic examination, gram-positive and gram-negative bacteria were also observed. Biochemical analysis such as IMVIC, indole, catalase, oxidase, carbohydrate test, Voges Proskauer, TSI and urease tests performed (Table 3). Among the 14 stool samples totally 56 isolates were identified as *Staphylococcus aureus, Vibrio cholerae*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *E. coli*, *Salmonella typhi, Shigella dysentriae,* and *Klebsiella sp*. All the identified pathogens were characterized as catalase positive and oxidase negative.

**Table 1. Qualitative detection of phytochemical constituents in *P. granatum* peel extracts**

| Phytochemical constituents | | Solvents | | |
|---|---|---|---|---|
| | | Aqueous | Ethanol | Chloroform |
| **Alkaloids** | Mayer's test | + | + | + |
| | Wagner's test | + | + | + |
| **Flavonoids** | | - | - | + |
| **Steroids** | Salkowski's test | - | - | - |
| **Saponins** | Foam test | + | + | + |
| **Tannins** | Lead acetate test | - | - | + |
| | Reaction with Fec13 | - | - | + |
| **Terpenoids** | | + | + | - |
| **Phenols** | Ferric chloride test - Colour with Fec13 | + | + | - |
| **Carbohydrates - Reducing sugars** | Molisch's test | + | + | - |
| | Fehling's test | + | + | - |
| | Benedict's test | + | + | - |
| **Amino acids** | Ninhydrin | + | + | + |

*+ indicates the presence of compound; '-' indicates the absence of compound*

**Table 2. Preliminary test for the isolation of enteric pathogens**

| Gram staining | Motility | Negative Staining | Isolates |
|---|---|---|---|
| G +ve Cocci in clusters | Non motile | Capsule absent | A |
| G –ve Rod | Non motile | Capsule Present | B |
| G –ve Rod | Swarming Motility | Capsule absent | C |
| G –ve Rod | Motile | Capsule absent | D |
| G –ve Rod | Motile | Capsule absent | E |
| G –ve Rod, Comma shaped | Motile | Capsule absent | F |
| G –ve Rod | Non-motile | Capsule absent | G |
| G –ve Rod | Motile | Capsule absent | H |

**Table 3. Biochemical characterization of the enteric pathogenic isolates**

| Isolates | Carbohydrate fermentation | | | | I | MR | VP | CIT | TSA | Urease | Proposed Organisms |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | G | L | S | M | | | | | | | |
| A | P | N | P | P | N | P | N | N | A/A, $G^-$, $H_2S^-$ | N | *Staphlylococcus aureus* |
| B | P | P | P | P | N | N | P | P | A/A, $G^+$, $H_2S^+$ | P | *Klebsiella sp.* |
| C | P | N | N | P | P | P | N | P | AK/A, $G^+$, $H_2S^+$ | P | *Proteus vulgaris* |
| D | P | N | N | P | N | N | N | P | AK/AK, $G^-$, $H_2S^-$ | P | *Pseudomonas aeruginosa* |
| E | P | N | P | P | N | P | N | N | AK/A, $G^-$, $H_2S^+$ | P | *Salmonella typhi* |
| F | P | N | P | P | P | N | P | P | AK/A, $G^-$, $H_2S^-$ | N | *Vibrio cholerae* |
| G | P | N | P | P | N | P | N | N | AK/A, $G^-$, $H_2S^-$ | N | *Shigella dysentriae* |
| H | P | P | P | P | N | P | N | N | A/A, $G^+$, $H_2S^+$ | P | *E. coli* |

*I; Indoel, MR; Methyl Red, VP; Voges Proskauer, CIT; Citrate, TSA; Triple Sugar Iron agar; G; Glucose, L; Lactose, S; Sucrose; M; Mannose, P-Postive, N-Negative, A-Acid, Ak-Alkaline, G-Gas, $H_2S$ –Hydrogen sulfide*

**Table 4. Antibiotic sensitivity test against the enteric pathogens (ZOI in mm)**

| Isolates | Te | | C | | Gm | | Co | | Va | |
|---|---|---|---|---|---|---|---|---|---|---|
| | ZOI | Inf | ZOI | Inf | ZOI | Inf | ZOI | Inf | ZOI | Inf |
| *Staphlylococcus aureus* | 27±1 | S | - | R | 20±1 | S | - | R | 18±1 | S |
| *Klebsiella sp.* | 15±1 | I | 14 | I | - | R | 16±1 | I | - | R |
| *Proteus vulgaris* | 16±1 | I | - | R | 15±1 | I | - | R | - | R |
| *Pseudomonas aeruginosa* | 15±1 | I | - | R | 16±1 | I | - | R | - | R |
| *Salmonella typhi* | 16±1 | I | 13 | I | 18±1 | S | - | R | - | R |
| *Vibrio cholerae* | - | R | 14 | I | - | R | 17 | S | - | R |
| *Shigella dysentriae* | - | R | - | R | 14±1 | I | - | R | - | R |
| *E. coli* | - | R | 17 | S | 18±1 | S | - | R | - | R |

