

Original Research Article**EXTRACTS OF *HYPERICUM ERICOIDES* POSSESS ANTIBACTERIAL ACTIVITY AGAINST CLINICAL ISOLATES OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA)
ANTI-MRSA ACTIVITY OF *HYPERICUM ERICOIDES****Zeb Saddiqe¹, Ismat Naeem², Abdul Hannan³, Yasir Cheema³

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ABSTRACT

The present study was carried out to evaluate the antimicrobial properties of extracts of aerial parts of *Hypericum ericoides*, against clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA). The extracts were screened for anti-MRSA activity using agar well-diffusion method for preliminary screening against a reference ATCC strain and agar dilution method against thirty clinical isolates for the determination of minimum inhibitory concentration (MIC). All the extracts were also subjected to phytochemical analysis. In agar diffusion method *n*-hexane fraction was the most active with diameter of zone of inhibition 12 mm at 1.875 mg/ml. The inhibitory effect was dose dependant with larger zones of inhibition observed at higher concentration. No activity was shown by ethyl acetate fraction. In agar dilution method significant antibacterial activity was observed for the crude methanolic extract and *n*-hexane and dichloromethane fractions with MIC values 2048, 1024 and 512 µg mL⁻¹ respectively. The study concluded that the *n*-hexane and dichloromethane fractions of *H. ericoides* have significant antibacterial potential against MRSA strains and can be a good source of isolating antibiotics with potential activity against multi drug-resistant bacterial strains.

Key words: *Hypericum ericoides*, *Staphylococcus aureus*, MRSA, antibacterial activity

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1 INTRODUCTION

Since the discovery of antibacterial drugs, the bacterial infections have been controlled remarkably. However, the emergence of drug resistance in these pathogens as well as appearance of undesirable side effects of certain antibiotics has led to the discovery of new antibacterial agents particularly from medicinal plants which are a potential source for new antimicrobial agents [1,2]. Medicinal plants have been used for centuries as remedies for human diseases and are still providing mankind with new remedies [3]. Even before the discovery of microorganisms causing diseases in man, many plants were believed to contain healing powers which are now characterized as antimicrobial principles [4]. In many developing countries plant materials continue to play a major role in primary health care as therapeutic remedies [5,6]. During the past decade there has been an increase in the use of folk medicine globally [1] which is attributed to better compatibility with the human body, better cultural acceptability and lesser side-effects [7,8]. The acceptance of traditional medicine in recent years as an alternative form of health care and the development of microbial resistance to the available antibiotics has led researchers and scientists to investigate the antimicrobial activity of many medicinal plants [6,9-11]. Research has proposed that antimicrobial compounds isolated from plants inhibit microorganisms by a different mechanism than the commonly used antibiotics and can be used in treatment of resistant microbial strains [12]. Plant extracts are a practically untapped source of antibacterial agents and remarkably, it has recently been shown that multi drug-resistant (MDR) strains of *S. aureus* are more susceptible to plant metabolites than wild type

strains [13]. This indicates the enormous potential of this source to provide antibiotic-leads, which target MDR strains.

Hypericum (Hypericaceae) is a large genus comprising of about 484 species which are naturally occurring on every continent in the world except Antarctica. In many areas of the world, various species of *Hypericum* have been a part of traditional systems of medicine used as healing agents for thousands of years due to their medicinal properties [14,15]. Many plants of the genus are used for the treatment of burns, bruises, depression, wounds and various forms of pain [16]. The plant extracts from various species of the genus have exhibited promising activity against resistant bacterial strains including MRSA [15,17-20]. The present research was undertaken to address the clinical problems of multidrug-resistant *S. aureus* by screening crude methanolic extract and fractions of *H. ericoides*. *H. ericoides* is a small shrub found exclusively in eastern and southeastern Spain and in North Africa. The leaves, stems and flowers of the plant are used in Valentian folk medicine. No previous reports are available for anti-MRSA activity of extracts of this plant.

2 MATERIALS AND METHODS

2.1 Plant material

The plants of *H. ericoides* were purchased from Perryhill Nurseries and were identified by Dr. Tahira Mughal, Associate Professor, Department of Botany, Lahore College for Women University, Lahore. Plants were grown in the green house of University of Portsmouth, UK for one year. Herbarium specimen of the species was lodged in the Herbarium of Hampshire County Council Museum Service, Winchester, Hampshire, UK (Index Herbarium code HCMS; accession number Bi 2000 16. 371).

