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In vitro analysis of Punica granatum against bacillary dysentery causing microbes

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Abstract— Continuous researches on the herbal drug as an alternative that can solve the problem of increasing the number of resistant bacterial strains are very important. This research work aims to explore the effectiveness of the Punica granatum L. aqueous extract against the bacterial strain of Shigella flexneri, Salmonella enterica, and E.coli. The MIC was calculated by using the broth dilution method, and the anti-bacterial activity is assessed by using agar well diffusion assay. The bactericidal activity was examined using the broth dilution method and the cytotoxicity was analyzed using the brine shrimp test. The obtained results show that the aqueous extracts of P. granatum showed antibacterial activity against bacillary dysentery, causing microbes, and were found non-toxic in brine shrimp lethality assay. The aqueous extracts of P. granatum also show the bactericidal effect and anti-bacterial effects against the bacterial strain of Shigella flexneri, Salmonella enterica, and E.coli, which are responsible for causing bacillary dysentery. The obtained evidence shows that the aqueous extracts of P. granatum have anti-bacillary activity.

Keywords: Punica granatum, Shigella flexneri, Salmonella enterica, E.coli, MIC.

INTRODUCTION

Shigellosis is also known as bacillary dysentery, caused by *Shigella* bacteria like *Shigella flexneri, Shigella dysenteriae,* and *Shigella sonnei* (1, 2). Among the *Shigella* strains, *S. flexneri* was the most frequent and common strain found in the case of developing countries, and other strains may include *Salmonella* and *E.coli* (3). The clinical characteristic of dysentery is blood or mucus in stool. *S. flexneri* infects the person's epithelial cells of the colon and terminal ileum and multiplies inside them. Annually, S. flexneri causes nearly about 590,000 cases of shigellosis among military personnel travelers and approximately 18,000 cases of severe shigellosis in the developed countries (4). Bacillary dysentery usually infects children below the age of 5 years and elderly peoples, but all humans of all ages are susceptible to some degree (5). A study shows that the resource-poor countries have the highest burden of shigellosis, with as many as 167 million cases of bloody diarrhea and approximately 1.2 million deaths occur annually. Several antibiotics (ciprofloxacin, tetracycline, sulphonamides, and ampicillin) which were used early for the treatment of bacillary dysentery infection of now become ineffective and narrow the choice of effective antibiotics (6). Shiga toxin released inhibits the fluid absorption in the intestine, which results in fluid secretion. This S. toxin kills the absorptive epithelial cells, which causes watery stool due to the results of blocking of absorption. To cause bacillary dysentery disease, bacteria (*Shigella, Salmonella,* and *E.coli*) must invade the intestinal epithelial lining and will have to multiply within the invading cells resulting in a bloody stool (bacillary dysentery) (7). With the aim of finding a new inhibitor for the treatment and prevention of bacillary dysentery, *Punica granatum* efficacy was investigated.

MATERIAL

PLANT COLLECTION

Dried powdered extract of *Punica granatum* was obtained from an authorized plant extract supplier. The extract was properly mixed and dissolved in an aqueous solvent and stored at -4^0 C for further use.

BACTERIA

The bacterial strain of *Shigella flexneri*, *Salmonella enterica,* and *E.coli* was procured from MTCC and was grown in nutrient broth media at 37^0 C, Ph. 7.0. When OD neared 1 at 600nm, the cultures were sub-cultured. Media including NB, TSB, was purchased from Himedia.

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MIC CALCULATION

The MIC (Minimum Inhibitory Concentration) of *Punica granatum* against *Shigella flexneri, Salmonella enterica,* and *E.coli* was analyzed by using the broth dilution method as per *NCCLS*, USA, 2006 guidelines (8). The cultures of *Shigella flexneri, Salmonella enterica,* and *E.coli* (OD 600nm = 1) were grown at 37^0C for 24 hours in the nutrient broth medium was mixed with varying concentrations of *Punica granatum* in a 96 ELISA well plate. The concentration, which totally inhibits the growth of *Shigella flexneri, Salmonella enterica,* and *E.coli,* was noted as the minimum inhibitory concentration for the *Punica granatum* extract, and further analysis was carried out at sub-MIC concentration (9).

TIME EFFECT RELATIONSHIP ASSAY

This assay was performed to analyze the bactericidal effect of *Punica granatum* extract. To perform this experiment, MHB (Mueller-Hinton broth) purchased from Himedia was added to each well of 96 ELISA plate along with the different concentrations of *Punica granatum* extract dissolved in an aqueous solvent (sub-MIC concentration), were inoculated with the 0.5OD culture of *Shigella flexneri, Salmonella enterica* and *E.coli* incubated at 37^0 C. Further, *Shigella flexneri, Salmonella enterica,* and *E.coli* cells absorbance were calculated by taking absorbance at 600 nm in ELISA microplate reader every hour (10).

