Holistic Approaches in Mental Health and Wellness

PRIME OPEN ACCESS

Volume 1, Issue 1

Research Article

Date of Submission: 04 May, 2025
Date of Acceptance: 29 May, 2025
Date of Publication: 05 June, 2025

Assessment of Antimicrobial Activity and Determination of Phy-toChemical Constituents of Herbs Used in Burn Wound Treatment

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Citation: Devaraju. V., Muthukrishnan. P and Ramasamy. R. (2025). Assessment of Antimicrobial Activity and Determination of PhytoChemical Constituents of Herbs Used in Burn Wound Treatment. *Holistic Appr Mental Health Wellness, 1*(1), 01-13.

Abstract
Burn wounds can be invasive as well as non-invasive as they are frequently exposed to microbial infections and this can lead to development of several complications as patients affected with burn wounds are in the state of immune suppression. Also, nosocomial infections play a major role in infecting the burn wounds. Apart from nosocomial infections, emerging multi-drug resistant microorganisms such as Methicillin-Resistant Staphylococcus aureus (MRSA) also contribute to burn wound infections. The development of fungal infections in burn wounds is caused by Candida albicans and Aspergillus sp., as they possess higher resistance to conventional azoles. To overcome the multi-drug resistance complication, the present study deals with screening of those herbal extracts that can treat burn wounds and microbial infections associated with them efficiently. The herbs collected for screening in this study were Rosmarinus officinalis, Myristica fragrans, Nigella sativa and Chrysopogon zizanoides. On completion of antimicrobial activity assessment, it was observed that Rosmarinus officinalis and Myristica fragrans possessed highest antimicrobial activity and were further used for in the form of combinatorial herbal extract. In case of combinatorial herbal extract highest antibacterial activity was observed against Klebsiella pneumoniae (19 mm) and combinatorial herbal extract showed activity against all the bacterial strains and in case of fungi the highest antifungal activity was against Candida albicans (16 mm). Apart from efficient antimicrobial activity both the extracts had efficient antioxidant activity which is required for wound healing also phytochemicals were present in a considerable quantity in ethanolic extracts of Rosmarinus officinalis and Myristica fragrans.

Keywords: Burn Wounds, Microbial Infections, Rosmarinus Officinalis, Myristica Fragrans, Nigella Sativa, Chrysopogon Zizanoides

Introduction
Wounds are referred as an injury to living tissues caused by many impacts leading to development of an incision on the injured site. Wounds are generally classified into two types based on the impact caused on the tissues; which are the wounds without tissue loss which develop in a surgery, and wounds with tissue loss which develop as a result of any kind of trauma, burns, diabetic ulcers, etc. Also, wounds can further be classified based on the destruction caused on the layers of the skin such as superficial wounds, partial-thickness wounds and full-thickness wounds [1]. As wounds are a kind of incisions hence, it is mandatory to protect them from external factors which can prove to be detrimental and be a major cause of delayed wound healing [2]. Wounds that are caused on the skin when skin comes in contact with fire, electricity, radiation and trauma are called as burn wounds [3]. Whereas, pressure ulcers are those wounds

which result from unrelieved pressure which in turn is in combination with friction and shear leading to formation of pressure ulcers [3,4]. When skin comes in contact with high temperature there is occurrence of protein denaturation, which leads to loss of integrity of plasma membrane and there is release of several inflammatory mediators such as histamine, nitric oxide, oxygen radicals, tumor necrosis factor, interleukins, etc. Patients affected with burns usually face a higher number of risk factors and this leads to increased chances of occurrence of pressure ulcers as well in body of the injured (burn wounds) patients. Factors which provoke development of pressure ulcers in burn patients are loss of skin integrity, edema and immobility also external factors which have been proved to be responsible in development of pressure ulcers are large volume resuscitation, repeated operations, splinting and immobilization [5]. Burn wounds can be invasive as well as non-invasive as they are frequently exposed to microbial infections and this can lead to occurrence of several complications as patients affected with burn wounds are in the state of immune suppression. Also, nosocomial infections play a major role in infecting the burn wounds. Coagulase-negative Staphylococci, Staphylococcus aureus and Enterococcus sp., are the most prevalent Gram-positive pathogens and Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae and Acinetobacter sp., are the most prevalent Gram-negative pathogens. Apart from nosocomial infections, emerging multi-drug resistant microorganisms such as Methicillin-Resistant Staphylococcus aureus (MRSA) also contribute to burn wound infections, sepsis and higher fatality rates [6].

In the year 1991, in a research study, Becker WK et al., reported the development of fungal infections in burn wounds, and Candida albicans and Aspergillus sp., were reported as prominent fungi associated with higher mortality rates and also possessed higher resistance towards conventional azoles. The applications of medicinal plants for relief from several ailments can be studied in the documents of early civilisation in China, India and Near East. As medicinal plants are rich source of antimicrobial agents and in many countries, they are applied in the form of potent and powerful drugs. Parts of plants such as roots, stems, leaves, fruits, twigs exudates are collected in the raw form and supplied to several herbal industries. Medicinal plants are used since ages in the treatment of various skin ailments as well as in case of dermatological disorders such as cuts, wounds and burns [7]. Although, there are availability of several topical applications for treatment of burn wounds in market, still there is inadequacy of suitable drugs as most of the available products are antimicrobial in nature rather than efficient wound healers, and also in some cases there is occurrence of cytotoxicity which is usually observed in case of silver sulfadiazine on fibroblasts. Medicinal plants can prove to be efficient wound-healing agents as they consist of vast variety of phytochemical constituents such as alkaloids, essential oils, flavonoids, tannins, terpenoids, saponins, fatty acids and phenolic compounds. Factors such as low cost, availability and fewer side effects also make utilisation of herbal remedies advantageous. Hence, several research studies have been focused all over the world to identify and isolate the active components of medicinal plants to treat burn wounds, pressure ulcers and microbial infections associated with them efficiently [8].

Materials and Methods

Collection of Chemicals and Medicinal Plants

Double distilled water from Millipore unit was utilised for the synthesis of microbial broth, media preparations as well as for some reagents. The chemicals and solvents used in the present study were purchased from HIMEDIA chemicals private limited, Mumbai, India.

