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RESEARCH ARTICLE

AN IN-VITRO STUDY OF THE ANTIMICROBIAL EFFECT OF SARACA ASOCA LEAF EXTRACT ON METHICILLIN SENSITIVE AND METHICILLIN RESISTANT STAPHYLOCOCCUS AUREUS IN COMBINATION WITH METHICILLIN

[2]Sarda, P., [3]Dr. Prsashar, K., [4]Dr. Gupta, S., [5]Dr. Damle, A., [6]Dr. Gaikwad, A. and [*,1]Warkhade, B.

[1,2,3]Department of Biotechnology, Badrinarayan Barwale Mahavidyalaya, Jalna, Maharashtra, India
[4]Government Institute of Forensic Science, Aurangabad, Maharashtra, India
[5,6]Department of Microbiology, Government Medical College, Aurangabad, Maharashtra, India

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ABSTRACT

Staphylococcus aureus is a species of bacterium commonly found on the skin and or in the noses of healthy people, although it is usually harmless at these sites, it may occasionally get into the body, through breaks in the skin such as abrasions, cuts, wounds, surgical infections, or indwelling catheters and cause infections. These infections may be mild (pimples or boils) or serious (infection of bloodstream, bones or joints). *Staphylococcus aureus* is one of the most important bacteria as a potential pathogen specifically for nosocomial infections. Methicillinresistant *Staphylococcus aureus*has been emerging worldwide as one of the most important hospital and community pathogen. Therefore, new agents are needed to treat MRSA. In the present study, antimicrobial activity of aqueous, ethanol and acetone extracts of *Saraca asoca* were evaluated against methicillin resistant *Staphylococcus aureus* (MRSA) and methicillin sensitive *Staphylococcus aureus* (MSSA).The ethanol extract demonstrated a higher antibacterial activity than the acetone and aqueous extract. These extracts were prepared from fresh *Saraca asoca* leaves. These extracts were evaluated for their part in increasing antibacterial activity of Methicillin against *S. aureus* (ATCC 25923) and MRSA (ATCC 43300). The antibacterial activity of Methicillin was enhanced against the test organism in the presence of these extracts. Methicillin in combination with these extracts showed maximum inhibition against MSSA and MRSA. Phytochemical analysis gave positive results for steroids, triterpenoids, glycosides, phenolic compounds, flavonoids, tannins, saponins, reducing sugar, carbohydrates, amino acids and proteins. Leaf extract of *Saraca asoca* contains pharmacologically bioactive constituents that may be responsible for its activity against test organisms.

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INTRODUCTION

The development of antibiotic resistance in bacteria is a major issue in the prevention of infectious diseases. Currently the spread of multi-drug resistant bacteria is not only through nosocomial infections, but also occur in the community (Talbot *et al.,* 2006, Gould, 2008). *Staphylococcus aureus* causes impetigo, furuncles, cellulitis, abscess, and wound infections among other diseases. Pathology results not only from tissue destruction and toxin production but also from its propensity to disseminate. This produces significant morbidities such as osteomyelitis, suppurative arthritis, pneumonia, deep abscesses, endocarditis and other disseminated infections (Wendy *et al.,* 2011).Methicillin-resistant *Staphylococcus aureus* (MRSA) is fast becoming a worrisome threat. Initially considered a purely nosocomial infection, it has now become a common, community-acquired infection in the United States. Treatment of such diseases entails considerable cost. Currently accepted standards for treatment include oral and intravenous oxacillin for susceptible *Staphylococcus aureus*, and vancomycin for MRSA. The organism flourishes in the hospital setting producing bloodstream and surgical wound infections (Haddadin *et al.,* 2002; Jones *et al.,* 2003). Methicillin was introduced 1959 to treat staphylococcal infections not responding to penicillin therapy. However only within a year some strains of *S. aureus* were reported to be resistant to it. These strains were named as 'Methicillin Resistant *Staphylococcus aureus'* (MRSA). During the past four decades MRSA has spread throughout the world and has become highly endemic in many geographic areas (Enright *et al*., 2002). As the bacteria that cause the

Corresponding author: **Warkhade, B.**
Department of Biotechnology, Badrinarayan Barwale Mahavidyalaya, Jalna, Maharashtra, India.

