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ANTIBACTERIAL ACTIVITY OF MURRAYA KOENIGII AGAINST *STAPHYLOCOCCUS AUREUS* AND *STREPTOCOCCUS PYOGENES*

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ABSTRACT

Evaluated the antibacterial effect of aqueous and ethanolic extract of the leaf, bark of *Murraya koenigii* L. against previously characterized *Staphylococcus aureus* and *Streptococcus pyogenes* was analyzed in this study. Aqueous and ethanolic extract of the leaf, bark of *Murraya koenigii* L. were prepared with help of soxhlet unit. Further, evaluated the antimicrobial activity of these extract were analyzed against *S. aureus* and *S. pyogenes*. Aqueous and ethanolic extract of the leaf and bark of *Murraya koenigii* showed significant antibacterial activity against *Staphylococcus aureus* and *Streptococcus pyogens*. 15 µg/ml extract of leave showed 47.05% more inhibition zone against *Staphylococcus aureus* as compared to 15µg/ml norflox (control drug) and 15, 20, 25, 30, 35 µg/ml extract showed 13.55, 27.11, 41.01, 47.47, 61.01% more antimicrobial activity against *Streptococcus pyogens* as compared to 15µg/ml norflox (control drug). Aqueous extract of bark *Murraya koenigii* showed significant antibacterial activity. 15 µg/ml extract showed 229.41% more inhibition zone against *Staphylococcus aureus* as compared to 15µg/ml norflox (control drug).

Key Words:- MIC, MBC, *Murraya koenigii*, *Staphylococcus aureus*, *Streptococcus pyogenes*.

INTRODUCTION

Murraya koenigii, commonly known as *curry* leaf or *kari patta* in Indian dialects, belonging to Family Rutaceae (Deshwal and Siddiqui, 2011). The *Murraya koenigii* plant is widely used as herb, spice, condiments and also used to treat various types of ailments in Indian traditional system and world's about 80% population relies upon herbal products, because they have been considered as safe, effective and economical (Arora *et al.*, 2011). *Murraya koenigii* originates from Pakistan, Sri Lanka and India east to China and Hainan and it has been widely cultivated in South-East Asia and some parts of the United States and Australia (Chauhan, 1999).

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The stem of *M. koenigii* is an aromatic and more or less deciduous shrub or small tree upto 6 meters in height and 15 to 40 cm in diameter. Petals five, free, whitish, glabrous and with dotted glands (Chopra *et al.*, 1999). The leaves of plant are use as tonic, stomachic, carminative, internally in dysentery, vomiting and plant used as antihelminthic, analgesic, cures piles, allays heat of the body, thirst, inflammation, itching and a scrutiny of literature reveals some notable pharmacological activities of the plant such as activity on heart, anti diabetic and cholesterol reducing property, antimicrobial activity, antiulcer activity, antioxidative property, cytotoxic activity, anti diarrhea activity, phagocytic activity and many more medicinal values (Khare, 2004).

The respiratory infection effects on major population of world wide. The human upper respiratory tract is the reservoir of a diverse community of commensals and potential pathogens (pathobionts),

including *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Staphylococcus aureus* which occasionally turn into pathogens causing infectious diseases (Juhn *et al.*, 2012).

The rapid division of bacterial cells causes them to evolve resistance to most treatments rather quickly and converted into resistance (Pray, 2008). Continuous use of drug makes the micro-organisms into multi drug resistant (MDR). The increasing prevalence of multidrug resistant strains of bacteria and the recent appearance of strains with reduced susceptibility to antibiotics raises the spectre of untreatable bacterial infections and adds urgency to the search for new infection-fighting strategies (Janovská *et al.*, 2003). In addition to this problem, antibiotics are sometimes associated with adverse effects on the host including hypersensitivity, immune-suppression and allergic reactions (Ahmad *et al.*, 1998).

Use of antibiotics is not safe so scientists are more focus on alternative. There is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action because there has been an alarming increase in the incidence of new and re-emerging infectious diseases (Parekh and Chanda, 2008).