Herbs used in this study such as leaves of Rosmarinus officinalis were procured through online shopping website Amazon from Neutraved company and seeds of Nigella sativa, mace of Myristica fragrans and roots of Chrysopogon zizanoides were procured from Sri Pazhamudir Nilayam, Coimbatore, India.

Isolation of Microbial Strains Associated with Burn Wounds and Pressure Ulcer Infections

The pathogenic bacterial and fungal strains such as Staphylococcus aureus, Methicillin-Resistant Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Proteus mirabilis, Klebsiella pneumoniae, Acinetobacter sp., Serratia marcescens, Candida albicans and Aspergillus niger; were isolated from wards of patients suffering from burn wounds and pressure ulcers in PSG Institute of Medical Sciences and Research, Coimbatore-641004, Tamil Nadu, India.

Extraction of the Phytochemicals from Herbs Using Soxhlet Extractor

The powdered form of herbs used in this study (leaves, seeds, mace and roots of the herbs Rosmarinus officinalis, Nigella sativa, Myristica fragrans and Chrysopogon zizanoides, respectively) were taken in10 grams of quantity in a filter paper and placed in the extraction chamber of soxhlet extractor and the solvents such as ethanol, chloroform, ethyl acetate, hexane and methanol were used for extraction of phytochemicals from the herbs. Finally, the phytochemicals were concentrated and removal of extra solvent was done through rotary evaporator. Later, the phytochemicals were scrapped off from the petri plates and were stored at 4ºC for further use [9].

Assessment of Antibacterial Activity of the Herbal Extracts (Agar Well Diffusion Method)

For antibacterial activity assessment of herbal extracts, 0.01gm of herbal extract was diluted with 1 ml of dimethyl sulfoxide solution (1%) and vortexed well for proper dissolution of the herbal extract in the solution. Then, bacterial inoculum of cell concentration 1.5–3.0 × 10^5 CFU/ml was evenly swabbed on the surface of nutrient agar plates and wells of adequate quantity were punctured. Into these wells, herbal extracts in 100µl quantity were added and then the petri plates were incubated at 37ºC for 18–24 hours. Finally, on completion of incubation period the petri plates were observed for occurrence of zones of inhibition. Standard antibiotic Tetracycline was used as reference for evaluation of

microorganisms's susceptibility [10].

Assessment of Antifungal Activity of the Herbal Extracts (Agar Well Diffusion Method)

For antifungal activity assessment of herbal extracts, 0.01gm of herbal extract was diluted with 1 ml of dimethyl sulfoxide solution (1%) and vortexed well for proper dissolution of the herbal extract in the solution. Then, fungal inoculum with spore concentration 0.4–5 × 10^4 CFU/ml was evenly swabbed on the surface of Czapek dox agar plates and wells of adequate quantity were punctured. Into these wells, herbal extracts in 100µl quantity were added and then the plates were incubated at 28ºC for 3–5 days. On completion of incubation period, the plates were observed for zones of inhibition. Standard antifungal agent Ketoconazole was used as reference for evaluation of microorganisms's susceptibility [11].

Determination of Minimum Bactericidal Concentration (MBC) of Ethanolic Extract of Rosmarinus Officinalis (Broth Macrodilution Method)

In broth macrodilution method, 12 sterile test tubes were taken, among them first 10 test tubes were labelled from 1-10 in number. The rest of the two test tubes were maintained for sterility control and growth control. 1ml of sterile nutrient broth was added into the series of test tubes. Then, herbal extract diluted with 1% of DMSO solution in the concentration of 0.01gm/ml was added to the first test tube and then it was serially diluted till test tube number 10 in such a way that the herbal extract concentration reduces by half in each dilution. The herbal extract concentration ranged from 5000µg/ml to 9.76µg/ml. After serial dilution of herbal extracts in broth, addition of 10µl of adjusted bacterial inoculum (1.5-3.0 × 10^5 CFU/ml) is done and test tubes were incubated at 37ºC for 18 hours and after incubation the minimum inhibitory concentration of herbal extract was determined through the spectrophotometric readings taken at 600 nm [12,13].

Determination of Minimum Fungicidal Concentration (MFC) of Ethanolic Extract of Myristica Fragrans

A stock solution of 5000µg/ml of herbal extract was prepared in 1% DMSO solution and this stock solution was further diluted to 1280µg/ml. Then, a three consecutive sets of test tubes named as A, B and C were made and in them 0.5 ml, 0.75 ml and 1.75 ml of sterile distilled water was added. Further, from the 2 ml of 1280µg/ml of stock solution, 0.5 ml, 0.25 ml and 0.25 ml of herbal extract was transferred to test tubes of set A. From last test tube of set, A again 0.5ml, 0.25 ml and 0.25 ml of herbal extract was transferred into the set B test tubes and the same step as mentioned above was repeated in case of set C test tubes and at last 1ml of herbal extract dilution was discarded from last test tube of set C. The concentration of the herbal extracts in the three sets of test tubes ranged from 1280µl to 2.5µl. After the dilution step, 20% v/v of herbal extract concentration was mixed with 40% v/v of medium (Sabouraud dextrose broth) to enhance the fungal spore suspension. Then, the final concentration of the herbal extract ranged from 256µl to 0.5µl. This is the herbal extract concentration range which is used to determine the minimum fungicidal concentration of herbal extract. In order to determine minimum fungicidal concentration 0.1 ml of herbal extract dilution and 0.1 ml of adjusted fungal spore suspension (0.4–5 × 10^4 CFU/ml) were added in the test tubes, incubated at 28ºC for 48 hours and were also covered with gas permeable plastic bags in order to prevent drying. Two separate test tubes were maintained for sterility and growth control. Finally, at the end of incubation absorbance was measured at 530 nm to determine the minimum inhibitory concentration of herbal extract against fungal strains [14].