infection was resistant to first-line antibiotics, treatment options are usually replaced with a second or third choice of antibiotics, which are generally much more expensive. Therefore, alternative antimicrobial agents are needed to be developed and employed to control multi-drug resistant bacteria. To face this challenge, there has been growing interests to find antimicrobial compounds from medicinal plant extracts as an alternative approach to discover new antimicrobial compounds. The antimicrobial activities of some herbal medicines against different pathogens have been reported from different countries (Rios *et al.,* 2005. Tomoko *et al.,* 2002). Ashoka is one of the most legendary and sacred trees of India. Ashoka tree universally known by its binomial Latin name *Saraca asoca* (Roxb.), de.wild or *Saraca indica* belonging family Caesalpinaceae (Biswas *et al.,* 1972; Warrier *et al.*, 2001). It is an evergreen tree called in English Ashoka tree. It is also known as Kankeli (Sanskrit), Ashoka (Assamese), Ashoka (Bengali), Ashoka (Gujrati), Ashoka (Hindi), Ashokadamara (Kannada), Ashok (Kashmiri), Asokam (Malayalam), Ashok (Marathi), Ashoka (Oriya), Ashok (Punjabi), Asogam (Tamil), Ashokapatta (Telugu) (Pradhan *et al.*, 2009). *Saraca asoca* has many uses mainly in the medicine to treat the women gynecological disorders, in all types of abnormal discharges per vagina, in uterine inertia, uterine pain, urinary calculus, dysurea, etc. *Saraca asoca* (ashoka) plant contains the presence of glycoside, flavonoids, tannins and saponins (Pradhan *et al.,* 2009). It is used as spasmogenic, oxytocic, antibacterial (Warkhade *et al.,* 2017), anti-tumor, anti-progestational, anti-estrogenic activity against menorrhagia and anticancer agent. The plant is useful in dyspepsia, fever, burning sensation, colic, ulcer, menorrhagia, leucorrhoea, pimples, etc. In current study, screening the bioactive components and the antibacterial effects of the ethanol, acetone and aqueous extract and the extracts were evaluated in combination with methicillin to assess their antibacterial activity against MRSA and MSSA.

MATERIALS AND METHODS

Sample collection

The fresh leaves of S*arasaasoca* were obtained from campus of Badrinarayan Barwale Mahavidyalaya, Jalna (MS). They were washed under running tap water to remove surface dirt and impurities, followed by distilled water. These leaves were air dried. After drying the leaves were chopped to get fine pieces. This chopped pieces were used for preparation of different plant extracts.

Preparation of leaf extracts

For aqueous extract 10gm of fine pieces of leaves was added to 100ml of distilled water and then boiled for 15min. after cooling this was filtered through Whatman's filter paper and filtrate was used as an aqueous extract. This extract was stored at 4^0C for further use. For ethanol extract absolute ethanol was taken 100ml and to this 10gm of fine pieces of leaves was added and kept overnight. After that this was filtered through Whatman's filter paper and filtrate was used as an ethanol extract. This extract was stored at 4^0C for further use. For acetone extract 100ml Acetone was taken and to this 10gm of fine pieces of leaves was added and kept overnight. After that this was filtered through Whatman's filter paper and filtrate was used as an Acetone extract. This extract was stored at 4^0C for further use.

Test organism

Bacterial cultures were selected from American type culture collection (ATCC). The strain used for the study were *staphylococcus aureus* (ATCC 25923) and MRSA (ATCC 43300) was taken from Government Medical College, Aurangabad. These were grown on selective media and purity was determined by morphological and biochemical characterization.

Inoculum preparation

Loopful of pure culture from selective media was picked up and inoculated in Muller Hinton Broth (Himedia). It was incubated at 37^0C for 3-7 hrs until moderate turbidity develops. Inoculum turbidity was compared with that of 0.5 McFarland standard.

Preparation of Disc

Whatman's filter paper no.1 was punched to get disc of 6mm diameter. These discs were sterilized under UV light. Each sterile disc was impregnated with ethanol extract, acetone extract, aqueous extract and excess of solvent was dried in controlled temperature.

Antimicrobial activity of extract

The antimicrobial activity of the extract was evaluated by standard disc diffusion method (Baur *et al*., 2012). Plates of Muller Hinton agar (Himedia) medium having media up to 4 mm were prepared. After solidification lawn of inoculum was prepared on to agar plates for each organism. Inoculum was taken by soaking the sterile swab (Himedia) in prepared inoculum of test organism i.e. *Staphylococcus aureus* and MRSA and spread over the agar plates for respective organism. Ethanol extract disc, acetone extract disc and aqueous extract disc of *Saraca asoca* was applied and incubated at 28-30^{o}C for 16-18 hours.

