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A RESEARCH ARTICLE ON EVALUATION OF ANTI FUNGAL ACTIVITY OF AQUEOUS AND ALCOHOLIC EXTRACT OF MADHUCA INDICA

J. Sushmitha[*1], C.H.V. Alekhya[1], Ch. Jyothirmai[1], A. Ashok Kumar[1]

[*1]Department of Pharmacology, NRK and KSR GUPTA College of Pharmacy, Tenali, Andhra Pradesh, India.
[2]Department of Pharmaceutics, NRK and KSR GUPTA College of Pharmacy, Tenali, Andhra Pradesh, India.

ABSTRACT

Fungal phytopathogens pose serious problems worldwide and cause a number of plants and animal diseases such as ringworm, athlete's foot, and several more serious diseases. Medicinal plants are gifts of nature to cure limitless number of diseases among human beings. In the present investigation the anti-fungal activity of *Madhuca indica* against *Rhizopus stolonifer* has been reported. One of the such finding is that reveals the use of plant *Madhuca indica* which has been used in Indian folk medicine as analgesic, anthelmintic, antimicrobial, diuretic, anti-ulcer, diabetic and also used to treat arthritis.

KEYWORDS

Phytopathogens, *Madhuca indica*, Analgesic, Antifungal activity and Anti-microbial activity.

Author for Correspondence:

Sushmitha J,
Department of Pharmacology,
NRK and KSR GUPTA College of Pharmacy, Tenali, Andhra Pradesh, India.

Email: sushmithajyothula11@gmail.com

INTRODUCTION[1-3]

Medicinal plants are gifts of nature to cure limitless number of diseases among human beings[4-6]. The abundance of plants on the earth's surface has led to an increasing interest in the investigation of different extracts obtained from traditional medicinal plants as potential sources of new antimicrobial agents[7].

Now a day's multiple drug resistance has developed due to the indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious diseases[8, 9]. In addition to this problem, antibiotics are sometimes associated with adverse effects on the host including hypersensitivity,

immune-suppression and allergic reactions[10]. This situation forced scientists to search for new antimicrobial substances. Therefore, there is a need to develop alternative antimicrobial drugs for the treatment of infectious diseases from medicinal plants[11,12]. Several screening studies have been carried out in different parts of the world. There are several reports on the antimicrobial activity of different herbal extracts in different regions of the worlds[13,14].

According to World Health Organization (WHO) more than 80% of the world's population relies on traditional medicine for their primary healthcare needs. Use of herbal medicines in Asia represents a long history of human interactions with the environment. Plants used in traditional medicine contain a wide range of ingredients that can be used to treat chronic as well as infectious diseases. A vast knowledge of how to use the plants against different illnesses may be expected to have accumulated in areas where the use of plants is still of great importance[15]. The medicinal value of plants lies in some chemical substances that produce a definite physiological action on the human body. The most important of these bioactive compounds of plants are alkaloids, flavonoids, tannins and phenolic compounds.

M*adhuca longifolia* (Koen.) Macbr. (Syn. *Bassia longifolia* J. Koenig ex. L. *M. longifolia* (Koen.) Macbr. var *longifolia*) is a large, shady, deciduous tree, both wild and cultivated, dotting much of the Central Indian landscape. The tree is valued for its flowers, fruits, seeds and timber. The expectorant flowers are used to treat chest problems such as bronchitis. They are also taken to increase the production of breast milk. The distilled juice of the flowers is considered a tonic, both nutritional and cooling. The tree wins in fame due to the liquor distilled from the flowers, which is used to make vinegar. The leaves are applied as a poultice to relieve eczema. In Indian folk medicine, the leaf ash is mixed with ghee (clarified butter) to make a dressing for wounds and burns. *Madhuca* preparations are used for removing intestinal worms, in respiratory infections, and in cases of debility and emaciation. The astringent bark extract is used for dental-related problems, rheumatism and diabetes.

In the present investigation the anti-fungal activity of *Madhuca indica* against Rhizopus stolonifer has been reported. One of the such finding is that reveals the use of plant *Madhuca indica* which has been used in Indian folk medicine as analgesic, anthelmintic, antimicrobial, diuretic, anti-ulcer, diabetic and also used to treat arthritis.

METHODOLOGY[16-18]

Collection of plant material

The flowers of *Madhuca indica* were purchased to a weight of 300 gms. In commercial Ayurvedic market at Tenali.

Preparation of extract

The air dried *Madhuca indica* flowers were powered and extracted with water and 50% ethanol by using soxhlet apparatus. The extracts were evaporated to dryness at a controlled temperature (45^{o}C).