Assessment of Antibacterial Activity of Combinatorial Herbal Extract (Rosmarinus Officinalis and Myristica Fragrans)

Assessment of antibacterial activity of combinatorial herbal extracts was done with agar well diffusion method. In case of combinatorial herbal extracts, 0.01gm of ethanolic extract of Rosmarinus officinalis and 0.01gm of ethanolic extract of Myristica fragrans were diluted with 1ml of DMSO solution (1%), separately. After this equal volume of the both the herbal extracts were added into the wells punctured in the nutrient agar plates [15].

Assessment of Antifungal Activity of Combinatorial Herbal Extract (Rosmarinus Officinalis and Myristica Fragrans)

Antifungal assessment of combinatorial herbal extract was done with agar well diffusion method.

Determination of Minimum Bactericidal Concentration (MBC) of Combinatorial Herbal Extracts (Rosmarinus officinalis and Myristica fragrans)

At first the phytochemical extracts of both the herbs were taken in a ratio of 1:1 and diluted with 1ml of DMSO solution (1%) [16]. The method for MBC breakpoint determination is the same as the method followed for MBC breakpoint determination in case of ethanolic extract of Rosmarinus officinalis [12,13].

Determination of Minimum Fungicidal Concentration (MFC) of Combinatorial Herbal Extract (Rosmarinus Officinalis and Myristica Fragrans)

MFC determination methodology is the same as mentioned in case of ethanolic extract of Myristica fragrans [14,16].

Phytochemical Analysis of the Herbal Extracts

For determination of the phytochemical constituents such as alkaloids, flavonoids, saponins, tannins, etc. performance

of a preliminary phytochemical study (Table 1) was done with the herbal extracts using the following procedure [17].

S. No	Test name	Extract	Reagents	Inference
1	**Alkaloids (Mayor's test)**	1ml of acidic aqueous herbal extract	Addition of few drops of Mayor's reagent	Development of white or pale-yellow precipitate
2	**Flavonoids**	5–10ml of diluted hydrochloric acid added to 0.5ml of aqueous herbal extract	Addition of a pinch of zinc or magnesium powder and further boiled for few minutes	Development of reddish pink or dirty brown coloured solution
3	**Saponins**	5ml of aqueous herbal extract	Addition of a drop of sodium bicarbonate solution and shaken vigorously	Development of honey comb-like froth
4	**Carbohydrates (Fehling's test)**	5ml of aqueous herbal extract	Addition of 1ml of Fehling's solution and boil for few minutes	Development of brick red colour precipitate
5	**Protein (Biuret's test)**	1ml of hot aqueous herbal extract	Addition of 5–8 drops of 10% sodium hydroxide solution and 1–2 drops of 5% copper sulphate solution	Development of red or violet colouration
6	**Phenols (Ferric chloride test)**	1ml of alcoholic herbal extract and add 2ml of distilled water to it	Addition of few drops of 10% aqueous ferric chloride solution	Development of blue or green colouration
7	**Steroids (Solkowsky's test)**	2ml of chloroform herbal extract	Addition of 1ml of concentrated sulphuric acid along the sides of the test tube	Development of red colouration above the chloroform layer
8	**Glycosides**	Small amount of aqueous herbal extract	Addition of few drops of aqueous sodium hydroxide solution	Development of yellow colour
9	**Thiols**	0.5ml of aqueous herbal extract	Addition of ammonium solution, 2–4 drops of sodium nitroprusside solution and 1–4 drops of concentrated nitric acid	Development of transient magenta colour
10	**Terpenoids**	5ml of aqueous herbal extract	Addition of 2ml of chloroform as well as 2ml of concentrated sulphuric acid along the sides of the test tube	Development of dark red colour
11	**Tannins**	1–2ml of alcoholic herbal extract	Addition of few drops of 5% ferric chloride solution and dilute sulphuric acid	Formation of bluish black colouration which disappears on addition of dilute sulphuric acid and changes to yellowish brown precipitate

Table 1: Phytochemical Analysis of Herbal Extracts

Antioxidant Activity Analysis of the Herbal Extracts (DPPH Assay)

The principle of DPPH (2,2-diphenyl-1-picrylhydrazyl) assay usually deals with the scavenging reaction that occurs between DPPH and an antioxidant agent (H-A)

$$(\text{DDPH}) + (\text{H-A}) \longrightarrow \text{DPPH} - \text{H} + (\text{A})$$

(Purple) (Yellow)

DPPH assay was performed by preparing 0.1mM concentration of DPPH solution in ethanol, which was stored in darkness. Also, both the herbal extracts and the standard used for comparison ascorbic acid were also dissolved in ethanol at the concentration ranging from 1mg/ml to 5 mg/ml. Further, these solutions were made up to 3ml in quantity using ethanol and to the 3ml of herbal extracts solution and standard solution 1ml of DPPH solution was added, vigorously shaken for proper mixing of reagent and extracts solution and then they were incubated in darkness for 30 minutes at room temperature. Finally, on completion of incubation period the solutions were subjected to spectrophotometric readings to determine the absorbance of the solutions at 517nm. For blank ethanol was used and for control 3ml of DPPH solution was used.

Percentage of DPPH Radical Scavenging Activity was Determined Using the Following Formula

DPPH radical scavenging activity (%) = $(A^{control} - A_{test} / A_{control}) \times 100$

where, $A_{control}$ refers to absorbance of the control and A_{test} refers to absorbance of reaction mixture. [18]

Gas Chromatography Mass Spectrometry Analysis of Herbal Extracts

Shimadzu GC-2010 was the instrument used in the present study to efficiently detect the chemical compounds present in the herbal extracts, where a fused silica column packed with 5% biphenyl and 95% dimethylpolysiloxane was used. The column's dimensions were 30m × 0.25mm ×250µm film and the separation of the chemical compounds was mediated by the helium gas which was made to flow at the rate of 1ml/min and the injection temperature was 280ºC. The herbal extracts were dissolved in ethanol and were injected in a quantity of 2µl followed by adjustments made in the oven temperature which ranged initially from 70ºC for 4 minutes then, raised to 280ºC at the rate of 5ºC/minute, where the extracts were held for 8 minutes. After this the conditions within the mass spectrometer were also adjusted in this the transfer lines temperature was 240ºC, ionisation mode electron impact was at 70eV and scan interval of 0.1 second was maintained. Finally, the fragments ranged from 40 to 600 Da in weight and their spectrum was compared with the databases of the spectrum of known components which are stored in the GC-MS National Institute of Standards and Technology library [19].