Plants are the richest resource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs Hammer *et al.*, 1999). So aim of present study is to evaluate the antimicrobial activity of *Murraya Koenigii* against *Staphylococcus aureus* and *Streptococcus pyogenes.*

MATERIALS AND METHODS

Testing of pathogens: The pathogenic samples had been collected from the hospitals at haridwar, Uttarakhand (India) and these characterized gram positive bacteria such as *Staphylococcus aureus* and *Streptococcus pyogenes* were selected for present study (Panwar and Bhatt, 2014).

Twenty *Staphylococcus aureus* strains and named as SAD-1, SAD-2, SAD-3, SAD-4, SAD-5, SAD-6, SAD-7, SAD-8, SAD-9, SAD-10, SAD-11, SAD-12, SAD-13, SAD-14, SAD-15, SAD-16, SAD-17, SAD-18, SAD-19 and SAD-20. And twenty *Streptococcus pyogens* named as SPD-1, SPD-2, SPD-3, SPD-4, SPD-5, SPD-6, SPD-7, SPD-8, SPD-9, SPD-10, SPD-11, SPD-12, SPD-13, SPD-14, SPD-15, SPD-16, SPD-17, SPD-18, SPD-19 and SPD-20 (Panwar and Bhatt, 2014).

Preparation of aqueous extraction: Approx. 30 grams of dried powder of medicinal plant were transferred into soxhlet unit. Extract was done at 95^{o}C for 24 hours. Bottom of soxhlet extraction unit contains plant extract which was filtered through 8 layers of muslin cloth and then stored at 4ºC.

Preparation of ethanol extraction: Approx. 30 grams of dried powder of medicinal plant were transferred into soxhlet unit. Extract was done at 45^{o}C for 72 hours. Bottom of soxhlet extraction unit contains plant extract which was filtered through 8 layers of muslin cloth and then stored at 4ºC.

Preparation of different concentration: The extracts were sieved through a fine mesh cloth and sterilized using a membrane filter (0.45-micron sterile filter). This extract was considered as the 100% concentration of the extract (Indu *et al.*, 2006). The concentrations such as 15, 20, 25, 30, 35 µg/ml were prepared and norflox 15 µg/ml worked as control drug.

Sterilization of extract: The dried extracts were exposed to ultra violet light (UV rays for 24 h to sterilize. Liquid extracts were sterilized using a membrane filter (0.45-micron sterile filter).

Sterility Test: The sterility was checked by streaking the extracts on nutrient agar plate and incubated at 37°C for 24 h. It was confirmed that there were no artifacts to contaminate the sensitivity testing.

Antibacterial Activity by disc diffusion method: The microorganism was activated by inoculating a loopful of the strain in the nutrient broth (30 ml) and incubated on a rotary shaker. Then 0.2 ml of inoculum (inoculum size was 10^8 cells/ml as per McFarland standard) was inoculated into the molten Muller Hinton agar media and after proper homogenization it was poured into the Petri plate. The test compound (0.1 ml) was introduced on the disc (0.7 cm) and then allowed to dry. Then the disc was impregnated on the seeded agar plate. The plates were incubated at 37ºC for 24 h. Microbial growth was determined by measuring the diameter of zone of inhibition. For each bacterial strain, controls were maintained in which pure solvents were used instead of the extract. The control zones were subtracted from the test zones and the resulting zone diameter is shown in the graph. The experiment was done three times and the mean values are presented.

Antibacterial Activity by serial dilution in tubes: Dry the extract of medicinal plant. This powder of medicinal plant was dissolved in sterilized Mueller-Hinton broth and sterilized by membrane filter method. Various concentration of medicinal plants such as 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100 µg/ml were prepared. The tubes were inoculated with 20 µl of the bacteria suspension per ml of broth, homogenised and incubated at 37ºC for 24 hours. After incubation, 50 µL

were taken from each tube and inoculated in a second tube containing 1 ml of sterile Mueller-Hinton broth, homogenised and incubated for another 24 hours at 37ºC. The Minimal Inhibitory Concentration (MIC) was determined as the lowest concentration of medicinal plant for growth was observed in second set of tubes. The Minimal Bactericidal Concentration (MBC) was determined as the lowest concentration of medicinal plant for which no growth was observed in the second set of tubes (Parekh *et al.*, 2005)

