

A study on few medicinal plant extracts against drug resistant *Streptococcus pyogenes* isolated from paediatric cases

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Abstract

Streptococcus pyogenes, a group-A *Streptococcus* (GAS) continues to be a significant public health problem worldwide causing Rheumatic fever (RF) and Rheumatic heart disease (RHD). Thirty five *S. pyogenes* isolates from repeat samples of 87 suspected cases belonging to the age group 5 to 15, were found to be sensitive to Penicillin G, Bacitracin, Cephalexin, Clindamycin, Amoxicillin, Ampicillin and Azithromycin antibiotics. However, two isolates BMERF/DP/S9 and S15 showed resistance for the antibiotics Erythromycin and Ciprofloxacin. The data were found to be statistically significant ($p = 0.007$). Ethanolic extracts of *Zingiber officinale*, *Allium sativum*, *Aloe vera*, and *Punica granatum* were tested for their antibacterial activity against the dual drug resistant GAS isolates. Among the herbal extracts, *Punica granatum* epicarp had a preponderant efficacy. Further phytochemical studies by HPLC revealed a predominant tannin fraction in the extract. It was concluded that *Punica granatum* epicarp extract could be an important alternate therapeutic agent in the management of drug resistant GAS.

Key words: GAS, RF, RHD, HPLC, drug- resistance, *Punica granatum*

Introduction

Rheumatic fever (RF) and Rheumatic heart diseases (RHD) caused by *Streptococcus pyogenes* has become one of the World's most widespread diseases of man targeting school children within the age group of 5 and 15 leading to high mortality and morbidity (Dipanwitha Ray et al. 2010). According to the WHO, at least 15.6 million children are affected with RHD. India is affected to a greater extent that, of the 5, 00,000 individuals who acquire rheumatic fever every year, 3,00,000 go on to develop RHD; and 2,33,000 deaths annually are attributable to RF or RHD (Mishra 2007).

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Though penicillin and its derivatives remain to be the drugs of choice for Streptococcal pharyngotonsillitis, high rates of clinical failure and penicillin hypersensitivity in patients allergic to penicillin are also reported. Other alternative antibiotics such as macrolides and quinolones due to unscrupulous usage have now been reported as resistant in different countries with varying frequencies (Yukthi Sharma et al. 2010). As a consequence, effective treatment and control of such drug resistant organisms remains to be an important challenge and has necessitated the need for a continued search for new antimicrobial compounds from other sources.

Plant extracts having known antibacterial activity such as *Aloe vera*, ginger, garlic and pomegranate could be tried against the antibiotic resistant bacteria. However, studies on these herbal extracts in the management of GAS are obscure. Such studies could provide a platform for the development of a novel formulation to combat the drug resistant GAS infections.

Materials and methods

Isolation and Identification

87 pediatric cases of the age group 5 to 15 years suspected for pharyngotonsillitis infection and receiving no antibiotics in antecedent for 7 days visiting Salem Hospitals and clinical laboratories were subjected for repeat throat samplings. All the isolates were taken for their identification study using routine lab diagnostic tools such as β – hemolysis on blood agar plates, gram staining, catalase test, bacitracin susceptibility and PYR test to type their possibility as GAS. A standard *S. pyogenes* strain MTCC 442 received from Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India was also included as control.

Antibiotic sensitivity Tests

All the isolates identified as GAS were tested for their sensitivity pattern with the routinely used antibiotics by Kirby-Bauer disk diffusion technique (Kirby et al. 1966) on Mueller-Hinton agar (MHA) with 5% sheep blood, as recommended by the Clinical and Laboratory Standards Institute, Wayne, USA (Dipanwitha Ray et al. 2010). The antibiotics tested were Penicillin G (10U), Bacitracin

(10U), Cephalexin (30 µg), Clindamycin (2 µg), Amoxicillin (30 µg), Ampicillin (10 µg), Azithromycin (15 µg), Erythromycin (15 µg) and Ciprofloxacin (5 µg). Two factorial ANOVA was applied by taking antibiotics and isolates as categorical factors and zone of inhibition as the dependent variable and was analyzed ($P < 0.05$) using Statistica software (version 6.0; StatSoft, Inc., USA).

Preparation of herbal extracts

A few common Indian medicinal plants including spices namely *Zingiber officinale* (ginger), *Allium sativum* (Garlic), *Aloe vera*, and *Punica granatum* (Pomegranate) were tested for their anti-*S. pyogenes* activity. The plant products were collected from the local market of Salem. They were dried under shade at room temperature for 10 days. The dried plant materials were ground into fine powder using electric blender and were filled in thimbles separately. Each thimble containing 8 g of dried powder was kept in a distillation unit with heating mantle at about 80°C. The extraction was carried out with ethanol and the residues were stored at 4°C for further use.

Antimicrobial Screening

The anti-GAS activity of ethanolic extracts at various concentrations (10, 20, 40, 80 and 160 µg/ml) was screened by well diffusion technique on sterile Muller Hinton Agar plates. Antibacterial activity in terms of zones of inhibition (mm) was recorded after 24 hours of incubation. The antagonistic action of the extracts was tested against the test organism in triplicates.

