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Preliminary Bioactive Compounds Screening and Antibacterial Activity of Methanolic Extract *of Hibiscus rosasinensis* against Selected Skin Pathogens.

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ABSTRACT

Various researches have been in progress to screen the bioactive potentials from floral source due to the negative impacts occurred during the intake of chemicals as a drug. So the present work aimed screen the bioactive potential of *Hibiscus rosasinensis*. The extracts prepared from the leaves of the Hibiscus were screened for antimicrobial activity against the pathogen isolated from skin. A portion of the extract was subjected to analyse various phytochemical present in the sample. Qualitative analysis was performed to find the presence of tannins, saponins, flavanoids, terpenoids, alkaloids and glycosides. In phytochemical studies the extracts of Hibiscus contains tannins, saponins, flavanoids, alkaloids and terpenoids. The Minimum Inhibitory Concentration (MIC) values showed the maximum of 13mm zone of inhibition at 80µl against *Escherichia coli* followed by 12mm against *Enterobacter aerogenes* and *Staphylococcus aureus* has been occurred. The present studies concluded the bioactive potential of methanolic extract of *Hibiscus rosasinensis* effective against various skin infecting pathogens.

Keywords: terpenoids, antioxidant, rhinoscleroma and skin infection.

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INTRODUCTION

Using plant based drugs as preventive and curative medicines is a rich heritage in India. In recent years, numbers of studies have been reported which screened the extracts of medicinal plants against pathogenic microbes (M Kannan, Singh, Kumar, Jegatheswari, & Subburayalu, 2007). This made applications in pharmaceuticals, alternative medicines and natural treatment (Marikani Kannan, 2010). Some of the active principles of bioactive compounds are preferred for therapy either singly or in combination to inhibit microbes (B. Dheeba, P. Sampathkumar, 2010),(Marikani Kannan, 2010).

In developing countries, low income people such as farmers, people of small isolate villages use folk medicine for treating common infections. They ingest plants as decoctions, teas, juice preparations to treat respiratory tract infections (Marikani Kannan Ranjit Singh and Narayanan, 2009). They are also made into a practice and applied directly on the infected wounds or burns (Aburjai, Darwish, Al-Khalil, Mahafzah, & Al-Abbadi, 2001). Many plants have been used due to their antimicrobial nature, which are due to the compounds synthesized during the secondary metabolism of the plant. The active substances include phenolic compounds which are part of the essential oils (I. Ahmad & Beg, 2001) as well as in tannin (V. U. Ahmad et al., 1994).

Skin disease is a major problem which may be caused due to bacteria, virus, fungi or other microscopic organisms. Bacteria like *Staphylococcus aureus, Streptococcus pyogenes, Enterobacter aerogenes, Pseudomonas aeruginosa, Klebsiella pneumonia* and *Escherichia coli* may also be a reason for so many skin infections. *Staphylococcus aureus* can cause a range of illness from minor skin infections such as pimples, impetigo, boils, cellulitis, folliculitis and carbuncles to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome, bacteremia and sepsis (Chomnawang, Surassmo, Nukoolkarn, & Gritsanapan, 2005).

Pseudomonas aeruginosa is the most common cause of burn injury infections and otitis externa. It may also cause “hot-tub dermatitis or rash” which is due to lack of proper, periodic attention to water quality. It is frequently associated with osteomyelitis involving puncture wounds of the foot, believed to result from direct inoculation with *Pseudomonas aeruginosa* through in the foam padding found in tennis shoes. *Enterobacter aerogenes* cause various community acquired infection, wound infection, skin and soft tissue infections. *Klebsiella* cause vaginitis, nasal mucosa atrophy and rhinoscleroma which are the mucosal skin infection (Crops, Agriculture, & Republic, n.d.).

Hibiscus rosasinensis is an ornamental plant classified under the family Malvaceae widely cultivated in tropical region. It is also used in traditional medicine to relieve from headache, fever and inflammation. The extracts prepared from the leaves, stems and flowers of the Hibiscus shown antimicrobial activity against methicillin resistant *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Klebsiella pneumonia*(M Kannan, Venkatesan, & Singh, 2004). It is a potent source of phenols, triterpenes, phytosteroids with antioxidant, antihypertensive, antiproliferative and cardioprotective functions (Lall & Meyer, 2000). Tannins possess antimicrobial and

antioxidant activities (Myrtaceae, Djadjo, & Delme, 2000)and saponins have antifungal activity (Mohan, Reddy, Subhash, & Sarma, 2010).

MATERIALS AND METHODS

Collection of Samples

Some of the skin infected in-patients were met and from them skin lesions and pus samples were collected by swabbing. After collection, the samples were transported to the laboratory in sterile peptone broth.