2.2 Extraction and fractionation of plant material

The fresh aerial parts of the plant were washed, dried and ground. The ground material was extracted thrice with methanol at room temperature. The combined solvent extracts were concentrated under reduced pressure to give crude methanolic extract. The crude extract was dissolved in double distilled water and successively partitioned between *n*-hexane, dichloromethane, ethyl acetate and *n*-butanol sequentially. The process afforded non-polar extracts of *n*-hexane and dichloromethane and polar extracts of ethyl acetate, *n*-butanol and water. The water fractions were freeze-dried to give aqueous fractions. All the extracts were weighed and stored in tightly sealed dark glass containers at 4°C for further analysis (Tables 1).

Table 1 Amount (g) and % yield of extracts of *Hypericum ericoides*

Fresh weight	Dry weight	MeOH extract (*)	<i>n</i> -Hexane (*)	CH ₂ Cl ₂ (*)	EtOAc (*)	<i>n</i> -BuOH (*)	Aqueous (*)
700	270	62 (22.00)	5.75 (9.27)	6.61 (10.66)	19.84 (32.00)	3.72 (6.00)	24.80 (40.00)

*% Values between parentheses indicate the percentage of mass in the corresponding extract, relative to the amount present in crude extract.

2.3 Phytochemical Analysis

The plant extracts were subjected to preliminary phytochemical analysis to test for the presence or absence of selected plant constituents, i.e., tannins, glycosides, steroids, triterpenes, flavonoids and saponins according to the standard methods [21].

2.4 Antibacterial Screening

2.4.1 Bacterial Strains

Thirty clinical isolates of methicillin-resistant *S. aureus* (MRSA-03, MRSA-04, MRSA-05, MRSA-06, MRSA-07, MRSA-08, MRSA-09, MRSA-10, MRSA-11, MRSA-12, MRSA-13, MRSA-14, MRSA-15, MRSA-17, MRSA-19, MRSA-20, MRSA-21, MRSA-22, MRSA-23, MRSA-24, MRSA-25, MRSA-26, MRSA-27, UHS-05, UHS-07, UHS-25, UHS-27, UHS-33, UHS-35 and UHS-201) were obtained from the Department of Microbiology, University of Health Sciences Lahore, Pakistan. These isolates were identified according to standard morphological (colonial and microscopic morphology), cultural and biochemical profile – coagulase production, haemolysis on blood agar plates, positive catalase and DNAase test (API-20E, bioMerieux, France) and serological identification (by antisera manufactured by BD Difco™, USA). Susceptibility was tested using Oxacillin discs (1µg). The ATCC strain of MRSA (25923) was used as standard strain.

2.4.2 Agar well-diffusion assay

Preliminary screening was carried out against standard MRSA strain (ATCC 25923) using agar well-diffusion method in accordance with the National Committee for Clinical Laboratory Standard [22]. All the extracts were tested at concentrations of 15, 7.5, 3.75 and 1.875 mg mL⁻¹ in dimethylsulfoxide (DMSO).

2.4.2.1 Preparation of inoculum

The bacteria stored at -70°C in micro-banks were sub-cultured on blood agar plates and incubated at 37°C for 24 hours. For preparing bacterial suspension four to five morphologically identical and isolated colonies were picked from the blood agar plate using a sterile inoculating loop and were transferred to a tube containing 4 mL sterile saline and vortex thoroughly. The suspension was compared and adjusted to 0.5 McFarland standards to have a culture stock containing about 1×10⁸ CFU mL⁻¹.

2.4.2.2 Inoculation of plates

The inoculum was evenly streaked on the entire surface of sterile Mueller Hinton agar plates using a sterile cotton swab. The plates were allowed to dry at room temperature for 15 min in a laminar flow chamber to let the inoculum set. Four wells (6 mm diameter) were made equidistance in each of the plates using a sterile cork borer. To each well was added 100 µL of the respective concentration of each sample using sterile micropipette. Three sets of control plates were also set. One was the organism control in which no extract was added to the inoculated plate to check the growth of the inoculum. In the second control set extracts were introduced in the wells of agar plate without any inoculum to check the sterility of the extracts used. In the third set the wells were filled with 100% DMSO (negative control) to check its antibacterial activity. All the plates were left at room temperature for 2 h for effective diffusion of solvent into the agar medium. Finally the inoculated plates were incubated for 24 h at 37°C. All the samples were tested in triplicate. The antibacterial activity was expressed as mean zone of inhibition diameters (mm) produced by each sample.