RESULTS

Aqueous extract of leaf *Murraya koenigii* showed significant antibacterial activity. 15 µg/ml showed 47.05% more inhibition zone against *Staphylococcus aureus* as compared to 15µg/ml norflox (control drug) and 35µg/ml extract showed 188.23% more inhibition zone against *Staphylococcus aureus* as compared to control. But 15, 20, 25, 30, 35 µg/ml extract showed 13.55, 27.11, 41.01, 47.47, 61.01% more antimicrobial activity against *Streptococcus pyogens* as compared to 15µg/ml norflox (control drug) (Table 1a,1b). Similarly, ethanolic extract of leaf of *Murraya koenigii* showed significant against *Staphylococcus aureus* and *Streptococcus pyogens*. 15µg/ml extract showed 51.72 % more inhibition zone against *Staphylococcus aureus* as compared to 15µg/ml norflox (control drug). But 15µg/ml showed less inhibition zone as compared to 15µg/ml norflox (control drug). Other 20, 25, 30, 35µg/ml showed more inhibition zone against *Streptococcus pyogens* by 0, 9.02, 13.54, 23.02% respectively as compared to 15µg/ml norflox (control drug) (Table 2a, 2b). Aqueous extract of bark *Murraya koenigii* showed significant antibacterial activity. 15 µg/ml extract showed 229.41% more inhibition zone against *Staphylococcus aureus* as compared to 15µg/ml norflox (control drug). 15 µg/ml extract showed 370.58% more inhibition zone against *Staphylococcus aureus* as compared to control. But 15, 20, 25 µg/ml extract did not show more antimicrobial activity against *Streptococcus pyogens* as compared to 15µg/ml norflox (control drug) and 35µg/ml extract showed more inhibition zone by 56.14% respectively against *Streptococcus pyogens* as compared to control (Table 3a, 3b).

Similarly, ethanolic extract of bark of *Murraya koenigii* showed significant against *Staphylococcus aureus* and *Streptococcus pyogens*. 20µg/ml extract showed 14.08% more inhibition zone against *Staphylococcus aureus* as compared to 15µg/ml norflox (control drug). 35 µg/ml extract showed 56.33% more more inhibition zone against *Staphylococcus aureus* as compared to 15µg/ml norflox (control drug). Similarly, 15, 20, 25, 30, 35µg/ml showed more inhibition zone against *Streptococcus pyogens* by 95.23, 126.98, 158.73, 190.47, 222.22% respectively as compared to 15µg/ml norflox (control drug) (Table 4a, 4b). Aqueous extract of leaf of *Murraya koenigii* showed least MIC (5µg/ml) against *Staphylococcus aureus* but MIC of extract was 10 µg/ml. MBC of aqueous and ethanolic extracts varies from 10 to 25 µg/ml

Table 1a. Effect of aqueous extract of leaf of *Murraya koenigii* against *Staphylococcus aureus*

Pathogen	Inhibition zone (mm)					
	Medicinal plant					control
	15µg/ml	20µg/ml	25µg/ml	30µg/ml	35 µg/ml	15µg/ml
SAD-1	6	8	9	10	12	4
SAD-2	6	8	9	10	12	4
SAD-3	6	8	9	10	12	4
SAD-4	7	9	10	11	13	5
SAD-5	6	8	9	10	12	4
SAD-6	7	9	10	11	13	5
SAD-7	6	8	9	10	12	4
SAD-8	7	9	10	11	13	5
SAD-9	6	8	9	10	12	4
SAD-10	6	8	9	10	12	4
SAD-11	6	8	9	10	12	4
SAD-12	6	8	9	10	12	4
SAD-13	6	8	9	10	12	4
SAD-14	7	9	10	11	13	5
SAD-15	6	8	9	10	12	4
SAD-16	6	8	9	10	12	4

SAD-17	6	8	9	10	12	4
SAD-18	6	8	9	10	12	4
SAD-19	7	9	10	11	13	5
SAD-20	6	8	9	10	12	4
Average	6.25	8.25	9.25	10.25	12.25	4.25
SD	0.433013	0.433013	0.433013	0.433013	0.433013	0.433013

SAD = *Staphylococcus aureus*

Table 1b. Effect of aqueous extract of leaf of *Murraya koenigii* against *Streptococcus pyogens*