Isolation of Skin Pathogens

The collected samples were plated on different selective agar plates like EMB agar, Mac Conkey agar, Mannitol Salt agar, Pseudomonas Isolation agar and Potato Dextrose agar all the above media were purchased from Hi Media, Mumbai, India. After inoculation of the sample all the plates were kept in incubation for 24 hours at 37^{o}C.

Identification of Pathogens

The isolated bacterial pathogens were identified by morphological and colouring pattern on selective agars, gram staining and various biochemical characteristics like TSI agar test, Indole production test, methyl red test, Voges-Proskauer test, citrate utilization test and Urease test. Fungal isolates were identified by growth pattern on Potato dextrose agar, hyphal and spore appearance on Lactophenol cotton blue staining, germ tube test, carbohydrate fermentation test and carbohydrate assimilation test.

Collection of Plant Source

Hibiscus rosasinensis leaves collected from our village was shade dried, powdered and 10g of it was placed in 100ml of methanol in a conical flask and kept in rotary shaker for 24h. After that, it was filtered, centrifuged at 5000rpm for 15 minutes and the supernatant collected was dried and used for the antimicrobial assay.

Antimicrobial Activity Testing

Muller-Hinton agar plates were prepared and 8mm diameter wells were punched for studying the activity of extract against bacteria. After solidification, 0.1ml of the test organisms was spreaded on the surface and filled with different concentrations (20µl, 40µl, 60µl and 80µl) of plant extract. The plates were incubated at 37˚C for 24 hours. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organism. 0.2 ml of the fungal isolates were swabbed uniformly on the surface of the Potato Dextrose agar and allowed to dry for 5 minutes and discs loaded with different concentrations (20µl, 40µl, 60µl and 80µl) of plant extract were placed on the culture swabbed agar surface. After 5 minutes, the plates were incubated at 27°C for 48 hours. Appearance of zone of inhibition around the discs was measured as zone of inhibition in millimeter. The procedure was carried out as triplicates.

Determination of the Minimum Inhibitory Concentration

A stock solution of 100mg/ml was prepared and serially diluted to obtain various ranges of concentrations from 20µg/ml to 80µg/ml. 0.5 ml of each and different concentration (20µl, 40µl, 60µl and 80µl) of it was transferred into sterile test tube containing 2ml of nutrient broth. To the tubes, 0.1ml of the most inhibited organism was then introduced and a set of test tubes containing broth alone were used as control. All the test tubes were incubated for 24 hours at 37° C. After the period of incubation, the test tube with least concentration of extract showing no visible sign of growth was considered as the minimum inhibitory concentration.

Qualitative Analysis of Phytochemicals

A portion of the extract was subjected to analyse various photochemical present in the sample. Qualitative analysis was performed to find the presence of tannins, saponins, flavanoids, terpenoids, alkaloids and glycosides.

TEST FOR TANNINS: 5ml of plant extract was added with few drops of 10% lead acetate solution. Formation of yellow or red precipitate indicates positive result (Sparg, van Staden, & Jäger, 2002).

TEST FOR SAPONINS: The extract was diluted with 20ml of distilled water and shaken in a graduated cylinder for fifteen minutes. Formation of foamy layer indicates positive result(Khanavi, Sharifzadeh, Hadjiakhoondi, & Shafiee, 2005).

TEST FOR FLAVANOIDS: 2-3ml of the phytoextract was added with a few drops of NaOH solution. Intense yellow colour formed becomes colourless on addition of few drops of dilute hydrochloric acid indicates the presence of flavonoids(Reid, Jäger, Light, Mulholland, & Van Staden, 2005).

TEST FOR TERPENOIDS: 5ml of extract was added with 2ml $CHCl_3$ in a test tube. 3ml of concentrated sulphuric acid was carefully added to the mixture to form a layer. Formation of interface with a reddish brown colouration indicates the presence of terpenoids(Charrouf & Guillaume, 1999).

TEST FOR ALKALOIDS: 2-3ml of the extract was added with a few drops of Dragendorff's reagent. Formation of orange brown precipitate indicates the presence of alkaloids(Maciel et al., 2000).

TEST FOR GLYCOSIDES: 1ml of concentrated sulphuric acid in test tube was added with 5ml of aqueous extract and 2ml of glacial CH_3CO_2H containing a drop of $FeCl_3$. The above mixture was carefully added to 1ml of concentrated H_2SO_4 so that the concentrated Sulphuric acid is underneath the mixture. If glycoside is present, a brown ring will appear(Ahmadiani, Javan, Semnanian, Barat, & Kamalinejad, 2001).