Results and Discussion

Extraction of the Phytochemicals from the Herbs Using Soxhlet Extractor

It was observed that highest yield of phytochemicals was in case of ethanol compared to other solvents. The yield ranged from 0.36–0.38g in case of ethanolic extracts, 0.24–0.25g in case of hexane extracts, 0.24–0.29g in case of methanolic extracts, 0.30–0.31g in case of chloroform extracts and 0.29–0.30g in case of ethyl acetate extracts.

Assessment of Antibacterial Activity of the Herbal Extracts (Agar Well Diffusion Method)

On examination of results it was observed that ethanolic extract of leaves of Rosmarinus officinalis has possessed highest antibacterial activity (Table 2) against six bacterial strains, which were Staphylococcus aureus (13mm), MRSA (14mm), Escherichia coli (14mm), Pseudomonas aeruginosa (13mm), Klebsiella pneumoniae (16mm) and Serratia marcescens (17mm). Least antibacterial activity of the herbal extracts was observed in case of methanolic extracts. Leaves of Rosmarinus officinalis have shown the highest antibacterial activity which is due to the presence of class of phytochemicals such as glycosides, flavonoids, turbines, alkaloids as well as saponins [20]. The strong antibacterial activity of ethanolic extract of leaves of Rosmarinus officinalis is due to the presence of the compounds which belong the class of phytochemicals such as tannins and saponins [21].

Rosmarinus officinalis possess strong antibacterial activity against the pathogenic microorganisms such as Staphylococcus aureus and Escherichia coli and is also found to be majorly responsible for the inhibition of mechanism of drug resistance in the several bacterial strains [22-24]. This is usually performed by the compounds of Rosmarinus officinalis extract by overcoming and reducing the impermeability of the bacterial membranes [23]. Presence of inhibitory compounds which are also found to be active against pathogenic bacteria are rosmarinic acid, epirosmanol, carnosic acid, rosmanol and carnosol which have ability to interact with cell membrane of bacteria and cause changes in their genetic material and nutrients [25].

Herbs	**Pathogenic test bacteria**	**Zone of inhibition (in mm) Extracts**					
		Methanol	**Ethanol**	**Chloroform**	**Ethyl acetate**	**Hexane**	**Tetracycline**
Rosamarinus officinalis leaves	Staphylococcus aureus	12	13	15	21	-	18
	MRSA	15	14	18	-	-	18
	Escherichia coli	-	14	-	-	-	13
	Pseudomonas aeruginosa	14	13	-	-	-	-
	Proteus mirabilis	-	-	-	-	-	-
	Klebsiella pneumoniae	-	16	-	-	-	17
	Acinetobacter sp.,	-	-	13	-	-	11
	Serratia marcescens	-	17	-	20	-	14
Nigella sativa seeds	Staphylococcus aureus	-	-	25	17	18	18
	MRSA	-	-	-	-	17	18
	Escherichia coli	-	-	-	-	-	13
	Pseudomonas aeruginosa	-	-	-	-	-	-

	Proteus mirabilis	-	12	-	-	-	-
	Klebsiella pneumoniae	-	-	-	-	-	17
	Acinetobacter sp.,	-	-	14	-	-	11
	Serratia marcescens	-	-	-	-	-	14
Myristica fragrans mace	Staphylococcus aureus	-	-	23	13	17	18
	MRSA	-	-	-	-	-	18
	Escherichia coli	-	-	14	-	-	13
	Pseudomonas aeruginosa	-	-	-	14	-	-
	Proteus mirabilis	-	-	14	-	-	-
	Klebsiella pneumoniae	-	-	-	-	-	17
	Acinetobacter sp.,	-	-	-	-	-	11
	Serratia marcescens	-	-	-	-	-	14
Chrysopogon zizanoides roots	Staphylococcus aureus	-	11	16	14	16	18
	MRSA	-	12	-	-	16	18
	Escherichia coli	-	-	-	-	-	13
	Pseudomonas aeruginosa	-	-	-	17	-	-
	Proteus mirabilis	-	-	-	-	14	-
	Klebsiella pneumoniae	-	-	15	-	-	17
	Acinetobacter sp.,	-	-	15	-	-	11
	Serratia marcescens	-	-	-	-	-	14
MRSA: Methicillin Resistant Staphylococcus aureus							

Table 2: Antibacterial Activity Assessment of Herbal Extracts Against the Pathogenic Test Bacteria

Assessment of Antifungal Activity of the Herbal Extracts (Agar Well Diffusion Method)

It was observed that the highest antifungal activity was exhibited by ethanolic extract of mace of Myristica fragrans (Table 3). In case of Candida albicans the zone of inhibition formed was 17mm in diameter and in case of Aspergillus niger it was 15mm. The antifungal activity of Myristica fragrans can be due to presence of lignan compounds such as erythroaustrobailignan-6, meso-dihydroguaiaretic acid and nectandrin-B. And Myristica fragrans is also a rich source of antimicrobial phenolic compounds such as α-pinene, β-pinene, carvacrol, etc [26]. These phenolic compounds possess mode of action against fungi by disrupting the cell membrane of fungi [27]. It has also been reported that carvacrol possess the ability to cross cell membranes, penetrate inside the cells and develops an interaction with the sites that perform antimicrobial activities. On other hand even β-caryophyllene has been reported for possessing antifungal and anti-inflammatory properties [28].