Pathogen	Inhibition zone (mm)					
	Medicinal plant					control
	15µg/ml	20µg/ml	25µg/ml	30µg/ml	35 µg/ml	15µg/ml
SPD-1	17	19	21	22	24	15
SPD-2	17	19	21	22	24	15
SPD-3	17	19	21	22	24	15
SPD-4	17	19	21	22	24	15
SPD-5	16	18	20	21	23	14
SPD-6	16	18	20	21	23	14
SPD-7	17	19	21	22	24	15
SPD-8	17	19	21	22	24	15
SPD-9	17	19	21	22	24	15
SPD-10	17	19	22	22	24	15
SPD-11	17	19	21	22	24	15
SPD-12	17	19	21	22	24	15
SPD-13	17	19	21	22	24	15
SPD-14	16	18	20	21	23	14
SPD-15	16	18	20	21	23	14
SPD-16	17	19	21	22	24	15
SPD-17	17	19	21	22	24	15
SPD-18	17	19	21	22	24	15
SPD-19	17	19	21	22	24	15
SPD-20	16	18	20	21	23	14
Average	16.75	18.75	20.8	21.75	23.75	14.75
SD	0.433013	0.433013	0.509902	0.433013	0.433013	0.433013

SPD= *Streptococcus pyogens*

Table 2a. Effect of ethanolic extract of leaf of *Murraya koenigii* against *Staphylococcus aureus*

Pathogen	Inhibition zone (mm)					
	Medicinal plant					control
	15µg/ml	20µg/ml	25µg/ml	30µg/ml	35 µg/ml	15µg/ml
SAD-1	17	20	22	25	27	11
SAD-2	18	21	23	26	28	12
SAD-3	18	21	23	26	28	12
SAD-4	17	20	22	25	27	11
SAD-5	18	21	23	26	28	12
SAD-6	17	20	22	25	27	11
SAD-7	17	20	22	25	27	11
SAD-8	17	20	22	25	27	11
SAD-9	17	20	22	25	27	11
SAD-10	18	21	23	26	28	12

SAD-11	18	21	23	26	28	12
SAD-12	18	21	23	26	28	12
SAD-13	18	21	23	26	28	12
SAD-14	18	21	23	26	28	12
SAD-15	17	20	22	25	27	11
SAD-16	18	21	23	26	28	12
SAD-17	18	21	23	26	28	12
SAD-18	18	21	23	26	28	12
SAD-19	18	21	23	26	28	12
SAD-20	17	20	22	25	27	11
Average	17.6	20.6	22.6	25.6	27.6	11.6
SD	0.489898	0.489898	0.489898	0.489898	0.489898	0.489898

SAD = *Staphylococcus aureus*

Table 2b. Effect of ethanolic extract of leaf of *Murraya koenigii* against *Streptococcus pyogens*

Pathogen	Inhibition zone (mm)					
	Medicinal plant					control
	15µg/ml	20µg/ml	25µg/ml	30µg/ml	35 µg/ml	15µg/ml
SPD-1	8	22	24	25	27	22
SPD-2	8	22	24	25	27	22
SPD-3	8	22	24	25	27	22
SPD-4	8	22	24	25	27	22
SPD-5	8	22	24	25	27	22
SPD-6	9	23	25	26	29	23
SPD-7	8	22	24	25	27	22
SPD-8	8	22	24	25	27	22
SPD-9	9	23	25	26	29	23
SPD-10	8	22	24	25	27	22
SPD-11	8	22	24	25	27	22
SPD-12	8	22	24	25	27	22
SPD-13	8	22	24	25	27	22
SPD-14	9	23	25	26	28	23
SPD-15	8	22	24	25	27	22
SPD-16	8	22	24	25	27	22
SPD-17	8	22	24	25	27	22
SPD-18	8	22	24	25	27	22
SPD-19	8	22	24	25	27	22
SPD-20	8	22	24	25	27	22
Average	8.15	22.15	24.15	25.15	27.25	22.15
SD	0.357071	0.357071	0.357071	0.357071	0.622495	0.357071

SPD= *Streptococcus pyogens*

Table 3a. Effect of aqueous extract of Bark of *Murraya koenigii* against *Staphylococcus aureus*