Disc diffusion assay to evaluate combined effects

Disc diffusion method was used to evaluate in-vitro antibacterial activity of Methicillin (5mcg, Himedia) against *Staphylococcus aureus* and MRSA on Muller Hinton Agar (Himedia). To determine combined effect, each standard paper disc was further impregnated with 20µl of each single extract. Muller Hinton Agar plates were inoculated with *Staphylococcus aureus* and MRSA. Standard antibacterial Methicillin disc were used as positive control and Methicillin disc impregnated with aqueous, ethanol, and acetone extract were place onto Muller Hinton Agar plate inoculated with test organisms. These plates were incubated 16-18 hours. After incubation, the zones of inhibition were measured. These assays were performed in triplicate.

Assessment of increase in fold area

The increase in fold area was assessed by calculating the mean surface area of the inhibition zone of each antibacterial agent (Methicillin) and Methicillin plus 20µl extract. The fold increase area of different test organism for Methicillin and for Methicillin plus extract was calculated by equation $(B^2-A^2)/A^2$, where A and B were zones of inhibition for Methicillin and Methicillin plusextract, respectively.

Phytochemical analysis

Phytochemical tests were done to find the presence of the active chemical constituents such as alkaloid, flavonoids, glycosides, triterpenoids, steroids, tannin and phenols, reducing sugar, carbohydrates and protein and amino acids by the following procedure. (Kokate, 2000; Harbone, 1999; Prashanth Tiwari *et al.*, 2011)

Tests for Alkaloids

To the extract, dilute hydrochloric acid was added, shaken well and filtered. With the filtrate, the following tests were performed.

Mayer's reagent test

To 3 ml of filtrate, few drops of Mayer's reagent were added along sides of tube. Formation of creamy precipitate indicates the presence of alkaloids.

Tests for Carbohydrates

Molisch test: 2 ml of aqueous extract was treated with 2 drops of alcoholic α-naphthol solution in a test tube and then 1 ml of concentrated sulphuric acid was added carefully along the sides of the test tube. Formation of violet ring at the junction indicates the presence of carbohydrates.

Tests for Reducing Sugars

Benedict's test: Equal volume of Benedict's reagent and extract were mixed in a test tube and heated on a water bath for 5-10 minutes. Solution appears green, yellow or red depending on the amount of reducing sugar present in the test solution which indicates the presence of reducing sugar.

Tests for Flavonoids

Alkaline reagent test: The extract was treated with few drops of sodium hydroxide solution separately in a test tube. Formation of intense yellow color, which becomes colorless on addition of few drops of dilute acid indicates the presence of flavonoids.

Tests for Glycosides

Borntrager's test: To 3 ml of test solution, dilute sulphuric acid was added, boiled for 5 minutes and filtered. To the cold filtrate, equal volume of benzene or chloroform was added and it was shaken well. The organic solvent layer was separated and ammonia was added to it. Formation of pink to red color in ammonical layer indicates the presence of anthraquinone glycosides.

Tests for Tannin and Phenolic compounds

Ferric chloride test: A small amount of extract was dissolved in distilled water. To this solution 2 ml of 5% ferric chloride solution was added. Formation of blue, green or violet color indicates presence of phenolic compounds.

Test for Saponin

Froth test: The extract was diluted with distilled water and shaken in a graduated cylinder for 15 minutes. The formation of layer of foam indicates the presence of saponins.

Tests for Protein and Amino acids

Ninhydrin test: 3 ml of the test solution was heated with 3 drops of 5% Ninhydrin solution on a water bath for 10 minutes. Formation of blue color indicates the presence of amino acids.

Tests for Triterpenoids and Steroids

Salkowski's test: The extract was treated with chloroform and filtered. The filtrate was added with few drops of concentrated sulphuric acid, shaken and allowed to stand. If the lower layer turns red, sterol is present. Presence of golden yellow layer at the bottom indicates the presence of triterpenes.

RESULTS AND DISCUSSION

The antibacterial activity of Acetone, Ethanol and aqueous extract of *Saraca asoca* against MRSA and *S.aureus* were shown in Table 1. Ethanol extract shows highest 10mm zone diameter against MSSA and 9mm zone against MRSA. Acetone extract shows 10mm zone diameter against MSSA and 8mm zone diameter against MRSA. aqueous extract shows no zone whereas standard antibiotic shows 20mm zone aginst MSSA and 2mm zone diameter against MRSA. (Figure 1, Graph 1).