Collected Aqueous Extract – 15gms

Collected Alcoholic extract- 19gms.

Investigation of Preliminary Qualitative Phytochemical Analysis

Phytochemical analysis of hydro alcoholic extract of *Madhuca indica* was carried out by using the standard procedures. Alkaloids, carbohydrates, flavonoids, glycosides, lactones, phytosterols, proteins, Saponins and triterpenoids were qualitatively analyzed.

TEST FOR PHYTOSTEROLS

Small quantity of decoction was dissolved in 5ml of chloroform separately. Then these chloroform layer subjected to,

Salkowski test

Libermann - Burchards test.

Salkowski test

To 1ml of the above prepared chloroform solutions, few drops of conc H_2SO_4 was added. Red color indicates the presence of phytosterols.

Libermann - Burchards test

The above chloroform solution was treated with few drops of conc H_2SO_4 followed by 1ml of acetic anhydride solution. Green color indicates the presence of phytosterols.

TEST FOR TRITERPENOIDS

Brieskorn and Binar test

To extract, add few drops of chlorosulphonic acid in glacial acetic acid. Appearance of red color within five minutes indicates presence of triterpinoids.

TEST FOR SAPONINS

Foam test

A small amount of extract was taken in a test tube with little quantity of water. Shake vigorously. No appearance of foam persisting for 10 minutes indicates absence of Saponins.

TEST FOR ALKALOIDS

Small amount of extract was stirred with a few ml of dil HCl and filtered. The filtrate was tested with various alkaloidal reagents such as Mayer's, Dragendroff's, Wagner's and Hager's reagent.

Mayer's test

To the small amount of filtrate add few drops of Mayer's reagent. White color indicates the presence of alkaloids.

Dragendroff's test (potassium bismuth iodide)

To the small amount of filtrate add few drops of Dragendroff's reagent. An orange red color precipitate indicates the presence of alkaloids.

Wagner's test:

To the small amount of filtrate add few drops of Wagner's reagent. A brown color indicates the presence of alkaloids.

Hager's test: (picric acid)

To the small amount of filtrate add few drops of Hager's reagent. A yellow crystalline precipitate indicates the presence of alkaloids.

TEST FOR CARBOHYDRATES

Small quantity of the extract was dissolved in distilled water and filtered. The filterate was subjected to

Molisch's test

Fehling's test

Barfoed's test

Benedict's test

Molisch's test

To the filtrate few drops of alcoholic α-napthol was added and 2ml of conc sulphuric acid was added slowly through the slides of the test tube. Purple colored ring at junction of the two layers, which indicates presence of carbohydrates.

Fehling's test

Small portion of the extract was treated with Fehling's solution I and II and then heated on water bath. Brick red color precipitate indicates presence of carbohydrates.

Barfoed's test

Small portion of the extract was treated with barfoed's reagent. Red precipitate indicates the presence of carbohydrates.

Benedict's test

Small portion of the extract was treated with benedicts' reagent. Boiling on water bath shows reddish brown precipitate formed, which indicates presence of carbohydrates.

TEST FOR FLAVONOIDS

The extract was dissolved in ethanol and then subjected to the following tests.

Ferric chloride test

To a small quantity of Methanolic solution of extract few drops of neutral ferric chloride was added. Blackish red color indicates the presence of flavonoids.

Shinoida's test

To the alcoholic solution a small piece of magnesium ribbon was added along with conc HCl. Magenta color indicates the presence of flavonoids.

Fluorescence test

Alcoholic solution was seen under ultra violet light. Green color fluorescence indicates the presence of flavonoids.

Reaction with alkali and acid

With sodium hydroxide solution the extracts gave yellow color. Extract gave orange color with conc H_2SO_4 indicates the presence of flavonoids.

Zinc, HCl reduction test

To a small quantity of extract, a pinch of zinc dust was added. Then add few drops of conc HCl. Magenta color was indicate the presence of flavonoids.

Lead acetate solution

To a small quantity of extract a few drops of 10% lead acetate solution was added. Yellow precipitate

indicates the presence of flavonoids.

TEST FOR LACTONES

Legal's test

To the extract mixtures add sodium nitroprusside and pyridine. Then the mixture is treated with NaOH. Appearance of deep red color indicates the presence of lactones.

TEST FOR PROTEINS

Small quantity of extract was dissolved in few ml of water and was subjected to million's, biuret and ninhydrin test.

Million's test

The extract was treated with million's reagent. White precipitate was produced, shows the presence of proteins and free aminoacids.