HPLC analysis

The dried extract of *Punica granatum* epicarp was re-suspended in 1.0 ml HPLC grade methanol by vortexing and filtered through ultra membrane filter (pore size 0.45 µm; Millipore India Private Limited, Bangalore.) before HPLC analysis as described earlier (Kuster et al. 2002). Tannic Acid, eugenol, and cinnamic acid (1 mg/ml) were used as standard markers. 20 µl of marker and the extract was injected into RP 18 column of HPLC (Shimadzu, Japan) instrument. Methanol: Water (2:1 ratio) formed the mobile phase and the flow rate was 1ml/min. Peak area percentage and extinction coefficient was calculated from chromatogram of the standard detected at 285 nm and the results were noted down.

Results and discussion

Among the 87 suspected cases with pharyngotonsillitis, only 35 children were confirmed to be harbouring GAS by routine lab diagnostic tools and 11 children with non-GAS isolates were excluded. All the 35 GAS isolates were identified as Gram positive cocci in chains exhibiting β-hemolytic activity on blood agar plates and sensitive to Bacitracin (0.04U). Apart from that the GAS isolates were catalase negative and showed positivity to PYR test. The present study revealed a high rate (40.2%) of *Streptococcus pyogenes* incidence from Salem children. The prevalence of GAS infection in other parts of India was earlier reported to lie in the range between 11.2 and 34%. Alarming increase in the rate of isolation from Salem needs attention of regular surveillance programmes to keep GAS infections and carriage in check for this region.

A battery of routinely used antibiotics was used for sensitivity analysis. All the isolates and the standard strain of GAS except two isolates typed as BMERF/DP/S9 and BMERF/DP/S15 were sensitive to Penicillin G, Bacitracin, Cephalexin, Clindamycin, Amoxicillin, Ampicillin, Azithromycin, Erythromycin and Ciprofloxacin. The S9 and S15 isolates were found to be resistant to the antibiotics Erythromycin and Ciprofloxacin (Fig 1). The zone of

inhibition obtained for all the isolates with different antibiotics were subjected for Two – Way ANOVA. The ‘F’ distribution values obtained for the antibiotics and the isolates were compared with the theoretical F-value at $F_{8,280}$ and $F_{35,280}$, calculated from the F distribution table. The F values for the antibiotics and the isolates were $F_{8,280} = 597.8 > 1.83$ and $F_{35,280} = 1.8 > 1.45$ respectively at $\alpha=0.05$ level of significance (Table 1). Hence the null hypothesis was rejected and it was concluded that the effect of antibiotics on the isolates were statistically significant.

The present study revealed that penicillin is still the drug of choice for the treatment of Streptococcal infections. The pattern of sensitivity to Penicillin found in this study was also reported in other parts of India (YukthiSharma et al. 2010). However, the finding of dual resistance for

Table 1: Analysis of Variance on the effect of antibiotics on the isolates

	SS	DF	MS	F	p
Intercept	176260.0	1	176260.0	129818.9	0.000000
Antibiotics	6493.6	8	811.7	597.8	0.000000
Isolates	83.2	35	2.4	1.8	0.007480
Error	380.2	280	1.4		

SS – Sum of squares; MS – Mean Square; DF – Degree of freedom; F– F-distribution Value at corresponding degree of freedom; P– p-distribution value at 0.05 level of significance

the antibiotics Erythromycin and Ciprofloxacin in BMERF/DP/S9 and BMERF/DP/S15 isolates amounting for 5.7% was a matter of concern in view of emergence of resistance against these safe drugs, which may further complicate the treatment of patients who are allergic to penicillin. Erythromycin resistance as observed in this study was reported by Dipanwitha Ray et al. (2010) with resistance in 3.5% of their GAS isolates.

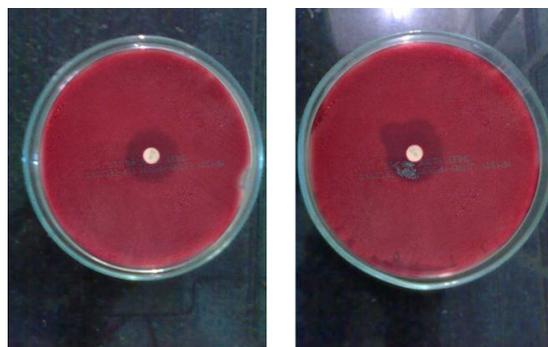


Figure 1: BMERF/DP/S9 exhibiting resistance to Ciprofloxacin & Erythromycin

Ciprofloxacin was the other drug found to be resistant (5.7%) among the two drug resistant GAS isolates of the present study. The result of this study was similar to Charmaine et al. (2006) findings who reported 8.3%, (5/60) of resistance towards Ciprofloxacin. The rate of co-resistance to macrolides and quinolones (5.7%) in the present study is of a greater concern, because new fluoroquinolones may be therapeutic alternatives for the treatment of infections due to macrolide-resistant strains in patients with β-lactam allergy. The co-resistance was also seen in Barcelona, Spain in the recent years (Carmen et al. 2010). Such dual resistance in GAS will further intricate the antibiotic resistance pattern of this region.