Pathogen	Inhibition zone (mm)					
	Medicinal plant					control
	15µg/ml	20µg/ml	25µg/ml	30µg/ml	35 µg/ml	15µg/ml
SAD-1	14	16	18	19	20	4
SAD-2	14	16	18	19	20	4
SAD-3	16	18	20	21	22	6
SAD-4	14	16	18	19	20	4

SAD-5	14	16	18	19	20	4
SAD-6	12	14	16	17	18	3
SAD-7	14	16	18	19	20	4
SAD-8	14	16	18	19	20	4
SAD-9	16	18	20	21	22	6
SAD-10	14	16	18	19	20	5
SAD-11	16	18	20	21	22	6
SAD-12	14	16	18	19	20	4
SAD-13	14	16	18	19	20	4
SAD-14	14	16	18	19	20	4
SAD-15	14	16	18	19	20	5
SAD-16	14	16	18	19	20	4
SAD-17	14	16	18	19	20	4
SAD-18	14	16	18	19	20	4
SAD-19	12	14	16	17	18	3
SAD-20	12	14	16	17	18	3
Average	14	16	18	19	20	4.25
SD	1.095445	1.095445	1.095445	1.095445	1.095445	0.887412

SAD = *Staphylococcus aureus*

Table 3b. Effect of aqueous extract of Bark of *Murraya koenigii* against *Streptococcus pyogens*

Pathogen	**Inhibition zone (mm)**					
	Medicinal plant					**control**
	15µg/ml	**20µg/ml**	**25µg/ml**	**30µg/ml**	**35 µg/ml**	**15µg/ml**
SAD-1	14	16	18	19	20	4
SPD-1	3	14	15	21	23	15
SPD-2	2	13	14	20	22	14
SPD-3	2	13	14	20	22	14
SPD-4	3	14	15	21	23	15
SPD-5	2	13	14	20	22	14
SPD-6	2	13	14	20	22	14
SPD-7	2	13	14	20	22	14
SPD-8	3	14	15	21	23	15
SPD-9	2	13	14	20	22	14
SPD-10	2	13	14	20	22	14
SPD-11	2	13	14	20	22	14
SPD-12	2	13	14	20	22	14
SPD-13	2	13	14	20	22	14
SPD-14	3	14	15	21	23	15
SPD-15	3	14	15	21	23	15
SPD-16	2	13	14	20	22	14
SPD-17	2	13	14	20	22	14
SPD-18	2	13	14	20	22	14
SPD-19	2	13	14	20	22	14
SPD-20	2	13	14	20	22	14
Average	2.25	13.25	14.25	20.25	22.25	14.25
SD	0.433013	0.433013	0.433013	0.433013	0.433013	0.433013

SPD= *Streptococcus pyogens*

Table 4a. Effect of ethanolic extract of Bark of *Murraya koenigii* against *Staphylococcus aureus*

Pathogen	Inhibition zone (mm)					
	Medicinal plant					control
	15µg/ml	20µg/ml	25µg/ml	30µg/ml	35 µg/ml	15µg/ml
SAD-1	5	16	17	18	22	14
SAD-2	5	16	17	18	22	14
SAD-3	5	16	17	18	22	14
SAD-4	5	16	17	18	22	14
SAD-5	6	17	18	19	23	15
SAD-6	6	17	18	19	23	15
SAD-7	5	16	17	18	22	14
SAD-8	5	16	17	18	22	14
SAD-9	5	16	17	18	22	14
SAD-10	5	16	17	18	22	14
SAD-11	5	16	17	18	22	14
SAD-12	5	16	17	18	22	14
SAD-13	5	16	17	18	22	14
SAD-14	5	16	17	18	22	14
SAD-15	6	17	18	19	23	15
SAD-16	6	17	18	19	23	15
SAD-17	5	16	17	18	22	14
SAD-18	5	16	17	18	22	14
SAD-19	5	16	17	18	22	14
SAD-20	5	16	17	18	22	14
Average	5.2	16.2	17.2	18.2	22.2	14.2
SD	0.4	0.4	0.4	0.4	0.4	0.4

SAD = *Staphylococcus aureus*

Table 4b. Effect of ethanolic extract of Bark of *Murraya koenigii* against *Streptococcus pyogens*

