EVALUATION OF EFFECT OF METHANOLIC AND AQUEOUS EXTRACTS OF *PUNICA GRANATUM* L. AGAINST BACTERIAL PATHOGENS CAUSING BOVINE MASTITIS

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ABSTRACT

Bovine Mastitis is an intra-mammary infection which is most common among the dairy cattle and continues to be the most costly disease to the dairy farmers. Presently, antibiotics are used for treatment of mastitis leading to the development of antibiotic resistant strains and consumer health problem. The ethno veterinary information about plants in Karnataka region to control Bovine mastitis was collected and the effect of different solvent extracts of *Punica granatum* L was investigated. Phytochemical analysis revealed the presence of bioactive compounds such as alkaloids, flavonoids, saponins, tannins, phenols, terpenoids etc. Each of the bioactive compounds were estimated and isolated separately by solvent-solvent extraction of *Punica granatum*. Saponins content was higher followed by flavonoids. All these bioactive compounds isolated from crude extracts were tested for antibacterial activity. Flavonoids of Methanolic extracts inhibits remarkable Zone against *S. uberis, S. aureus, E. coli* and *Coagulase negative S. aureus* was 11 mm, 12 mm, 14 mm, 16 mm and for water extracts it was 16 mm, 12 mm, 15 mm and 13 mm respectively.

KEYWORDS: *Punica granatum*, Phyto-chemicals analysis, Antibacterial activity, flavonoids, methanolic extracts, aqueous extracts

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INTRODUCTION

Mastitis is a persistent, inflammatory reaction of the udder tissue in cows. Bacteria secrete toxins which damage the milk-secreting tissue and various ducts throughout the mammary gland. Bovine mastitis may also be indicated by abnormalities in milk such as watery appearance, flakes, clots, or pus. An increased somatic cell count is observed in cows suffering from bovine mastitis. It is considered and continues to be the costliest disease in the dairy industry all over the world (Adaobi 2011). The repeated use of antibiotics to treat Mastitis for a long period may cause multidrug resistivity in causative organisms which requires high doses of antibiotics, which may leads to accumulation of large amount of antibiotics in milk and its products, again a potential hazard (Annapoorani Chockalingam 2007). Knowledge of medicinal plants has been accumulated in course of many centuries. Even today, 85% of Indians use higher plants as effective antimicrobials for the treatment of various diseases. The aim of this work was to collect ethno veterinary information about plants used in the prevention and control of Bovine mastitis in Karnataka region. There were no reports available relating to In-vitro applications of P. granatum extracts in Bovine mastitis studies. Therefore, the present study was designed to investigate antibacterial activity of the leaves of P. granatum and identification of particular bioactive compound as potential drug for the treatment of Bovine Mastitis.

About the plant

Punica granatum L. commonly known as Pomegranate belongs to the Family Punicaceae. Punica granatum is a shrub or small tree with several upright, thorny stems, the leaves are elliptic, roughly 2 x 1 inches. In the Indian subcontinent's ancient Ayurveda system of medicine, the pomegranate has extensively been used as a source of traditional remedies for thousands of years. The plant has also been used as an antispasmodic and antihelmintic. Pomegranate juice (of specific fruit strains is also used as eye drops as it is believed to slow the development of cataracts (Vasant Lad 2002). Pomegranate has been used as a contraceptive and abortifacient by means of consuming the seeds, or rind, as well as by using the rind as a vaginal suppository.

MATERIAL AND METHODS

All the solvents and reagents used in the study were analog grade sourced from Hi media.

Collection and Extraction of plant material

The plant was collected in the month of March-2011 from Acharya Institute of technology campus, Soladevanhalli, Bangalore. The plant with leaves was rinsed with sterilized water and leaves were removed and separated. The leaves were air dried for 3 weeks and then crushed with mortar and pestle and kept in air tight glass container at 4°C until further use (Harborne JB, 1973; Jamine. R Daisy, 2007)

Preparation of crude extracts

Aqueous extract was prepared by using 50 g of crushed leaves and 500 ml of distilled water in soxhlet apparatus and the apparatus was allowed to run for 10 h. Similarly the methanol extract was prepared (C.K. Hindumathy, 2011).

Bacterial strains

Bacterial strains used in this study were isolated from clinical cases of Bovine mastitis namely Staphylococcus aureus, Streptococcus uberis, Escherichia coli and coagulase negative Staphylococcus aureus. All the strains were confirmed by cultural and biochemical studies (Gopinath. S. M., 2011) and maintained in nutrient agar slants at 4ºC for further use.