Herb	**Pathogenic test fungi**	**Zone of inhibition (in mm) Extracts**					
		Methanol	**Ethanol**	**Chloroform**	**Ethyl acetate**	**Hexane**	**Ketoconazole**
Rosamarinus officinalis leaves	Candida albicans	-	-	13	7	-	21
	Aspergillus niger	-	-	-	16	-	20
Nigella sativa seeds	Candida albicans	13	15	15	12	13	21

	Aspergillus niger	-	-	11	-	11	20
Myristica fragrans mace	Candida albicans	12	17	15	13	-	21
	Aspergillus niger	-	15	-	-	-	20
Chrysopogon zizanoides roots	Candida albicans	15	-	14	14	14	21
	Aspergillus niger	-	-	13	-	12	20

Table 3: Antifungal Activity Assessment of Herbal Extracts Against the Pathogenic Test Fungi

Determination of Minimum Bactericidal Concentration (MBC) of Ethanolic Extract of Rosmarinus Officinalis Leaves (Broth Microdilution Method)

As ethanolic extract of leaves of Rosmarinus officinalis has exhibited highest antibacterial activity hence, it was subjected for its MBC determination as well. The MBC breakpoints of the bacterial strains are given in Table 4

Pathogenic bacteria	**Concentration of Rosmarinus officinalis leaves extract (µg/ml)**									
	5000 µg/ml	2500 µg/ml	1250 µg/ml	625 µg/ml	312.5 µg/ml	156.25 µg/ml	78.12 µg/ml	39.06 µg/ml	19.53 µg/ml	9.76 µg/ml
Staphylococcus aureus	0.117	0.194	0.241	0.419	0.508	**0.566**	0.693	0.870	1.002	1.017
Methicillin resistant Staphylococcus aureus	0.166	0.209	0.374	0.399	**0.458**	0.619	0.858	1.003	1.018	1.100
Escherichia coli	0.281	0.392	0.497	0.512	**0.570**	0.679	0.815	1.008	1.011	1.019
Pseudomonas aeruginosa	0.232	0.416	0.471	**0.556**	0.592	0.725	0.834	0.933	1.009	1.101
Proteus mirabilis	0.178	0.300	0.368	0.477	**0.519**	0.757	0.838	0.891	1.002	1.014
Klebsiella pneumoniae	0.191	0.369	0.433	**0.522**	0.598	0.614	0.638	0.777	1.016	1.118
Acinetobacter sp.,	0.191	0.258	0.430	0.501	**0.571**	0.743	0.832	0.861	1.004	1.034
Serratia marcescens	0.206	0.321	**0.418**	0.591	0.630	0.744	0.873	0.994	1.031	1.114

Table 4: Determination of Minimum Bactericidal Concentration of Ethanolic Extract of Rosmarinus Officinalis Leaves

Determination of Minimum Fungicidal Concentration (mfc) of Ethanolic Extract of Myristica Fragrans Mace

As mentioned earlier the ethanolic extract of mace of Myristica fragrans possessed highest antifungal activity and it was subjected for determination of MFC in case of pathogenic fungi. The MFC breakpoints of the fungal strains are given in Table 5

Pathogenic fungi	**Concentration of Myristica fragrans mace extract (µg/ml)**									
	256 µg/ml	128 µg/ml	64 µg/ml	32 µg/ml	16 µg/ml	8 µg/ml	4 µg/ml	2 µg/ml	1 µg/ml	0.5 µg/ml
Aspergillus niger	0.191	0.241	0.456	0.490	**0.555**	0.770	0.832	0.997	1.001	1.021
Candida albicans	0.179	0.233	0.388	**0.566**	0.599	0.677	0.812	0.895	0.905	1.006

Table 5: Determination of Minimum Fungicidal Concentration of Ethanolic Extract of Myristica Fragrans Mace

Assessment of Antibacterial Activity of the Combinatorial Herbal Extracts (Rosmarinus Officinalis and Myristica Fragrans)
In case of combinatorial herbal extract, it was observed that highest zone of inhibition was formed in case of Klebsiella pneumoniae and combinatorial herbal extract has possessed antibacterial activity against all the pathogenic bacterial strains. And on comparison with Tetracycline it was observed that the tetracycline antibacterial activity ranged slightly higher than combinatorial herbal extract (Figure 1 and 2). The occurrence of efficient antibacterial activity of combination of ethanolic extract of Rosmarinus officinalis leaves and Myristica fragrans mace can be due to the presence of several

chemical compounds such as rosmarinic acid, epirosmanol, carnosic acid, rosmanol and carnosol in Rosmarinus officinalis 25 and myristicin, safrole, trimyristin, mace-lignan, terpenes, neolignane, α and β- pinene in Myristica fragran [29]. As these compounds are majorly involved in protection of plants against pathogens and act widely against human pathogenic microorganisms as well.