Pathogen	Inhibition zone (mm)					
	Medicinal plant					control
	15µg/ml	20µg/ml	25µg/ml	30µg/ml	35 µg/ml	15µg/ml
SPD-1	13	15	17	19	21	7
SPD-2	13	15	17	19	21	7
SPD-3	12	14	16	18	20	6
SPD-4	12	14	16	18	20	6
SPD-5	12	14	16	18	20	6
SPD-6	13	15	17	19	21	7
SPD-7	12	14	16	18	20	6
SPD-8	12	14	16	18	20	6
SPD-9	12	14	16	18	20	6
SPD-10	12	14	16	18	20	6
SPD-11	12	14	16	18	20	6
SPD-12	12	14	16	18	20	6
SPD-13	13	15	17	19	21	7
SPD-14	12	14	16	18	20	6
SPD-15	13	15	17	19	21	7
SPD-16	12	14	16	18	20	6
SPD-17	12	14	16	18	20	6
SPD-18	12	14	16	18	20	6

SPD-19	12	14	16	18	20	6
SPD-20	13	15	17	19	21	7
Average	12.3	14.3	16.3	18.3	20.3	6.3
SD	0.458258	0.458258	0.458258	0.458258	0.458258	0.458258

SPD= *Streptococcus pyogens*

DISCUSSION AND CONCLUSION

Staphylococcus aureus and *Streptococcus pyogenes* are pathogenic gram positive cocci and responsible for common respiratory tract disease. Previously, total 381 *Streptococcus pyogenes* and Erythromycin A-resistant strains were characterized for the underlying resistance genotype, showing 55.6% had the efflux type *mef*(A), 31.5% had *erm*(A), and 13.0% had *erm*(B) (Reinert *et al.*, 2004). Further, It has been provided that antibiotics has side effect and now scientists are focus on alternative of antibiotics. Plants based products have been in use for medicinal therapeutic or other purposes right from the drawn of history.

Even now, contrary to common belief, drug from higher plants continue to occupy an important niche in modern medicine. On the global basis, at least 130 drugs, all single chemical entities extracted from the higher plants, or modified further synthetically, are currently in use, though some of them are now being made synthetically (Newman *et al.*, 2000).

The Aqueous and ethanolic extract of leaf of *Vitex negundo* showed 10µg/ml MIC against *Staphylococcus aureus* and *Streptococcus pyogens*. Similar observation has been observed by (Ríos and Recio, 2005; Chaisawadi *et al.*, 2005). The antimicrobial potential of seventy-seven extracts from twenty-four plants was screened against eight bacteria and four pathogenic fungi, using microbroth dilution assay (Dabur *et al.*, 2007). Further, antimicrobial activity of ethanolic extract of various plants against certain pathogens (Gacche *et al.*, 2011). Further, Medicinal plants for antimicrobial activity of *Asphodelus tenuifolius* Cav., *Asparagus racemosus* Wild., *Balanites aegyptiaca* L., *Cestrum diurnum* L., *Cordia dichotoma* G. *Forst*, *Eclipta alba* L., *Murraya koenigii* (L.) *Spreng*., *Pedalium murex* L., *Ricinus communis* L. and *Trigonella foenum graecum* L against certain pathogens and all eight medicinal plants (*A. tenuifolius*, *A. racemosus*, *B. aegyptiaca*, *E. alba*, *M. koenigii*, *P. murex*, *R. communis* and *T. foenum graecum*) showed significant antimicrobial activity ($P < .05$) against most of the isolates (Panghal *et al.*, 2011). Another report showed the antimicrobial activity of methanol extract of *Ocimum americanum*, *Syzygium cumini*, *Murraya koenigii*, *Eucalyptus maculata*, *Lawsonia inermis*, *Adhatoda vasica*, *Tridax procumbens*, *Prunus amygdalus*, *Aazardirecta indica*, *Syzygium aromaticum* on *E. coli*, *S. aureus*, were evaluated by well diffusion method (Borde *et al.*, 2013). All these report suggested that medicinal plants can be alternative source of antibiotics and during the tenure of the present research, angiospermic plant - *Murraya koenigii* had been found to inhibit the growth of the isolated pathogens.

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CONFLICT OF INTEREST:

The authors declare that they have no conflict of interest.

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