Biuret test

To the extract equal volume of 5%w/v NaOH and four drops of 1%w/v $CuSO_4$ solution were added. Pink or purple color indicates the presence of proteins.

Ninhydrin test

The extract was treated with ninhydrin reagent. Purple color indicates the presence of proteins.

TEST FOR GLYCOSIDES

A small amount of the extract was hydrolyzed with hydrochloric acid for one hour on a water bath and hydrolysate was subjected to

Legal's test

Balget's test

Borntrager's test

Modified borntrager's test

Legal's test

To the hydrolysate 1ml pyridine few drops of sodium nitroprusside solution was added and then made alkaline with NaOH solution. Pink color indicates the presence of glycosides.

Balget's test

To a solution of extract sodium picrate solution was added. Yellowish orange color indicates the presence of glycosides.

Borntrager's test

Hydrolysate was treated with chloroform and the chloroform layer was separated. To this equal quantity of dilute ammonia solution was added. Pink color indicates the presence of glycosides.

Modified borntrager's test

The extracts were boiled with few ml of dil HCl and 5ml of ferric chloride solution. The contents are cooled and shaken with organic solvent. Organic layer was separated and to this equal volume of ammoniacal solution was added. The ammoniacal layer showed pink color. In this test, addition of ferric chloride was added to break the C – C linking of glycosides which is a stronger than C = O linkage.

Tannins

The extract will be treated with 10% lead acetate solution. Formation of a white colour indicates the presence of tannins.

Test for phenolic compounds

Extract treated with neutral ferric chloride formation of a violet colour indicates the presence of phenolic compounds. Extract will be treated with 10% NaCl solution formation of a cream colour indicates the presence of phenolic compounds.

Qualitative Anti-Fungal Studies[4-6]

The *in-vitro* antifungal activity by agar well diffusion method was standardized using clopromazole. This method is based on diffusion of antifungal component from reservoir hole to the surrounding inoculated Sabouraud's dextrose agar medium, so that the growth of fungus is inhibited as zone around the hole. Two fungi were selected *Rhizopus stolonifer.*

***In vitro* Model**

Agar well diffusion method[7].

Nutrient Medium

Sabouraud's dextrose agar medium (Hi Media) was used for preliminary antifungal activity. The medium was prepared by dissolving in water and autoclaving at 121^0C for 15 minutes. The contents showed in Table No.1.

The pH of the medium plays an important role for the growth of fungi. Acidic medium favour the growth but excess of acid prevent solidification of agar. Hence the pH of medium was adjusting using 0.1% w/v lactic acid.

Mould growing on the bread can be microscopic fungi belonging to different species like Penicillium,

Rhizopus, Aspergillus, Monascus and Fusarium. They are of different shapes and colors depending on the species. *Rhizopus stolonifer* is the most common and fast growing bread mould. It is also known as black mould as it appears dark green or black in color. It causes rotting of some fruits and some infections in humans.

For fungal culture Saboraud Dextrose Agar was prepared and transferred into sterile Petri plates and solidified. The medium plates were then swabbed with fungal culture. Then this culture media was incubated at 25^0C for 24 hrs.

The sterilized paper discs were soaked in the prepared solutions of the extracts with different solvents and were dried at 50^0C. The dried paper disc was then placed Saboarud Dextrose agar seeded with test microorganisms. The plates were incubated at room temperature for 48 hours and the zones of inhibition were measured.

RESULTS AND DISCUSSION

Phytochemical screening is the process of tracking plant constituents by performing certain tests and procedures to the intended extracts. One of the most important and fundamental considerations in designing a Phytochemical screening procedure is the selection of proper solvent. It is often difficult to follow general solubility rule for a given class of phyto constituents since there are substances of unknown characters present in crude plant extracts that effect solubility.

Investigation of Preliminary Qualitative Phytochemical Analysis.

Current Study: Anti-fungal activity

Antifungal activity of aqueous and alcoholic extract of *Madhuca indica* flowers was determined by measuring diameter of inhibition zone in millimeters. This is evident in following Table No.3.

Aqueous and Alcoholic extracts of flowers of *Madhuca indica* were screened for antifungal activities against *Rhizopus stolonifer* at dose level ranging from 50 µg/ml to 250 µg/ml.

Aqueous and Alcoholic extracts of *Madhuca indica* flowers effectively inhibited growth of the fungi (*R. stolonifer*).

Among the two extracts, Alcoholic extract of *Madhuca indica* flowers have shown effective inhibition against fungi.

