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RESEARCH ARTICLE

IDENTIFICATION OF ANTI MRSA (METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS*) COMPOUNDS FROM ETHANOLIC LEAF EXTRACTS OF *AEGLE MARMELOS* BY GC - MS

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ABSTRACT

Due to increase in occurrence of antibiotic resistant bacterial pathogens, there is a pressing need to develop effective alternate antibacterial compounds. Medicinal plants are the rich source of secondary metabolites that serve as potential antibacterial agents. The aim of the present study is to fractionate screen the crude ethanolic leaf extract of *Aegle marmelos* screen for the antibacterial activity against Methicillin resistant *Staphylococcus aureus* (MRSA) and identify the compounds present in the active fraction. Column chromatography of the crude ethanolic extract yielded a total of 10 fractions. One candidate fraction showed highest antibacterial activity. Gas chromatography mass spectral studies identified the presence of seven phytochemical compounds in the active fraction. Among them, the three leading compounds are alphamonostearin followed by stigmast–5–en–3–ol and pyrrolidino piperazine 3, 6 dione.

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INTRODUCTION

As a result of indiscriminate use of antimicrobial drugs in the treatment of infectious diseases, microorganisms have developed resistance to antibiotics (Cowan, 1999). To overcome the problems of drug resistance alternative synthetic drugs have been explored and antimicrobial activities of many natural products are yet to be explored (Upadhyay *et al*., 2010). *Staphylococcus aureus* is one of the prominent medically important bacterial pathogens. Its potential to cause wide sprectrum of pyogenic lesions involving several organs, hospital outbreaks and community acquired infections are well recognized. *Staphylococcus aureus* infections are associated with resistance to several beta lactum antibiotics used in hospitals. These strains are known as MRSA (Methicillin resistant *Staphylococcus aureus*) (Arunava Kali, 2015). The prevalence of Methicillin resistant Staphylococcus aureus (MRSA) varies from 20% to 54.8% in different parts of India (Mathanraj *et al*., 2009). Recent study shows 29.1% prevalence in South India (Pai *et al*., 2010). It is important to discover new therapeutic compounds from natural biological sources like medicinal plants to treat infectious diseases caused by drug resistant bacteria. Antimicrobial compounds found in plants may prevent bacterial infections by different mechanisms than the commercial antibiotics and therefore may have clinical value in treating resistant microorganisms (Eloff, 1999). Leaves of *Camelia sinensis, Triphala, Lawsonia inermis*, *Azhadiracta indica, Holarrhena antidysentrica, Punica granatum, Hemidesmus indicus, Plumbago zeylanica* have shown invitro activity against MRSA (Aqil *et al*., 2006; Mehrotra *et al*., 2010; Aqil *et al*., 2005; Hena and Sudha, 2011; Sarmiento *et al*., 2011). As per Charaka (1500 BC) no tree has been longer and better known or appreciated by people of Indians than Bael (Phulan Rani *et al*., 2013). It's a slow growing, medium sized tree, upto 12- 14 m tall with short trunk, thick bark, alternate leaves, borne singly or in group composed of 3 to 5 oval, pointed leaflets (Rama Dahiya and Rajesh, 2010). The leaves, roots, bark, seeds and fruits of *Aegle marmelos* are edible astringent, expectorant and useful to treat ophthalmia, inflammations and diabetes (Shaili Yadav *et al*., 2015). Leaf is considered to be one of the highest accumulatory parts of the plant containing bioactive compounds which are synthesised as secondary metabolites (Cowan, 1999). Leaves of *Aegle marmelos* are used in abscess, abdominal disorders, vomiting, cuts and wounds (George *et al*., 2003). Several research work on *Aegle marmelos* antibacterial, antifungal, antifungal,

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antidiabetic, anti inflammatory activities have been reported. However scientific data of the potential of *Aegle marmelos* particularly with reference to antibiotic resistant pathogens has not been much explored. Hence, present study was undertaken to investigate the antibacterial and phytochemical properties of *Aegle marmelos* against MRSA in particular. In our previous studies, we investigated the antibacterial activity of crude ethanolic leaf extract against MRSA and the findings showed active antibacterial activity (Srikala Ganapathy and Karpagam, 2016). Therefore, crude ethanolic leaf extract was used for further studies namely fractionation and identification of active compounds using Gas chromatography- Mass spectrometry (GC MS) study. GC- MS is used as a valuable tool for identification of phytocompounds (Sampath Kumar and Ramakrishnan, 2011).

MATERIALS AND METHODS

Fractionation of the extract

The active crude ethanolic leaf extract of *Aegle marmelos* was subjected to column chromatography for further isolation. Silica gel column (150- 200 mesh) was used as a stationary phase and mobile gradient of increasing polarity solvent system (diethyl ether, ethyl acetate, methanol and water) were used to elute the constituents. About 5 ml of ethanolic extract was subjected to separation. Flow rate was standardised to be 1 ml/minute and eluants were collected in test tubes. All the fractions were labelled and stored at – 20 C in air tight bottles and used within one week (Udgire and Pathade, 2014; Davies Don and Johnson Todd, 2007). Each of the eluted fractions were checked for antibacterial efficacy against MRSA.