*R–Resistance, I –Intermediate, S–Sensitivity, ZOI–Zone of Inhibition, Inf–Inference; Te; Tetracycline, C; Chloramphenicol, Gm; Gentamycin, Co; Cotrimaxazale and Va; Vancomycin,*

**Table 5. Antimicrobial activity of *Punica granatum* against the enteric pathogens**

| Enteric pathogens | *Punica granatum* peel extracts (ZOI in mm) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Aqueous extract (in mcg) | | | Ethanol extract (in mcg) | | | Chloroform extract (in mcg) | | |
| | 25 | 50 | 75 | 25 | 50 | 75 | 25 | 50 | 75 |
| *Staphlylococcus aureus* | 25±1 | 30±1 | 35±1 | 29±1 | 33±1 | 36±1 | - | 12±1 | 16±1 |
| *Klebsiella sp.* | 22±1 | 24±1 | 26±1 | 21±1 | 25±1 | 27±1 | - | - | 13±1 |
| *Proteus vulgaris* | 21±1 | 27±1 | 29±1 | 22±1 | 23±1 | 31±1 | - | - | 13±1 |
| *Pseudomonas aeruginosa* | 22±1 | 28±1 | 31±1 | 23±1 | 26±1 | 33±1 | - | - | 14±1 |
| *Salmonella typhi* | 23±1 | 27±1 | 30±1 | 21±1 | 23±1 | 32±1 | - | - | 12±1 |
| *Vibrio cholerae* | 26±1 | 29±1 | 33±1 | 21±1 | 23±1 | 27±1 | - | - | 15±1 |
| *Shigella dysentriae* | 22±1 | 26±1 | 29±1 | 23±1 | 24±1 | 27±1 | - | - | 15±1 |
| *E. coli* | 19±1 | 23±1 | 28±1 | 22±1 | 26±1 | 31±1 | - | - | 13±1 |

## 3.3 Antibacterial Activity

According to the antibacterial activity results, the sensitive zone of inhibition for Tetracycline and vancomycin was observed on *Staphylococcus aureus* (27 mm and 18 mm) only, Chloramphenicol was sensitive to *E. coli* (17 mm), Gentamycin was sensitive to *Staphylococcus aureus* (20 mm), *Salmonella typhi* (18 mm) and *E. coli* (18 mm). The zone of inhibition showed by the antibiotic against the 8 isolates and the results are measured and tabulated (Table 4 & Fig. 1). Most of the organisms showed a multidrug-resistant pattern.

Significant activity of ethanolic and aqueous peel extract in 25 mcg, 50 mcg and 75 mcg concentration

shows effective zone of inhibition in the range of 13-38 mm was observed on *Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella typhi, Vibrio cholerae, Proteus vulgaris, Shigella dysentriae, E. coli* and *Klebsiella sp.* Chloroform extract shows the zone of inhibition in the range of 13-16 mm at high (75 mcg) concentration only. The zone of inhibition of *Punica granatum* peel extract was given in Table 5 & Fig. 2.

## 4. DISCUSSION

Require innovative antimicrobial agents of plant source which are secure, protective and economical for the enhance of antibiotic resistance as well as the adverse side effects of man-made drugs (tooth staining on long-term use, unpleasant taste, and also they are elevated in expenditure). Really, the peel of *Punica granatum* is extensively used as antimicrobial agents [29] and its antibacterial properties were studied in the past. The existence of a wide-ranging spectrum of antibacterial compounds operates in opposition to the enteric pathogens (Gram + ve and Gram –ve bacteria) [30,31] indicated by the metabolic toxins or novel bioactive compounds in *P. granatum* peel extract. Besides, all three diverse solvent extracts tested also showed antibacterial actions.