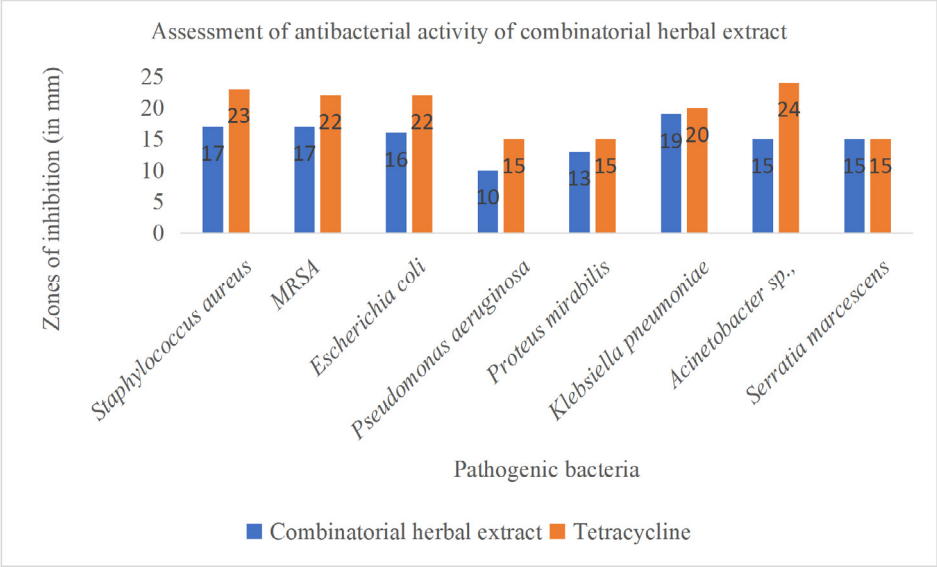

Figure 1: Antibacterial Activity of Combinatorial Herbal Extract

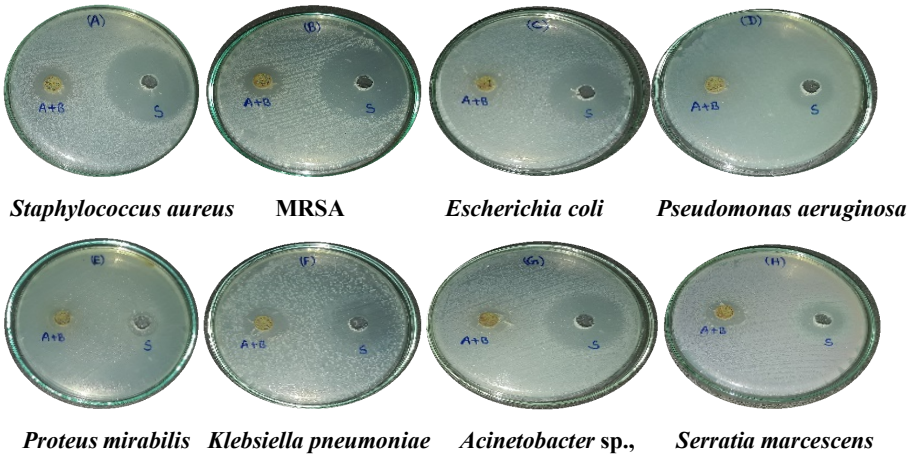

Figure 2: Antibacterial Activity of Combinatorial Herbal Extract (Agar Well Diffusion Method)

Note: A+B refers to combinatorial herbal extract and S refers to Tetracycline

Assessment of Antifungal Activity of the Combinatorial Herbal Extracts (Rosmarinus Officinalis and Myristica fragrans)

Highest antifungal activity occurred in case of Candida albicans and on comparison of antifungal activity of herbal extracts and Ketoconazole it was observed that Ketoconazole exhibited higher antifungal activity against the pathogenic fungal strains (Figure 3 and 4). The antifungal activity of combinatorial herbal extract was due to the presence of chemical compounds such as erythroaustrobailignan-6, meso-dihydroguaiaretic acid, nectandrin-B, α-pinene, β-pinene, and carvacrol etc. in case of Myristica fragrans [26,30].

Whereas, in case of Rosmarinus officinalis the antimicrobial activity majorly depends on its essential oil composition and through several studies it has been observed that Rosmarinus officinalis can inhibit the adhesion of Candida albicans by denaturing its cellular structure and altering membrane permeability [31]. Several research studies have proved that coating of nanoparticles with Rosmarinus officinalis essential oil can inhibit the development of Candida fungal biofilms and this strategy can be efficient in developing an alternate for utilising traditional medicines against drug-resistant fungal strains [32].

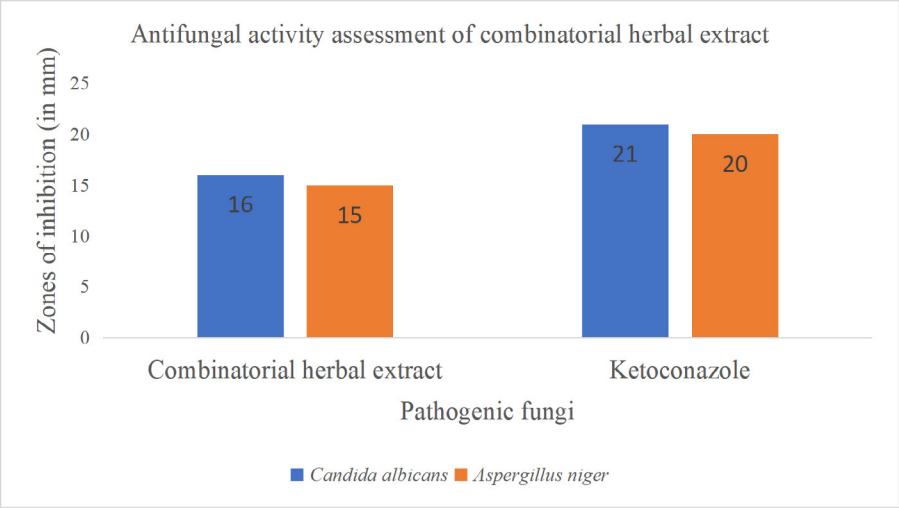

Figure 3: Antifungal Activity Assessment of Combinatorial Herbal Extract

Candida albicans *Aspergillus niger*

Figure 4: Antifungal Activity Of Combinatorial Herbal Extract (Agar Well Diffusion Method)

(**Note:** A+B refers to combinatorial herbal extract and S refers to Ketoconazole)

Determination of Minimum Bactericidal Concentration (MBC) of Combinatorial Herbal Extracts (Rosmarinus Officinalis and Myristica Fragrans)

MBC breakpoints for combinatorial herbal extracts are given in Table 6

Determination of Minimum Fungicidal Concentration (MFC) of Combinatorial Herbal Extracts (Rosmarinus Officinalis and Myristica Fragrans)

MFC breakpoints for combinatorial herbal extracts are given in Table 7

Pathogenic bacteria	**Concentration of combinatorial herbal extract (µg/ml)**									
	5000 µg/ml	2500 µg/ml	1250 µg/ml	625 µg/ml	312.5 µg/ml	156.25 µg/ml	78.12 µg/ml	39.06 µg/ml	19.53 µg/ml	9.76 µg/ml
Staphylococcus aureus	0.212	0.240	0.369	0.517	**0.555**	0.659	0.681	0.849	0.992	1.001
Methicillin resistant Staphylococcus aureus	0.251	0.341	0.421	0.514	**0.534**	0.592	0.679	0.719	0.901	0.999
Escherichia coli	0.221	0.277	0.389	**0.427**	0.619	0.665	0.779	0.832	0.912	1.011
Pseudomonas aeruginosa	0.106	0.321	0.407	0.450	0.497	**0.581**	0.733	0.774	0.899	1.009
Proteus mirabilis	0.159	0.217	0.333	0.491	0.516	**0.571**	0.700	0.847	0.999	1.035
Klebsiella pneumoniae	0.217	0.323	0.466	**0.576**	0.590	0.661	0.713	0.990	1.010	1.045
Acinetobacter sp.,	0.221	0.291	0.300	0.347	**0.508**	0.711	0.899	0.923	1.001	1.012
Serratia marcescens	0.121	0.271	0.408	0.459	**0.500**	0.649	0.771	0.901	0.948	1.001