Antibacterial activity

The antibacterial assay of aqueous and methanolic extracts was performed by agar disc diffusion method (Harborne JB., 1973; Jamine.R.Daisy, 2007). The molten Mueller
Hinton agar was inoculated with 100µl of the inoculums (1*10^6 CFU/ml) and poured into the petriplate (Himedia). For agar disc diffusion method, the disc (0.7 cm), (Himedia) was saturated with 100 µl of the test compound, allowed to dry and was introduced on the upper layer of the seeded agar plate. The plates were incubated overnight at 37ºC. Microbial growth was determined by measuring the diameter of the zone of inhibition of each bacterial strain.

**Phytochemical analysis**

Phytochemical analysis for major phytoconstituents of the plant extracts was undertaken using standard qualitative methods as described by various authors (Leite JR, 1986; Parekh, J, 2007). The plants extracts were screened for the presence of biologically active compounds like glycosides, alkaloids, phenolics, tannins, flavonoids, saponins and steroids.

**Estimation and extraction of phyto-compounds**

**Alkaloids**

Isolated crude sample was extracted with solvent ether and alcohol mixture (4:1) and ammonia solution (5% v/v). To, this 1N H_2SO_4 followed by 0.5N H_2SO_4 and alcohol mixture (3:1) was added and the acid layers were separated until the aqueous layer is colorless. This acid layer was then washed with chloroform. Further this chloroform layer was washed with acid alcohol mixture. This layer was then added with 5% v/v ammonia solution in excess. This was then extracted with chloroform and washed with water. The chloroform layer was filtered through a layer of anhydrous sodium sulfate in pre-weighed beaker. The chloroform was allowed to evaporate followed by addition of alcohol which was then dried at 105ºC in hot air oven, with alkaloids been left in the beaker. The beaker was then weighed to know the content alkaloids isolated.

**Flavonoids**

Isolated crude sample was dissolved in water washed with hexane to remove oil content. The aqueous layer was washed with chloroform followed by warming the aqueous layer. This warmed aqueous layer was extracted with ethyl-acetate into pre-weighed beaker. The ethylacetate extracted layer was concentrated and dried at 105ºC in hot air oven and the beaker was weighed again. (Wynn GS. 2001; Prasad, N.R., 2008)

**Saponins**

Isolated crude sample was extracted with 90% methanol and further concentrated to more than half of the original. This concentrated extract was then extracted with petroleum ether followed by chloroform. The obtained aqueous layer was washed with 90% methanol and again allowed to concentrate. This was then added into pre-weighed beaker containing acetone drop by drop to form saponin precipitates. This was then filtered through pre-weighed filter paper. The pre-weighed beaker and filter paper were then allowed to dry at 105º C in hot air oven.

**Tannins**

The material was extracted with mixture of distilled water and 8% Sodium carbonate in a boiling flask under reflux for two hours having used a liquor / crude extract ratio of 15:1. This was repeated again and again to produce more of tannins. After extraction, the material was filtered under vacuum using a Büchner funnel. Finally the filtrate in pre-weighed beaker was dried in hot air oven at 105º C (Williamson G., 2005; Klastrup O, 1975)

**Phenolic compounds**

Isolated crude sample was extracted with 20 mL of the extracting ethanol in a conical flask. Conical flask was covered with parafilm and aluminium foil to prevent light exposure. The mixture was shaken at constant rate using a water bath shaker for 2 h at 50ºC. The ethanol
extracted was then filtered through a Whatman No. 1 filter paper into a pre-weighed beaker, and the filtrate was evaporated at 105° C (Williamson G., 2005; Klastrup O., 1975).

Terpenoids

Terpenoids were isolated in the form of essential oils. Isolated crude sample was extracted with solvent- hexane. This was then washed with alcohol and the hexane layer was evaporated in water bath to concentrate and then evaporated in hot air oven at 105°C.

RESULTS AND DISCUSSION

Crude methanolic and aqueous extracts of leaves of plant *P. granatum* was prepared and then analyzed for phyto-compounds present in them.