WELL DIFFUSION ASSAY

Agar well diffusion is one of the widely performed and commonly used methods for assessing the herbal plant's antimicrobial activity. To perform the experiment, a nutrient agar plate was prepared, and on the prepared NB agar plate surface, the of *Shigella flexneri, Salmonella enterica,* and *E.coli* inoculums were spread by using a sterile plastic spreader. Then, aseptically a well of diameter 8 mm is cut with the help of a sterile tip, and an aqueous extract of *Punica granatum* of volume (100 µL) was placed into the created well. Further, these NB agar plates are incubated at 37^0C for 24 hrs. After the incubation period is over, a clear inhibition zone was observed around the wells, which shows the antibacterial activity of the *Punica granatum* against the *S. flexneri, Salmonella enterica,* and *E.coli* bacterial strain. The diameter of the zone was measured in mm (9).

BRINE SHRIMP LETHALITY ASSAY

Currently, to analyze the cytotoxicity effect of herbal compounds, the brine shrimp (artemia Salina) test (BST) is generally performed. BST is used for analyzing the toxicity of plant extract, fungal toxins, heavy metals, pesticides, and performed by the method described by *Meyer et al.* with some modifications (11). In brief, 1 $mg.ml^{-1}$ stock solution of the *Punica granatum* was prepared, and different *Punica granatum* concentrations of (1µg, 10µg, 30µg, 60µg, 120µg, 240µg, 1000µg, and 2500µg) were prepared from this stock solution. Put the *Punica granatum* solution in the labeled tubes along with the 10 nauplii. Using artificial seawater (Himedia), the final volume of each tube was adjusted to 5 ml. The negative control tube contains only the nauplii along with the artificial seawater and sterile Milli-Q. Afterward, the tubes were incubated under a light source for 24 hours. After 24 h, the dead nauplii were counted in every tube. The percentage of death was calculated, and LC50 values were examined using prism software (11).

STATISTICAL ANALYSIS

In triplicates, all the experiments were performed. The values are represented as mean ± SEM (Standard Mean Error). Statistical analysis of the obtained results was analyzed using the software Graph pad

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prism, and further significant values were calculated by one-way; only values at $p<0.05$ were considered significant.

RESULTS

THE *PUNICA GRANATUM* ANTI-BACTERIAL ACTIVITY

Then the antimicrobial activity of the *Punica granatum* was examined against the *Shigella flexneri, Salmonella enterica,* and *E.coli,* and a clear halo inhibitory zone was observed (Figure 1).

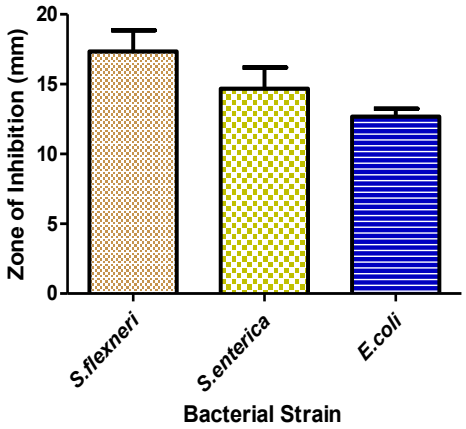

Figure 1. Anti-bacterial activity of *Punica granatum* against *Shigella flexneri, Salmonella enterica,* and *E.coli* by agar well diffusion method.

DETERMINATION OF MIC OF *PUNICA GRANATUM*

According to the obtained result, the MIC value of the *Punica granatum* was found 1.6 $mg.ml^{-1}$, 1.6 $mg.ml^{-1}$, 2.1 $mg.ml^{-1}$against the *Shigella flexneri, Salmonella enterica,* and *E.coli* respectively.

DETERMINATION OF TIME–EFFECT RELATIONSHIP ASSAY

Time–effect relationship assay performed at and below the sub-MIC values (0.8 $mg.ml^{-1}$ and 0.4 $mg.ml^{-1}$ against the *Shigella flexneri,* 0.8 $mg.ml^{-1}$ and 0.4 $mg.ml^{-1}$ against *Salmonella enterica* and 1.0 $mg.ml^{-1}$ and 0.5 $mg.ml^{-1}$ against *E.coli*). This assay was performed to assess the effect of *Punica granatum* on the proliferation of bacteria at a different time interval. The absorbance was noted at 600nm. Bacterial cells untreated with *Punica granatum* were used as the control, and the result shows the bactericidal effect of the *Punica granatum*, and the graph was shown in Figure 2.

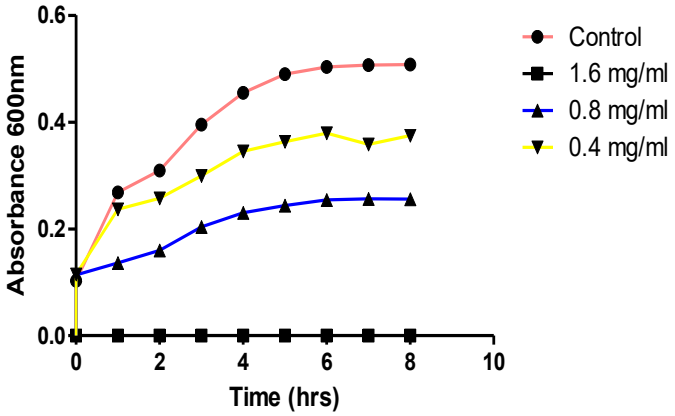

Figure 2. Time effect relationship assay. Effect of *Punica granatum* on the proliferation of *Shigella flexneri*. Cells untreated with *Punica granatum* were taken as the control. $P<0.01$ were considered significant (one-way ANOVA).