Table 6: Minimum Bactericidal Concentration of Combinatorial Herbal Extract

Pathogenic fungi	Concentration of combinatorial herbal extract (µg/ml)									
	256 µg/ml	128 µg/ml	64 µg/ml	32 µg/ml	16 µg/ml	8 µg/ml	4 µg/ml	2 µg/ml	1 µg/ml	0.5 µg/ml
Aspergillus niger	0.206	0.237	0.356	0.447	0.478	**0.574**	0.709	0.867	0.999	1.011
Candida albicans	0.111	0.271	0.400	0.482	0.517	**0.556**	0.677	0.890	1.003	1.020

Table 7: Minimum Fungicidal Concentration of Combinatorial Herbal Extracts (Rosmarinus Officinalis and Myristica Fragrans)

Phytochemical Analysis of the Herbal Extracts (Rosmarinus Officinalis and Myristica Fragrans)

The natural bioactive agents present in the plants are called as phytochemicals and commonly occur in each and every part of plant ranging from vegetables, fruits, leaves, roots, etc. and they usually interact with nutrients and fibres to act as a defence system against diseases or any adverse environmental conditions [33]. In the present study it was observed leaves of Rosmarinus officinalis possessed phytochemicals such as alkaloids, saponins, carbohydrates, phenols, steroids and tannins. In several studies it has been proven that the active constituents present in Rosmarinus officinalis are phenolic acids, steroids, triterpenes and essential oils also condensed form of tannins are usually present in a large quantity in the ethanolic extract of Rosmarinus officinalis [21]. In case of Myristica fragrans it was observed that it is rich in class of phytochemicals such as saponins, carbohydrates, steroids, glycosides and tannins. In several studies it was observed that alcoholic extract of Myristica fragrans are usually rich in alkaloids, saponins, tannins, flavonoids, phenols as well as carbohydrates [34]. Hence, the phytochemical analysis of the antimicrobial efficient herbal extracts proved that they possess those class of bioactive compounds which can be used for medicinal applications and the class of phytochemicals present are involved in wound healing (Table 8)

Phytochemical tests	Herbal extracts	
	Myristica fragrans	Rosmarinus officinalis
Alkaloids	-	+
Flavanoids	-	-
Saponins	+	+
Carbohydrates	+	+
Proteins	-	-
Phenols	-	+
Steroids	+	+
Glycosides	+	-
Tannins	+	+
Thiols	-	-

Table 8: Phytochemical Analysis of the Selected Herbal Extracts

Determination of Antioxidant Activity of the Herbal Extracts (DPPH Assay)

On observation of the results of antioxidant activity analysis of ethanolic extracts of Rosmarinus officinalis and Myristica fragrans it was observed that the Rosmarinus officinalis extract possessed higher and adequate level of antioxidant activity compared to Myristica fragrans (Figure 5). Also, the level of antioxidant activity of herbal extracts was compared with the standard antioxidant agent ascorbic acid. In this case the extract of Rosmarinus officinalis possessed equal level of antioxidant activity as the standard whereas, Myristica fragrans possessed slightly lower level of antioxidant activity compared to standard. Since, both the extracts have Possessed antioxidant activity in a considerable level hence it proves that their utilization can be done in the treatment of burn wounds and microbial infections associated with them. In case of any diseased state or ageing process the antioxidant ability must be at higher level in order to defend immune system and make function very efficiently against any homeostatic disturbances by stimulating the functions of immune cells [35]. Antioxidant agents such as tannins, saponins, terpenoids, phenols and ascorbic acid as well are present in several plants and are highly efficient in scavenging free radicals. Due to harmful effects of synthetic antioxidant agents the studies have been focused utilization of antioxidant agents that are extracted from plant sources and can be applicable for human usage [36]. In the present study the expected results in case of Rosmarinus officinalis can be due to presence of class of phytochemicals such as, phenolic acids, flavonoids and diterpenoids and in case of Myristica fragrans it can be due to presence of class of phytochemicals such as vitamins, carotenoids, lignans, phenols, etc [37,38].