2.4.3 Agar dilution assay (Determination of MIC)

The MIC of all the extracts was determined by using agar dilution method (CLSI [23]). The extracts were tested at concentrations of 4096, 2408, 1024, 512, 256, 128, 64, 32, 16 and 8 µg mL⁻¹. The results obtained were used to have a quantitative estimation of antimicrobial activity in terms of % inhibition. To ensure reproducibility of the results experiments were performed in triplicates.

2.4.3.1 Preparation of sample incorporated Muller-Hinton agar plates

For preparation of sample incorporated plates, 20 ml of Mueller-Hinton agar was added to thirty flasks for each sample (for ten concentrations of each sample in triplicate) and autoclaved. The agar was allowed to cool to a temperature of 50°C and in each flask the extract was added from the stock solution (30 mg mL⁻¹) to attain the desired concentration. The contents of each flask were shaken and poured into 90 mm sterilized Petri plates. The plates were allowed to solidify at room temperature and then stored at 4°C.

2.4.3.2 Inoculation of plates

The inoculum for each MRSA strain was prepared as described in 2.4.2.1. Each bacterial suspension was then diluted to give 1×10⁷ CFU mL⁻¹ by adding 0.5 mL of suspension in 4.5 mL of saline solution. Then 600

μL of each bacterial suspension were transferred to the wells of inoculum replicating apparatus. Pre-heated plates were inoculated within 30 min of standardizing the inoculum to avoid changes in inoculum density. Three control plates were also set up. The first control plate without any extract was inoculated at the start of inoculation to confirm the validity of the bacterial cultures. A second control plate without inoculation was set to confirm the sterility of the medium. The third control plate contained the medium with plant extract without inoculation to check the sterility of the plant extract. All the plates were inoculated with multipoint inoculator. The replicator pins (2.5 mm in diameter) transferred about $1\mu\text{l}$ of inoculum (10^4 CFU/spot). The inoculum spots were allowed to dry at room temperature before incubating the plates. The plates were incubated at 37°C in air for 18 h. The MIC was taken as the lowest concentration of the extract that completely inhibited the visible growth of all the microorganisms as judged by the naked eye disregarding any single colony of a thin haze within the area of the inoculated spot.

3 RESULTS

The phytochemical analysis of the crude extract and fractions of *H. ericoides* revealed the presence of bioactive compounds including glycosides, triterpenes, steroids, flavonoids and phenolics while tannins were not detected in all the extracts. Polar extracts were rich in polar compounds i.e., steroids, glycosides, flavonoid and phenolics, while non-polar fractions were rich in non-polar substances such as terpenes (Table 2). Terpenes, flavonoids and phenolics present in plant extracts have been shown to possess antibacterial activity against MRSA strains [24-28] so their presence in the active fractions of the plant justifies the antibacterial activity of these extracts in the present study against clinical isolates of MRSA.

Table 2 Phytochemical analysis of extracts and fractions of *H. ericoides*

Extractant	<i>H. ericoides</i>					
	Tannins	Glycosides	Triterpenes	Steroids	Flavonoids	Phenolics
MeOH	+	+	-	-	+	++
<i>n</i> -Hexane	-	-	++	-	-	-
CH_2Cl_2	+	-	++	-	-	-
EtOAc	-	++	-	+	++	++
<i>n</i> -BuOH	-	++	-	+	++	++
Aqueous	-	+	-	-	+	++

-, not detected; +, minimum content; ++, moderate content; +++, maximum content

The extracts exhibited activity against MRSA strains both in terms of diameter of zone of inhibition (ϕ) and in terms of MIC values (Table 5). In agar well-diffusion method highest activity was of *n*-hexane fraction which was active at all the tested concentrations with zones of inhibition between 12-14 mm. Dichloromethane fraction was active at concentrations of 15 and 7.5 mg mL^{-1} with zones of inhibition 16 and 15 mm respectively while no activity was observed at 3.25 and 1.875 mg mL^{-1} . Both the fractions were more active than the crude methanol extract indicating the antibacterial potential of these fractions and the components present in them. *n*-Butanol and aqueous fractions were active only at 15 mg mL^{-1} with growth inhibition zone of 9 and 11 mm respectively and were inactive at 7.5 , 3.75 and 1.875 mg mL^{-1} . The ethyl acetate fraction was inactive at all the concentrations tested (Figure 1).