Table 1. Antibacterial activity of Ethanol, Acetone and Aqueous extract of *Saraca asoca*

Test Organism	Zone of inhibition of *Saraca asoca* Extract in mm			Zone of inhibition of Methicillin in mm
	Ethanol	Acetone	Aqueous	
MRSA	09	08	00	02
MSSA	10	10	00	20

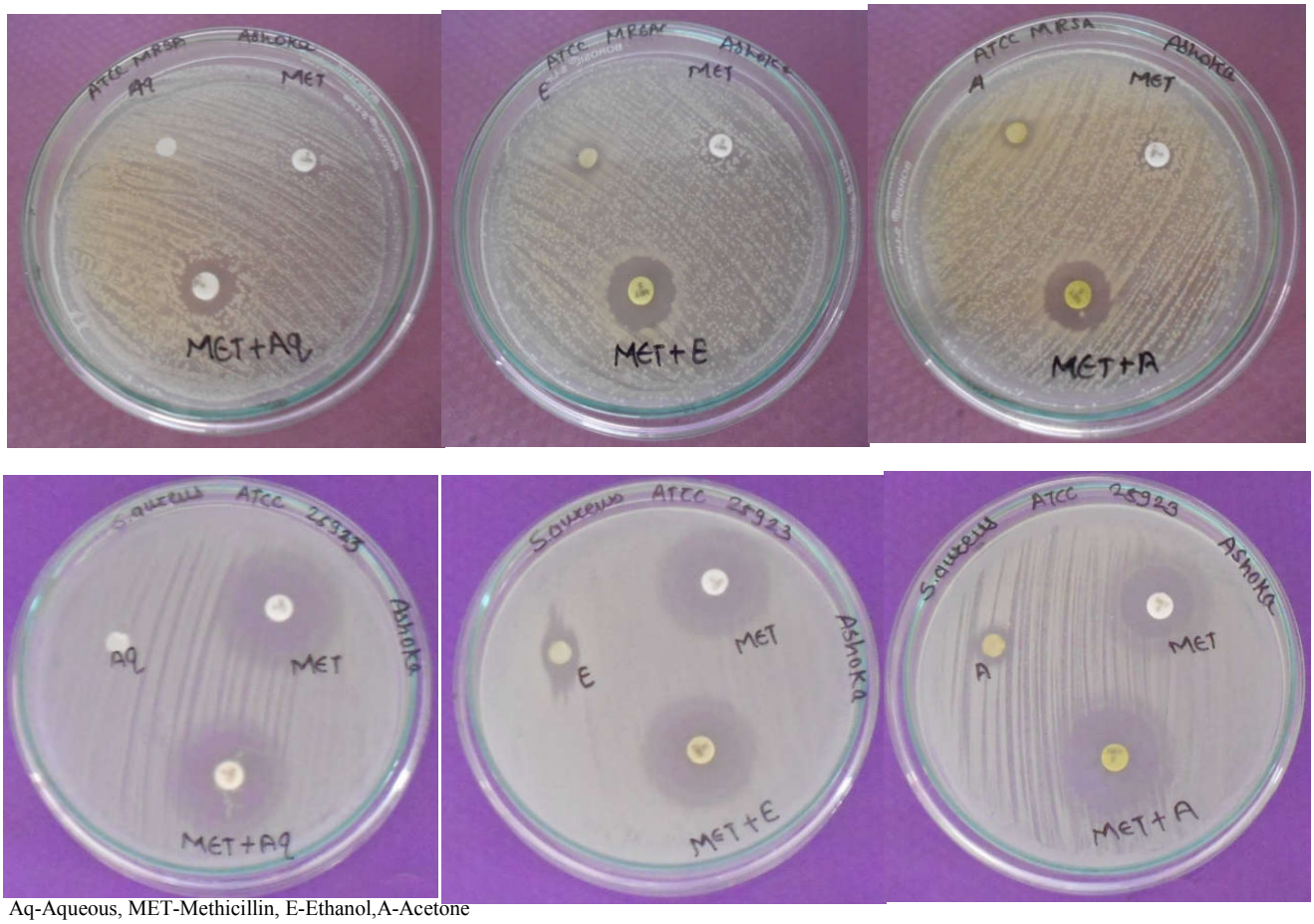
In the in-vitro antibacterial activityofMethicillin, an antibacterial agent that is widely used against staphylococcal infection, was used as positive control for comparison with *Saraca asoca* extracts. The diameter of zone of inhibition and increase in fold area for all the test organism was measured. The antibacterial activity of methicillin increased significantly in presence of ethanol, acetone and aqueous extract of *Saraca asoca*.