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RESULT

The aseptically collected skin lesions were plated on various selective agars like EMB agar, Mac Conkey agar, Mannitol Salt agar, Pseudomonas Isolation agar, Potato Dextrose agar grown seven types of colonies were tableted (Table.1). The isolated Colonies were further analyzed for morphological and biochemical analysis helped to identify the organisms (Table.2, 3 and 4). Antibacterial activity for methanolic extract of *Hibiscus rosasinensis* was screened against the seven isolated skin pathogens *Pseudomonas aeruginosa, Escherichia coli, Enterobacter aerogenes*, *Staphylococcus aureus, Streptococcus pyogenes, Candida albicans* and *Aspergillus niger shown* varied zone of inhibition. The maximum zone of inhibition 13mm was observed against *Escherichia coli* followed by 12mm against *Enterobacter aerogenes* and *Staphylococcus aureus* at 80µl concentration. Of the two fungi obtained, the maximum activity of 9mm was observed against *Aspergillus niger* and 7mm against *Candida albicans* (Table.5). The most inhibited *Escherichia coli* was tested by the extract for identifying the minimum inhibitory concentration which was found to be 80µl. Qualitative analysis of phytochemicals in extract reported the presence of tannins, saponins, flavanoids, alkaloids and terpenoids which may be the reason for the bioactivity (Table.6).

Table.1 IDENTIFICATION OF THE BACTERIAL AND FUNGAL PATHOGEN ON SELECTIVE AGAR

S.No.	SELECTIVE AGAR	COLONY MORPHOLOGY	PATHOGENS
1	Pseudomonas Isolation Agar	Bluish-green colonies	*Pseudomonas aeruginosa*
2	Eosine Methylene Blue	Metallic sheen	*Escherichia coli*
3	Eosine Methylene Blue	Pink	*Enterobacter aerogenes*
4	Mannitol Salt Agar	Turned the medium yellow	*Staphylococcus aureus*
5	Blood Agar	β - hemolysis	*Streptococcus pyogenes*
6	Potato Dextrose Agar	Cream colonies	*Candida albicans*
7	Potato Dextrose Agar	Black	*Aspergillus niger*

Table.2 THE MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF ISOLATED PATHOGENS

S.No.	Tests	*P.a*	*E.c*	*E.a*	*S.a*	*S.p*
1	Gram staining	-	-	-	+	+
2	Shape	Rod	Rod	Rod	Grape cluster	Chained cocci
3	Motility	Motile	Motile	Motile	Non-motile	Non-motile
4	Indole	_	+	_	_	_
5	+MR	_	+	_	+	_
6	VP	_	_	+	_	_
7	Citrate	+	_	+	_	+
8	TSI	k/a	a/a	a/a	a/a	a/a
9	Urease	_	+	_	+	+

P.a - ***Pseudomonas aeruginosa, E.c- Escherichia coli, E.a- Enterobacter aerogenes, S.a - Staphylococcus aureus and S.p - Streptococcus pyogenes. + = Positive, - = Negative, k/a* = Alkaline slant (red) and acid butt (yellow), *a/a* = Acid slant (yellow) and acid butt (yellow).**

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Table.3 THE FOLLWING TESTS WERE USED TO IDENTIFY THE FUNGI *Candida Albicans*

S.No.	TESTS	RESULTS
1	Germ tube test	Germ tubes were observed
2	On Cornmeal agar	True hyphae were observed
3	Carbohydrate assimilation	Glucose, Maltose and Galactose were assimilated
4	Carbohydrate Fermentation	Glucose and Maltose were fermented
5	Indian ink preparation	Capsulated white coloured cells observed under dark background

Table. 4 THE FOLLWING TESTS WERE USED TO IDENTIFY THE FUNGI *Aspergillus niger*

S.No.	TESTS	RESULTS
1	Hyphae	Septate
2	Conidial head	Brown black
3	Conidiophore	Smooth walled and brown near the vesicle
4	Sterigmata	Brown on entire surface produced in two series

Table. 5 ANTIMICROBIAL ACTIVITIES OF METHANOLIC EXTRACT OF *Hibiscus rosasinensis* Leaves

S.No.	Pathogens	Zone of Inhibition (in mm ± S.D.)			
		20µl	40µl	60µl	80µl
1	*Pseudomonas aeruginosa*	9±0	11± 0	11± 0	11.3± 0.57
2	*Escherichia coli*	5.6±0.57	8.3 ±0.57	9.3± 0.57	13± 0
3	*Enterobacter aerogenes*	7.6± 0.57	8.6± 0.57	9± 0	12± 0
4	*Staphylococcus aureus*	7 ±0	7.6± 0.57	10.3± 0.57	12.3± 0.57
5	*Streptococcus pyogenes*	5± 0	5.3± 0.57	6± 0	7± 0
6	*Candida albicans*	4.6± 0.57	5.3± 0.57	6.3± 0.57	6.6± 0.57
7	*Aspergillus niger*	7± 0	8.3± 0.57	9± 0	9.3± 0.57