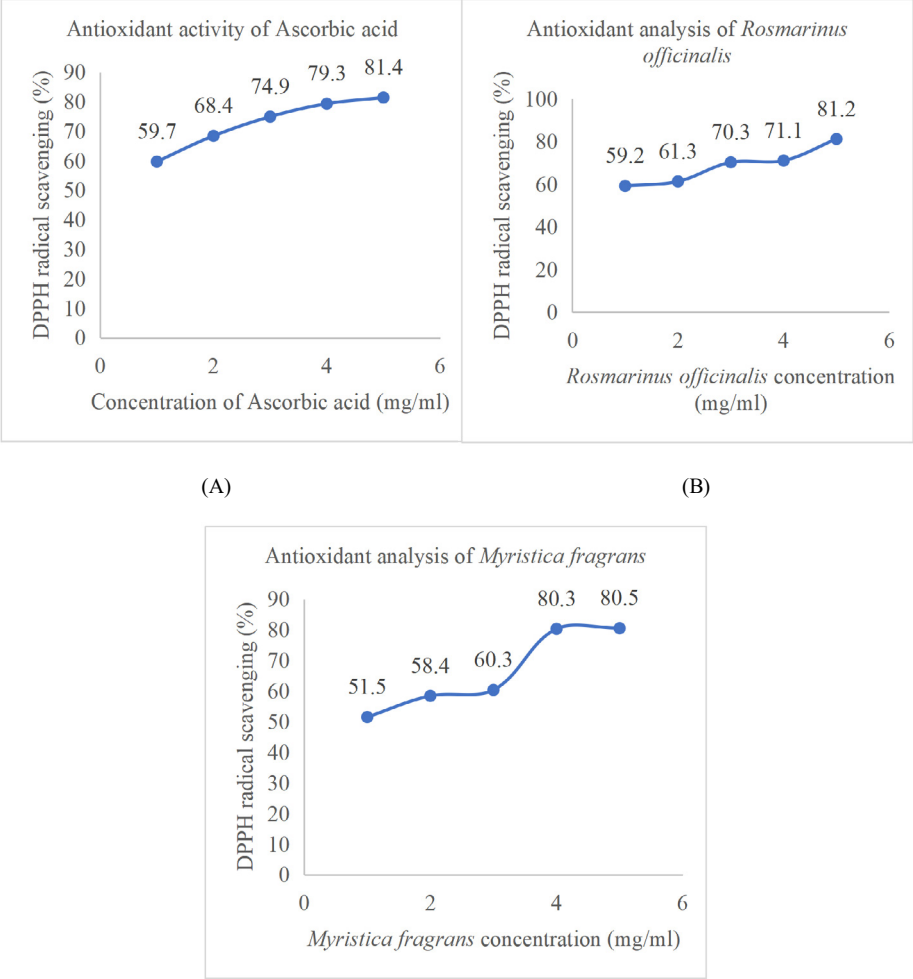

Figure 5: Antioxidant Activity of Ascorbic Acid, Rosmarinus Officinalis and Myristica Fragrans

Gas Chromatography Mass Spectrometry (GC-MS) Analysis of Herbal Extracts (Rosmarinus Officinalis and Myristica Fragrans)

The ethanolic extracts of leaves of Rosmarinus officinalis (Table 9) and mace of Myristica fragrans (Table 10) possessed highest antimicrobial activity individually as well as in combination and were further subjected for detection of chemical compounds present within them due to which there was occurrence of antimicrobial and antioxidant activity.

The phytochemicals present in plants play a very important role in defense mechanism of plants as well as the essential activities possessed by the plants for wound healing and combating microbial infections associated with the wounds.

S. No	Retention Time	Compound name	Area (%)
1	2.835	α-Pinene	1.68
2	7.860	2-Oxabicyclo (2.2.2) octane,1,3,3-Trimethyl	2.69
3	9.175	Propane,1,1,3-triethoxy	-3.86
4	10.783	Camphor	2.48
5	11.666	Bicyclo(2.2.1)heptan-2-ol,1,7,7-trimethyl	2.83
6	12.590	Bicyclo(3.1.1)heptane-3-en-2-0ne. 4,6,6-trimethyl	2.67
7	13.132	Benzaldehyde, 2-methyl-	6.03
8	15.568	2-methoxy-4-vinylphenol	5.40
9	16.792	Phenol, 2-methoxy-4-(2-propenyl)-	2.23
10	18.435	1-pentanol, 4.-methyl-2-propyl-	2.23
11	30.878	Hexadecanoic acid	4.06

12	38.258	Isocarnosol	2513410
13	39.400	Ferruginol	3.73
14	40.944	7-Hydroxy-1-methyl-8-methylenegibba-1,3,4A(10A)-triene-10-carboxylic acid	3.46

Table 9: Gc-Ms Analysis of Ethanolic Extract of Leaves of Rosmarinus Officinalis

S. No	Retention Time	Compound name	Area (%)
1	6.349	Bicyclo (3.1.0) hexane,4-methylene-1-(1-methiethyl)-	4.93
2	7.541	1,3-Cyclohexadiene,1-Methyl-4-(1-methylethyl)	3.38
3	8.712	1,4-Cyclohexadiene1-methyl-4-(1-methylethyl)-	2.84
4	12.074	3-Cyclohexane-1-ol.4-methyl-1-(1-methylethyl)-	4032653
5	12.388	(+)-Alpha-terpineol(P-menth-1-en-8-ol)	2.56
6	17.949	Benzene,1,2-Dimethoxy-4(2-propenyl)	2.67
7	19.184	Phenol,2-methoxy-4-(1-propenyl)-	3.17
8	20.947	Croweacin	5944303
9	23.892	Benzene, 1,2,3-Timethoxy-5-(1-propenyl)-	2.88
10	26.893	Tetradecanoic acid	5.07
11	27.743	2-Oxetanone,4-(2,6,8-trimethyl-1,5-nanodienyl)-	2.83
12	28.282	2-Benzofuranmethanol, 2.4.5.6.7.7a-hexahydo-4,47a-trimethyl-	6.56
13	36.693	Hexadecanoic acid, methyl ester	3.10
14	31.121	1-(+)-Ascorbic acid2,6-dihexadecanoate	4375631
15	33.432	9-Octadecenoic acid (Z), methyl ester	2.85
16	34.396	Oleyl alcohol, trifluoroacetate	4947349
17	40.733	Adamantane-1-carboxylic acid(2-methyl-4-thiocyanato-phenyl)-amide	3.90
18	41.005	Hydrazine, (2,3,5,6-tetrafluro-4-pentyl(1,1-Biphenyl)-4-yl-	4.09
19	42.638	Phenol, 4-(2,3-dihydro-7-methoxy-3-methyl-5-(1-propenyl)-2	7.66
20	43.097	Phenol,4,4-methylenebis(2-(1.1-dimethylethyl)6-methyl-	4.74
21	43.267	Carbazole,2,4,7-trimethyl-(alkaloids)	3.81
22	44.650	Phenol, 2,6-Dimethoxy-4-(2-Propenyl)-	4.06

Table 10: Gc-Ms Analysis of Ethanolic Extract of Mace of Myristica Fragrans

Conclusion

From the results of the present study it is observed that among the four selected herbs it was only ethanolic extract Rosmarinus officinalis and Myristica fragrans which showed highest antimicrobial activity. Due to this result their ethanolic extracts were combined together in a ratio of 1:1 and further assessed for antimicrobial activity determination which also provided fruitful results. Apart from antimicrobial activity the extracts even had considerable antioxidant activity and when phytochemical tests were performed on the extracts it was found that there was presence of class of phytochemicals such as alkaloids, saponins, carbohydrates, tannins, steroids, etc. which are potential wound-healing agents and highly active against microbial infections. Finally, it is concluded that the combinatorial herbal extract of Rosmarinus officinalis and Myristica fragrans can be used to develop potential wound dressings in order to treat burn wounds, pressure ulcers and microbial infections associated with them.

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