In view of contriving an alternate therapeutic agent against drug resistant isolates, ethanolic extracts of *Zingiber officinale*, *Allium sativum*, *Aloe vera*, and *Punica granatum* were tried for their anti GAS activity. The extracts showed various levels of inhibitory zones against the drug resistant GAS isolates. *Punica granatum*

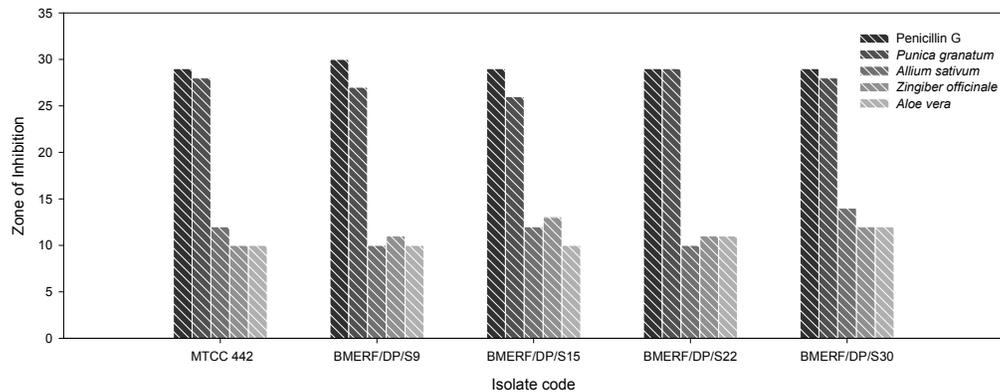


Figure 2: Comparative anti GAS activities of the herbal extracts

epicarp extract (80 µg/ml) produced 27 mm and 26 mm sized zone of inhibition against the drug resistant isolates BMERF/DP/S9 and BMERF/DP/S15 respectively. The inhibition zone of Penicillin antibiotic against BMERF/DP/S9 and BMERF/DP/S15 isolates was 30 mm and 29 mm respectively. Even the lowest concentration of the *Punica granatum* epicarp extract (10 µg/mL) had a zone of inhibition greater than 12 mm, whereas the maximum zone size for the other extracts, even at a higher concentration (160 µg/mL) was less than 13 mm.

Inferior zone of inhibition was observed for *Zingiber officinale* (13 mm), *Allium sativum* (12 mm), and *Aloe vera* extract (10 mm) even at the highest concentration (160 µg/mL). Although Fani *et al.* (2007) showed the efficacy of *Allium sativum* against multi drug resistant *S. mutans*, their effect on dual drug resistant GAS of this study was not very significant. Thus among the various extracts tested for its antibacterial activity against the isolates, the epicarp extract of *Punica granatum* had a preponderant efficacy against the drug resistant isolates with a maximum zone of inhibition (Fig 2).

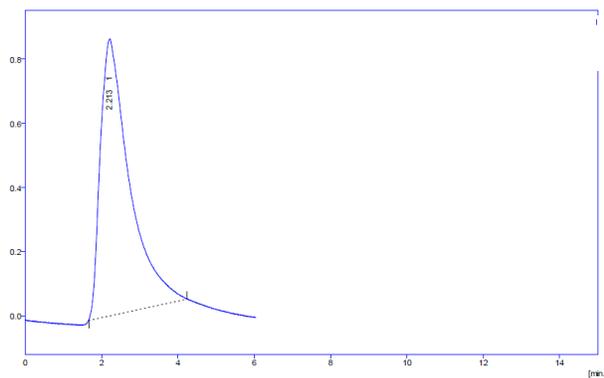


Figure 3: Determination of active principle of *Punica granatum* epicarp extract by HPLC studies

Therefore further phytochemical studies were carried out on *Punica granatum* epicarp extracts. The HPLC analysis of the extract and the Tannic acid standard exhibited significant peaks. Standard marker peak value was 2.52, whereas the extract of *Punica granatum* had a peak value at 2.21 (Fig 3). Among the different standards, namely eugenol, cinnamic acid and tannic acid the peak value was close to the tannin standard only. Thus the active principle present in the extract responsible for its anti GAS activity perhaps could be Tannin moiety. Tannic acid as the active principle in various medicinal plants against many bacterial pathogens has been documented by other earlier workers (Kuster *et al.* 2002). Though *Punica granatum*

exhibited excellent anti-GAS activity it cannot supplant the orthodox therapeutic agents for the RF and RHD cases, but can be used as an adjuvant to manage the drug resistance.

Conclusion

It was concluded that to at least this region of India, group A *Streptococcus* has become an important matter of concern because of their higher preponderance of isolation and emergence of drug resistance in them. The anti-GAS efficacy of *Punica granatum* epicarp on the drug resistant strains could possibly be the first report of its kind. Based upon the present finding it was concluded that *P. granatum* could be an excellent alternate therapeutic agent for the better management of drug resistant GAS causing RF and RHD. However, further studies are needed on the purification of the active principle of the extract before recommending it as a drug.

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