Table 2. Zone of inhibition of Methicillinagainst test organism in absence and in presence of *Saraca asoca* extract at content 20µl per disc

Test Organism	*Saraca asoca* Extract	Methicillin +Extract (B)	Methicillin (A)	Increase in Fold B^2-A^2/A^2 Area
MRSA	Ethanol	16mm	2mm	63
	Acetone	16mm	2mm	63
	Aqueous	13mm	2mm	41.25
MSSA	Ethanol	22mm	20mm	0.21
	Acetone	22mm	20mm	0.21
	Aqueous	22mm	20mm	0.21

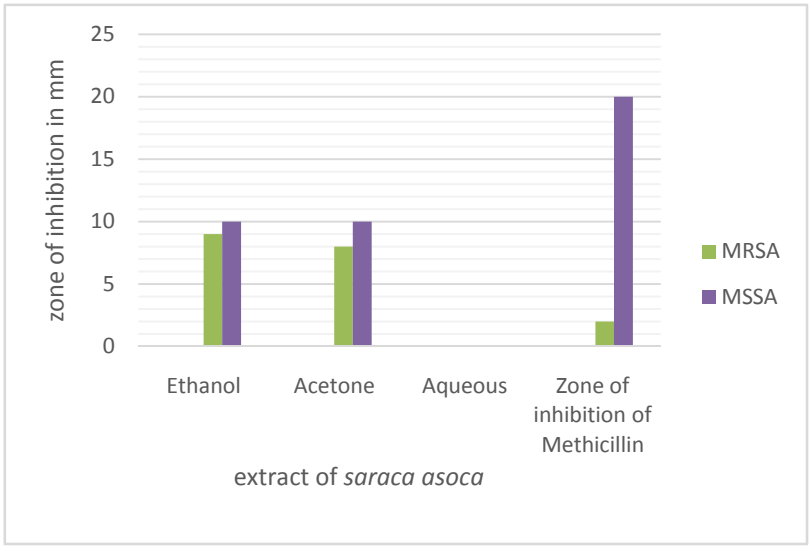
Phytochemical analysis

The phytochemical analysis of plant extracts using acetone, ethanol and aqueous was showed in Table 3. From the phytochemical analysis, the Ethanol extract of *Saraca asoca* showed the presence of glycosides, flavonoids, saponins, carbohydrates, reducing sugar, triterpenoids and steroids. Amino acids and proteins, flavonoids, tannin and phenolic



Aq-Aqueous, MET-Methicillin, E-Ethanol,A-Acetone

Figure 1. Antimicrobial activity of *Saraca asoca* against MRSA and MSSA in combination with methicillin



Graph 1. Antibacterial activity of Ethanol, Acetone and Aqueous extract of *Saraca asoca* and Methicillin

Table 3. Phytochemical analysis of plant extracts

Phytochemical test	Ethanol extract	Acetone extract	Aqueous extract
Tests for Alkaloids	-	-	-
Tests for Carbohydrates	+	-	+
Tests for Reducing Sugars	+	-	+
Tests for Flavonoids	+	+	+
Tests for Glycosides	+	-	+
Tests for Tannin and Phenolic compounds	-	+	+
Test for Saponins	+	-	+
Tests for Protein and Amino acids	-	+	-
Tests for Triterpenoids and Steroids	+	-	+

(+) indicates presence while (–) indicates the absence of the components

compound were observed in acetone extract of *Saraca asoca* but there was absence of carbohydrates, reducing sugar, triterpenoids and steroids. Glycosides, flavonoids, saponins, tannin and phenolic compound, reducing sugar, carbohydrates, triterpenoids and steroids were found in presence of aqueous extract of *Saraca asoca*. Ethanol, Acetone and Aqueous extract of *Saraca asoca* shows absence of alkaloids.Control of infections acquired in hospitals and communities caused by multi-drug resistant bacteria has become a major problem not only in developing countries but also in developed countries. In the past few decades, MRSA become an increasingly important pathogen in both hospitals and community settings (Radji *et al.,* 2011; Kollef*et al.,* 2006).The preliminary phytochemical tests performed were of qualitative type and from the phytochemical investigations it was observed that tannins and phenolic compounds, flavonoids, terpenoids and steroids, saponins, Glycosides, carbohydrates, proteins and amino acids and reducing sugars were present in the extracts. *Saraca asoca* leaves possessed good anti-bacterial activity confirms the presence of bioactive compounds responsible for the antibacterial activity. The search for new ways to treat MRSA infections stimulates the investigation of natural compounds as an alternative treatment of these infections. In present study the analysis of the growth inhibition activity by disc diffusion method showed that *Saraca asoca* may be use to treat infection cause by MRSA.

Conclusion

The combined effects of a standard antibacterial agent (Methicillin) with extracts against MRSA is similarly a new finding. Further, it can be concluded that extract alone or their formulations (combination) can be used as effective agents against human bacterial pathogen (MRSA).

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