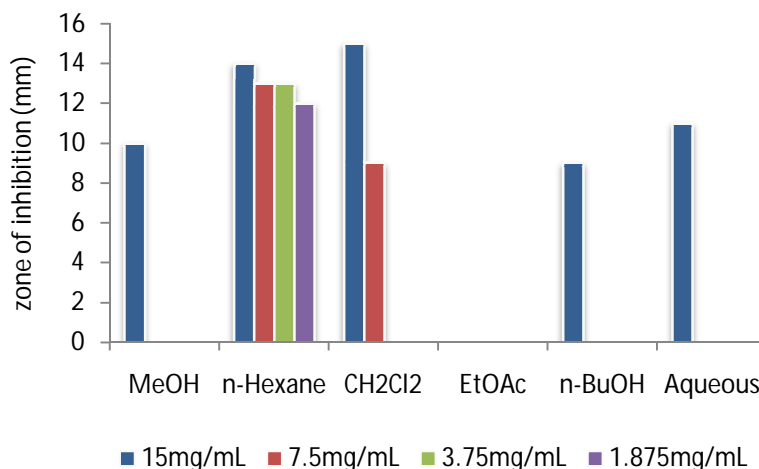


Figure 1 Extract concentration versus diameter of zone of inhibition in *H. ericoides*

In agar dilution assay the results were much more significant than agar diffusion method. The crude methanol extract produced 100% inhibitory effect at 2048 $\mu\text{g mL}^{-1}$. The hexane fraction showed 100% inhibition at 1024 $\mu\text{g mL}^{-1}$. Dichloromethane fraction showed 100% inhibition at 512 $\mu\text{g mL}^{-1}$. The ethyl acetate fraction was not so active against the tested MRSA strains and 74% inhibition was observed at 4096 $\mu\text{g mL}^{-1}$ (MIC >4096). The *n*-butanol fraction exhibited only 55% inhibition at 4096 $\mu\text{g mL}^{-1}$ and was inactive at all other concentrations tested (MIC >4096). The aqueous fraction did not show any significant activity and only 13% strains were inhibited at 4096 $\mu\text{g mL}^{-1}$ (MIC >4096) (Table 3).

Table 3 Minimum inhibitory concentration (MIC) of *H. androsaemum* and *H. ericoides* extracts against clinical isolates of MRSA*

MRSA	**MIC ($\mu\text{g mL}^{-1}$)					
	MeOH	<i>n</i> -Hexane	CH ₂ Cl ₂	EtOAc	<i>n</i> -BuOH	Aqueous
N = 31	2048	1024	512	>4096	>4096	>4096

*N = 31; **MIC = minimum concentration at which 100% inhibition was observed

4 DISCUSSION

Medicinal plants are practically an untapped source of antibiotics. Plants living in a changing environment have to face many challenges including microbial infections. They have innate ability to synthesize novel compounds active against the pathogenic fungi and bacteria which attack these plants. These compounds are generally secondary metabolites which are accumulated in different parts of the plants [29]. Many plant derived chemical including flavonoids, terpenes and phenolic compounds have been found to possess antimicrobial activity against resistant bacterial strains causing infections in humans [24,30,31]. These phytochemicals are free from side effects normally associated with synthetic chemicals used as antibiotics. In the present study the crude extracts and fractions of the two *Hypericum* species significantly inhibited the growth of clinical isolates of MRSA. Infections due to MRSA are increasing day by day with more and more antibiotics becoming less effective against these microorganisms [32,33]. Antibacterial activity was more concentrated in non-polar fractions of the plants since the lowest MIC values were determined for the *n*-hexane and dichloromethane fractions. These fractions mostly contain non-polar compounds including terpenes and flavonoid aglycones. Both the fractions were more active than the crude methanol extract further indicating the antibacterial potential of these fractions and their components. Many *Hypericum* species have shown activity against MRSA strains and in most of these studies the active fractions were *n*-

hexane and chloroform fractions which supports our findings [17,18, 20]. The results thus indicated that dichloromethane fraction *H. ericoides* can be a good source of bioactive compounds with MIC values less than $1 \mu\text{g mL}^{-1}$ since these are the crude extracts containing a large number of compounds.

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