Table.6 PRELIMINARY PHYTOCHEMICAL ANALYSIS OF *Hibiscus rosasinensis*

S.No.	Test	Appearance	Result
1	Tannins	Red precipitate	+
2	Saponins	Foamy layer	+
3	Flavanoids	Intense yellow colour	+
4	Terpenoids	Reddish brown interface	+
5	Alkaloids	Orange brown precipitate	+
6	Glycosides	Absence of brown ring	_

Table.7 ZONE OF INHIBITION OF *Hibiscus rosasinensis ON ISOLATED PATHOGENS*

S.No	Pathogens	DIAMETER OF ZONE IN mm			
		20µl	40µl	60µl	80µl
1	*Pseudomonas aeruginosa*	9	11	11	11
2	*Escherichia coli*	5	8	9	13
3	*Enterobacter aerogenes*	7	8	9	12
4	*Staphylococcus aureus*	7	8	10	12
5	*Streptococcus pyogenes*	5	5	6	7
6	*Candida albicans*	5	5	6	7
7	*Aspergillus niger*	7	8	9	9

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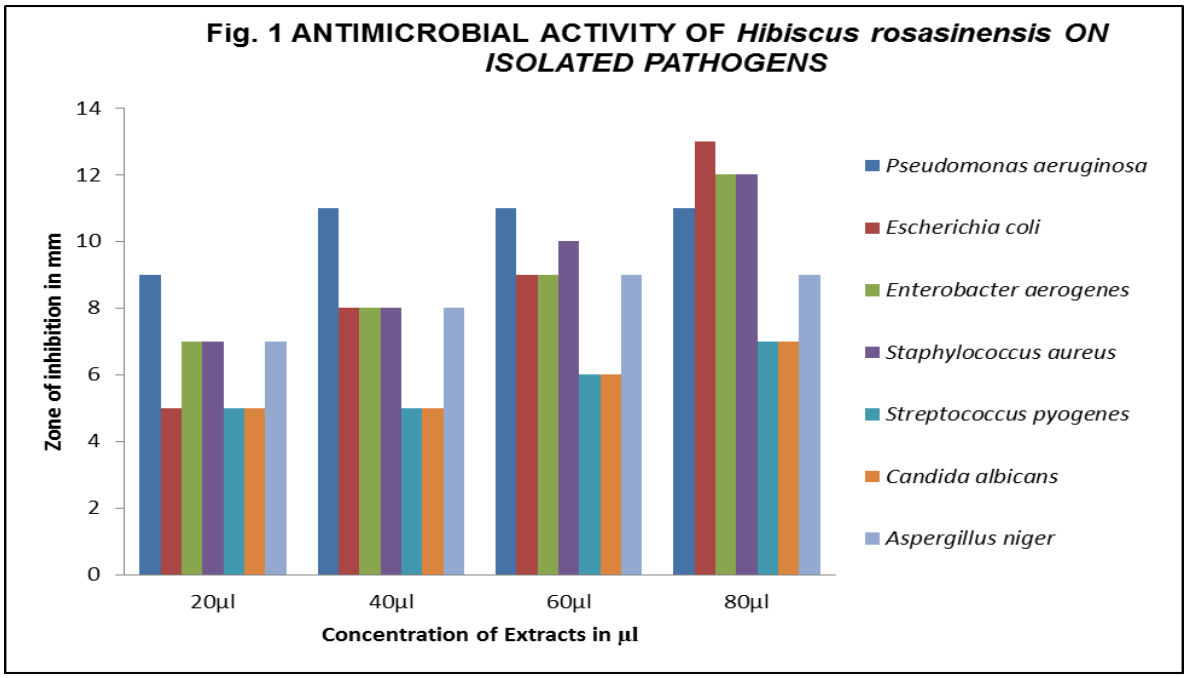

The methanolic extract of various parts of *Hibiscus rosasinensis* was most effective against various skin infection causing microorganisms like *Escherichia coli, Staphylococcus aureus, Streptococcus pyogenes, Candida albicans* and *Pseudomonas aeruginosa* (Chandrasekaran & Venkatesalu, 2004). In the present study, the pathogens obtained from skin infections were inhibited by the methanolic extract of *Hibiscus rosasinensis* leaves (Table.7) (Fig. 1). The reason for the activity may be due to the secondary metabolites produced which may either kill or prevent growth. Chemical compounds synthesized by plants help them to prevent them from a wide variety of predators. Some of this compound possesses the positive effects when used to treat human diseases. The compounds responsible for the activity may be due to tannins, saponins, flavonoids, terpenoids or alkaloids. They may be manipulated for further studies for the development of new pharmaceutical therapeutics. The present study concludes the bioactive potential of methanolic extract of *Hibiscus rosasinensis* against various skin infecting pathogens. The maximum inhibition was reported against *Escherichia coli* followed by *Enterobacter aerogenes* and *Staphylococcus aureus*. The activity may be due to the presence of tannins, saponins, flavanoids, alkaloids and terpenoids in the extract.

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