Most of the secondary metabolites were identified in the polar extracts (Table-1) Alkaloids are one of the characteristic secondary metabolite in leaves of this genus found in aqueous extract. Tannins are water soluble polyphenols known as tannic acid which acts as antimicrobial agents. Presence of tannins is to prevent the development of microorganism by precipitating microbial proteins. Phyto-therapeutically, Flavonoids are known to be synthesized by plants in response to microbial infection. Hence it should not be surprising that they have been found to be effective as antibacterial substances against a wide array of infectious agents (Tyler V., 1994). Alkaloids, Flavonoids, Saponins, Tannins, Phenols, Terpenoids were isolated separately and the content of each was found by Content,

\[
\text{Content (\%)} = \frac{\text{Weight of phyto-compounds}}{\text{weight of crude extract}} \times 100.
\]

Antibacterial activity was performed for these isolated samples such as alkaloids, flavonoids, saponins, tannins, phenols (Table-3; Fig. 2). Flavonoids isolated from methanolic and aqueous extracts of *P. granatum* showed antibacterial activity against the causative organisms of Bovine mastitis. The highest inhibition zone was observed by methanolic extracts of *P. granatum* against Coagulase negative *Staphylococcus aureus* (CONS) and *S. uberis* (16 mm) and the least was observed by methanolic extracts of *P. granatum* (11 mm). Phyto-compounds isolated from water extracts have shown higher inhibition zones than methanolic extracts. Saponin was found to be higher in content with highest in aqueous extracts (16%). (Table – 2; Fig. 1) Followed by this is flavonoids, tannins and phenols were higher in content. The highest content of flavonoids was found in aqueous extracts of *P. granatum* (13.83%). The least is alkaloids in water extracts of *P. granatum* (0.162%).

| Table-1 Phytochemical analysis of *Punica granatum* in Methanol and Water extracts. |
|----------------------------------|-----------------|-----------------|------------------|
| **Compound**                      | **Methanol**    | **Water**       |                  |
| Steroids                          | −               | −               |                  |
| Terpenoids                        | +               | +               |                  |
| Alkaloids                         | +               | +               |                  |
| Flavonoids                        | +               | +               |                  |
| Saponins                          | +               | +               |                  |
| Tannins                           | +               | +               |                  |
| Phenolic compounds                | +               | +               |                  |
| Catechin                          | −               | −               |                  |
| Anthraquinone                     | −               | −               |                  |
| quinone                           | +               | +               |                  |

+ indicates the presence of Phytocompound  
− Indicates the absence of phytocompound
Table – 2. Phyto-compounds present in methanol and aqueous extracts of *Punica granatum*

<table>
<thead>
<tr>
<th>PHYTOCOMPOUNDS</th>
<th>CONTENT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLANTS</td>
<td><em>PUNICA GRANATUM</em></td>
</tr>
<tr>
<td>SOLVENTS</td>
<td>METHANOL     WATER</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>0.181        0.162</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>13.55        13.83</td>
</tr>
<tr>
<td>Saponins</td>
<td>14.7         16</td>
</tr>
<tr>
<td>Tannins</td>
<td>10.1         10.4</td>
</tr>
<tr>
<td>Phenolic compounds</td>
<td>11.2     10.5</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>0.178        0.198</td>
</tr>
</tbody>
</table>

+ indicates the presence of Phytocompound
− Indicates the absence of phytocompound

Table-3 Antibacterial activity of different phytocompounds of *Punica granatum*

<table>
<thead>
<tr>
<th>Causative organisms</th>
<th>Alkaloids M W</th>
<th>Flavanoids M W</th>
<th>Saponins M W</th>
<th>Tannins M W</th>
<th>Terpenoids M W</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. uberis</em></td>
<td>0 0</td>
<td>11 16</td>
<td>0 0</td>
<td>0 0</td>
<td>0 0</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>0 0</td>
<td>12 12</td>
<td>0 0</td>
<td>0 0</td>
<td>0 0</td>
</tr>
<tr>
<td><em>E.coli</em></td>
<td>0 0</td>
<td>14 15</td>
<td>0 0</td>
<td>0 0</td>
<td>0 0</td>
</tr>
<tr>
<td>CONS</td>
<td>0 0</td>
<td>16 13</td>
<td>0 0</td>
<td>0 0</td>
<td>0 0</td>
</tr>
</tbody>
</table>

Fig-1. Phyto-compounds present in methanol and aqueous extracts of *Punica granatum*
CONCLUSION

Methanol and water extracts of flavonoids from *Punica granatum* have antibacterial potential against the causative organism of Bovine mastitis. Water as solvent is better for extraction of bioactive compounds. Further, flavonoids can be studied to find specific compound which can further act as a drug and solve the problem of antibiotic drug resistance by causative organisms of Bovine mastitis.

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