Antibacterial activity of the fractions

All the fractions were subjected to antibacterial sensitivity assay. The bacterial culture used in the study was pure clinical isolates of Methicillin resistant *Staphylococcus aureus* procured from Private Hospital, Chennai. Muller Hinton Agar (MHA) medium was used to study antibacterial activity. Prior to antibacterial screening, the bacterial culture was cultured in Muller Hinton Broth for about 4 hrs at 37°C. Antibacterial testing was carried out by Kirby Bauer disc diffusion method (Bauer *et al*., 1986.) The bacterial culture was inoculated as lawn culture using sterile swab over the agar surface. The filter paper discs impregnated with 100 microl of plant extract fraction (1mg/ml)were placed on the seeded agar plates. Dimethyl sulfoxide (DMSO) served as negative control and Streptomycin (10 microg) as reference. The plates were then labeled and incubated at 37^{0}C for 24 hours. After incubation, the plates were examined for clear inhibition zone and zone diameters were measured and recorded. The active fraction was selected for the identification of active phytoconstituents by Gas Chromatography- Mass spectrophotometer (GC MS) analysis.

Gas Chromatography- Mass spectrophotometer (GC MS) analysis

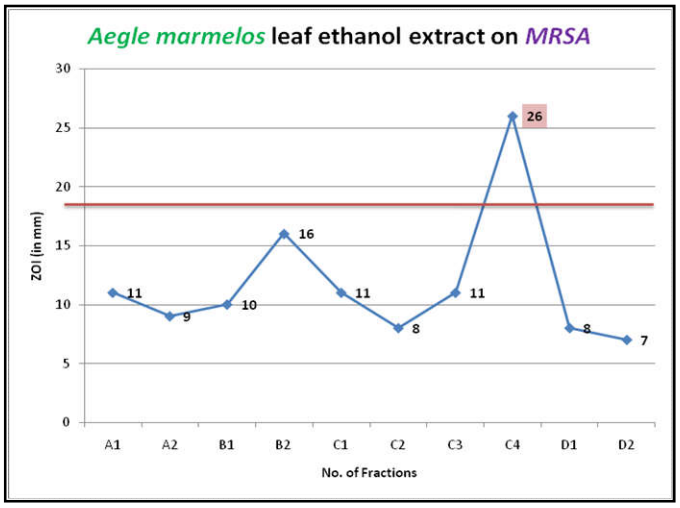
The active methanolic fraction was used for GC MS analysis to obtain information on active components present in the fraction. The analysis was performed in Sargam labs, Chennai with automated instrument available. 2 microl of methanolic extract was injected into the injection port of the instrument. Column oven temperature was set at 70 C. Injector temperature – 240 C. Column flow of helium was 1.50ml/min. Carrier gas helium 99.9995% purity was used and diameter of the column was 0.25mm. Ion source temperature was kept at 200 C. The mass range from 40 to 100m/z was scanned. MS Start time was 5 minutes and end time was 35 minutes. After obtaining the GC MS spectrum the compounds were identified by MS and Wiley libraries. Compounds identified with the area percentage and retention time by GCMS analysis are given in the result section.

RESULTS AND DISCUSSION

Total of 10 fractions were collected after column chromatography and their zones of inhibition against MRSA are presented in the graph (Graph 1). With respect to antibacterial efficacy, among the four solvents systems, methanol fraction showed the most promising antibacterial activity. The fraction C4 showed maximum zone of inhibition diameter of 26mm as compared to standard antibiotic Streptomycin which showed 18mm. Another fraction B2 showed moderate activity with zone of inhibition diameter 16mm while rest of the fractions showed diminished zone of inhibition.

Graph 1

The compounds identified from the promising bioactive fraction C4 is illustrated in chromatogram (Fig 1 & 2).



GC MS analysis of methanolic fraction of leaves of *Aegle marmelos* indicated the presence of various secondary metabolites. Knowledge of chemical constituents of plant is desirable as this information will be of value in the synthesis of drugs using complex chemical substances (Shailesh Kumar *et al*., 2013). In the present study, a mixture of 7 chemical compounds responsible for the antibacterial activity against MRSA were identified.