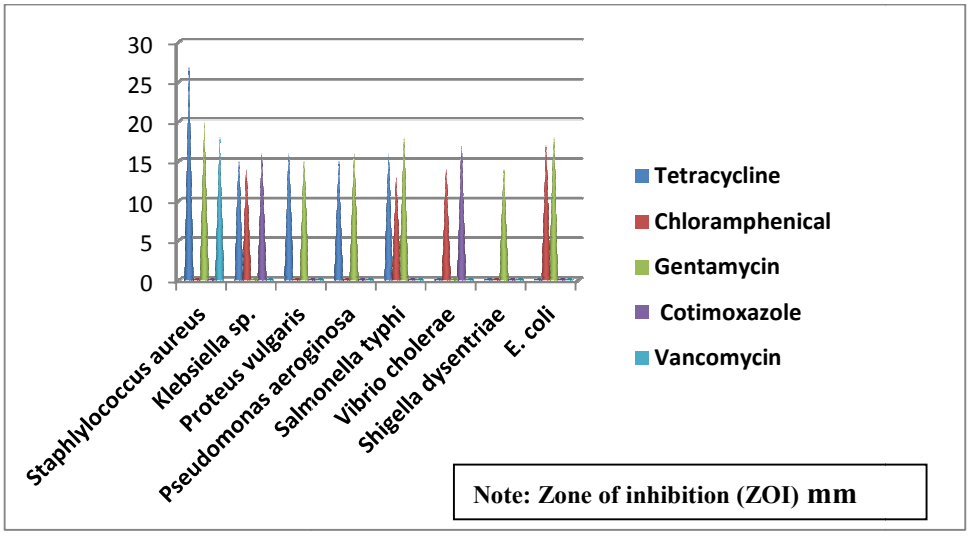


**Fig. 1. Antibiotic sensitivity test against the enteric pathogens**

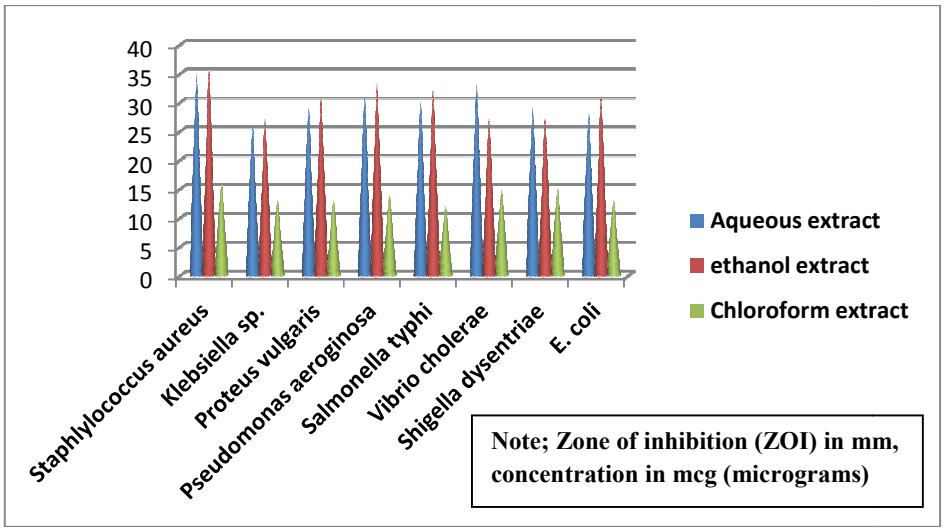


**Fig. 2. Antimicrobial activity of *Punica granatum* against the enteric pathogens**

Dynamic inhibitors in pomegranate fruit peels are exposed via phytochemical investigation as persuasive constituents and phytochemicals in the peel extract of *Punica granatum* implies as anti-bacterial agents effortlessly reachable resource of natural antioxidant, this analogous interpretation was reported [32]. The preeminent resolution for the systematic society engaged in drug invention and progress is the new dynamic constitutes which are explicitly conscientious for the antibacterial activity, recognized by a multidisciplinary approach. The immense exertion exposes latent of the plant for restorative cure manage of enteric pathogens; which is appropriate a menace to human being existence. By *in vitro* study, all targeted microorganisms and its inhibitory outcome in Pomegranate peel extracts [11] can also be used as preservatives in food goods to control enteric pathogens.

## 5. CONCLUSION

Necessity for the development of novel antimicrobial compounds because of the alarming increase in antibiotic resistance among microorganisms, these *Punica granatum* is an imperative component and relied as healing agents in the conventional medicinal system. The aqueous extracts of the peel extract have antidiarrhoeal activity, increasing resistance to universally employed antibiotics and universal source of many enteric infections of the aforementioned microorganisms. The peel of *Punica granatum* has been in use for many years as decoctions or infusions arranged in water to treat different extra ailments., home-made remedies, potential application in treating gastrointestinal disorders and safe alternatives to man-made antimicrobial drugs.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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