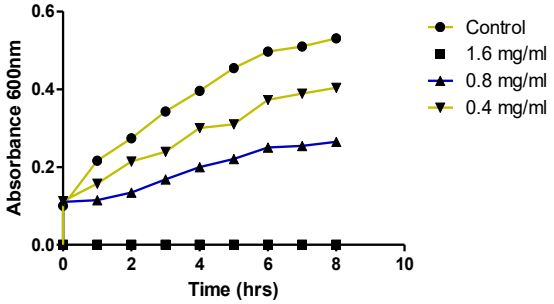

Figure 3. Time effect relationship assay. Effect of Punica granatum on the proliferation of *Salmonella enterica*. Cells untreated with *Punica granatum* were taken as the control. $P<0.01$ were considered significant (one-way ANOVA).

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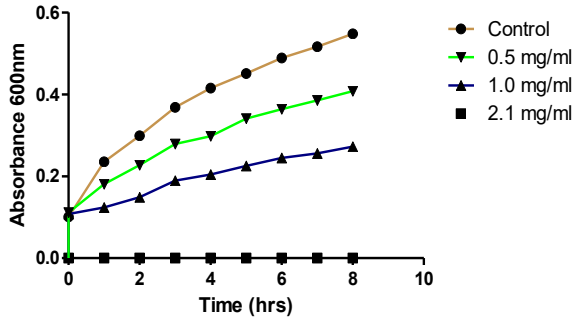

Figure 4. Time effect relationship assay. Effect of *Punica granatum* on the proliferation of *E.coli*. Cells untreated with *Punica granatum* were taken as the control. P<0.01 were considered significant (one-way ANOVA).

CYTOTOXICITY ANALYSIS BY USING BRINE SHRIMP TEST

According to *Meyer*, if the LC50 value is more than 1 mg.ml$^{-1,}$ then the plant extract is considered non-toxic. The results show that the *Punica granatum* was non-toxic to the nauplii (LC50 value above 1000 mg.ml^{-1}), as shown in Table 1. The tube having only nauplii is taken as control.

Table 1 The obtain LC50 value of different Punica granatum s

S.No	Sample	Regression Equation	LC50 µg/ml	Regression Coefficient (r^2)
1.	*Punica granatum*	Y=37.03x + 12.03	> 1mg (1000µg/ml)	0.9330

DISCUSSION

Bacillary dysentery continues to emerge as a disaster worldwide, with an increasing infectivity rate. It is emerging as a serious problem in developing countries because it causes nearly 70% of deaths, and out of 70% of deaths, about 60% of deaths in children under five years of age. *Shigella flexneri, E.coli, and Salmonella* are among the main bacterial strains responsible for causing bacillary dysentery (12). Because of the action of Shiga-toxin, which destroys the host epithelium, various symptoms such as blood in the stool, fever, and stomach cramps were seen (13). For many decades, our ancestors are using different herbal plants, extracts, and oils to treat and prevent various diseases such as diarrhea and dysentery (14, 15). So the inhibitory activity of *Punica granatum* was analyzed against different bacillary dysentery causing microbes. In this study, the anti-dysenteric activity of *Punica granatum* was investigated on *Shigella flexneri, E.coli, and Salmonella* growth along with its bactericidal property. The *in-vitro* results reveal that *Punica granatum* inhibits the *Shigella flexneri, E.coli and Salmonella* growth with a MIC value of 1.6 mg.ml^{-1}, 2.1 mg.ml^{-1}, 1.6 mg.ml^{-1} respectively. The clear halo inhibitory zone (17.33 mm, 14.66 mm, and 12.66 mm against *Shigella flexneri, Salmonella, and E.coli*) was observed, which shows the antimicrobial activity of the *Punica granatum* (10). The result suggests that *Punica granatum* may contain some bioactive compounds that will directly affect bacteria's destruction (14, 15). The cytotoxicity study of the plant extracts by using a brine shrimp is a widely recognized method. According to Meyer *et al.,* if the LC50 value is greater than 1 mg.ml^{-1}, the plant extract is considered non-toxic (11). The results show that the *Punica granatum* extract was non-toxic to the shrimp nauplii (LC50 value higher 1 mg.ml^{-1}) (16).

CONCLUSION

In conclusion, the *Punica granatum* extract has anti-dysenteric activity. The overall findings reveal that the *Punica granatum* also has anti-bactericidal activity and were also found non-toxic. The obtained *in vitro* results suggest that the *Punica granatum* can be effectively used for treating bacillary dysentery. However, maybe in the future,

some *in-vivo* and clinical investigation studies should be performed to further supports its efficacy.

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