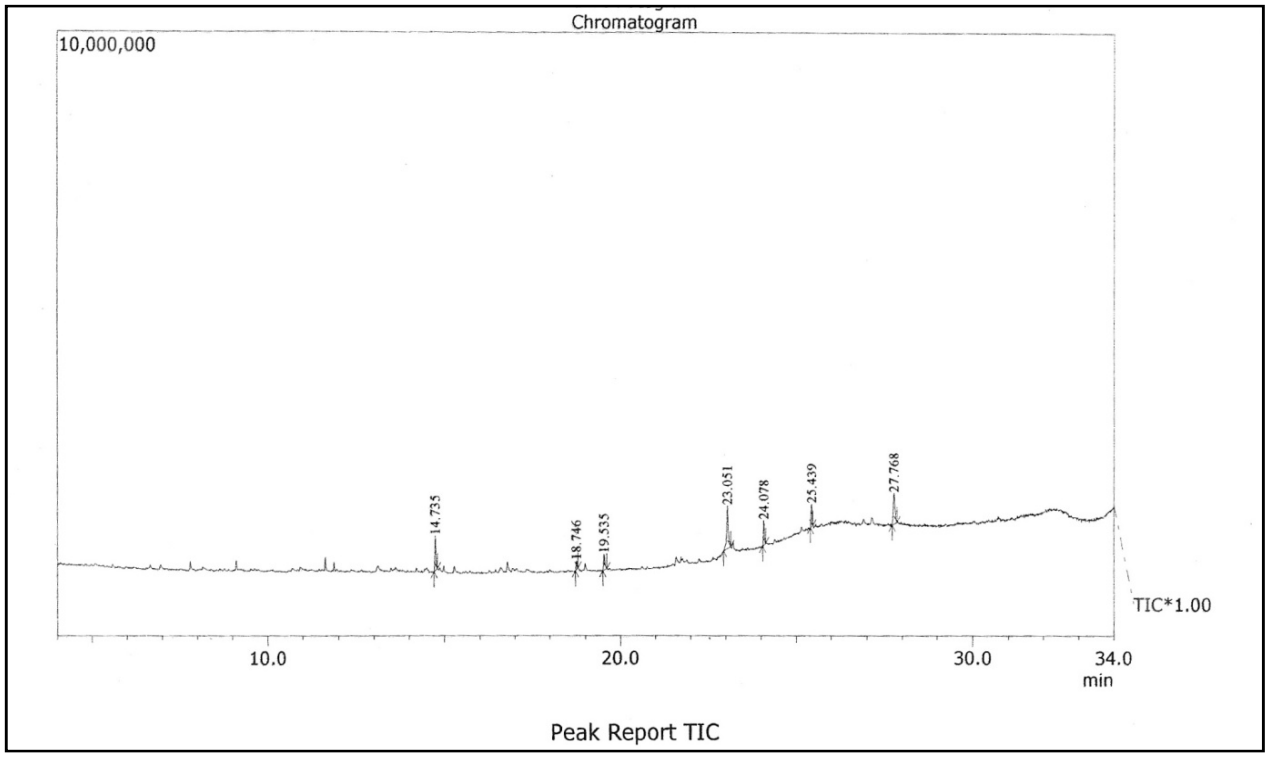


Fig.1. GC MS chromatography spectrum of active methanolic fraction of *Aegle marmelos* crude ethanolic leaf extract

PEAK#	R.TIME	AREA	AREA%	NAME
1	14.735	1284958	15.08	Pyrrolidino[1,2-a]piperazine-3,6-dione
2	18.746	318591	3.74	3,6-DIISOBUTYL-2,5-PIPERAZINEDIONE
3	19.535	699256	8.21	(2E)-2,7-DIMETHYL-2,7-OCTADIEN-1-AMINE
4	23.051	2486621	29.19	.alpha.-Monostearin
5	24.078	1099613	12.91	13,15-Octacosadiyne
6	25.439	781898	9.18	5,16-ANDROSTADIEN-3.BETA.-OL
7	27.768	1848422	21.70	STIGMAST-5-EN-3-OL
		8519359	100.00	

23/08/2014

Fig.2. Identification of antibacterial compounds by GC MS from *Aegle marmelos*

Three major phytochemical constituents present in the leaf extract were: Alphamonostearin with the peak area 29% followed by Stigmast–5-en–3-ol with peak area 21% and pyrrolidino – piperazine 3, 6 dione with the peak area 15% suggesting the inhibitory effect could be attributed to these compounds. The potent inhibitory activity of the fraction is due to the presence of these phyto compounds as secondary metabolites (Reshmi *et al*., 2010). One of the major bioactive compounds, Stigmas-5-en–3–ol is reported by previous researchers as anti inflammatory and anti oxidant compound. Also, earlier reports on Stigmasterol as a strong oxidant having antibacterial activity against multidrug resistant (MDR) Mycobacteria are reported (Hadan *et al*., 2011). To the best of our knowledge, this report is first of the kind to identify the phytochemical constituents of ethanolic leaf extract of *Aegle marmelos* against MRSA.

Conclusion

The results of the present study concludes that *Aegle marmelos* will be a potential source of valuable therapeutic alternative option for MRSA infections in the future and contributes to the current knowledge of the plant in the area of pharmacology. The plant can be further considered for investigations of these

bioactive principles for their purification, mechanism of action, toxigenicity assay and clinical trials for therapeutic applications.

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