According to research finding of Kalaivani. M *et al.*, in their Title "Antimicrobial activity of alcoholic extract of leaves and flowers of *Madhuca longifera"* Antibacterial and antifungal activities of alcoholic extract of flowers of *Madhuca indica* could be attributed to the presence of biological compounds like 2-Furan methanol, 4H pyran 4-one, 2,3-dihydro 3,5-dihydroxy-6-methyl, Thiophene, 2-Furancarboxyaldehyde-5- (hydroxymethyl) and 1,4-tetra decanediol.

The use of medicinal plants play a vital role in covering the basic health needs in developing countries and these plants may offer new sources of antibacterial, antifungal and antiviral agents with significant activity against infective microorganisms.

Table No.1: Sabouraud's dextrose agar medium

S.No	Ingredients	Weight (ing)
1	Dextrose	40
2	Peptone	10
3	Agar	20
4	Distilled water	q. s to 1000
5	P^H	5.6

Table No.2: Qualitative Phytochemical Analyses

S.No	Test	Ethanolic and Aqueous Extract of *Madhuca Indica*
1	Alkaloids	+
2	Carbohydrates	+
3	Flavanoids	-
4	Glycosides	-
5	Lactones	-
6	Phenolic compounds	-
7	Phytosterols	-
8	Proteins	+
9	Saponins	-
10	Triterpenoids	-
11	Tannins	+

(+) Indicates positive result (–) Indicates negative result

REPORT

Preliminary phyto chemical study of extracts of *Madhuca indica* confirmed strong presence of desired phyto chemicals in 50% ethanolic and aqueous extracts. Hence ethanolic and aqueous extracts of *Madhuca indica* have been selected for current studies.

Table No.3: Anti-fungal activity of aqueous and alcoholic extract of flowers of *Madhuca indica*

S.No	Name of extract	Diameter of inhibition Zone (in mm) at different concentrations (in µg/ml)					
		10	50	100	150	200	250
1	Aqueous extract of *Madhuca indica*	-	5	5	7	8	9
2	50% Ethanolic extract of *Madhuca indica*	-	7	7	8	9	11
3	Standard drug (Clotrimazole)	10	-	-	-	-	-

REPORT

Aqueous and Alcoholic extracts of *Madhuca indica* flowers effectively inhibited growth of fungi *Rhizopus stolonifer* using clotrimazole as standard.

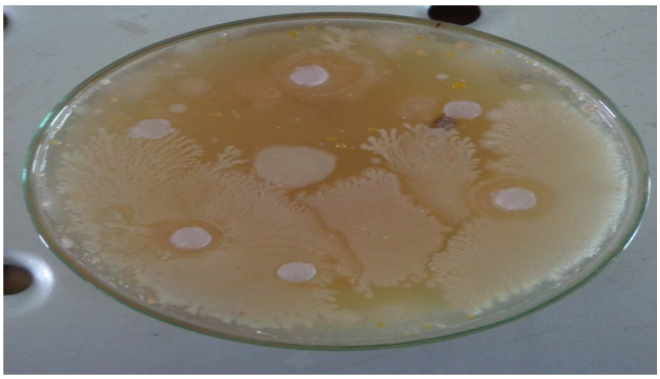

Figure No.1: Zone of inhibition of aqueous extract of *Madhuca indica*

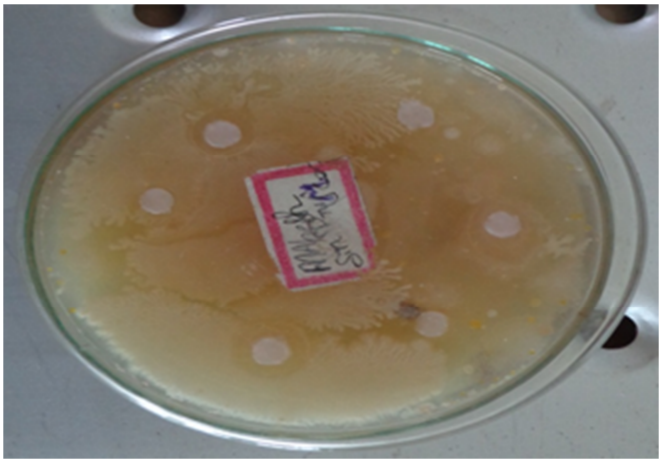

Figure No.2: Zone of inhibition of alcoholic extract of *Madhuca indica*

CONCLUSION

The present study indicates that *Madhuca indica* extracts have broad inhibitory activity on fungal growth and potential to act as anti-fungal agent from natural sources. In general, commercial antibiotic and antifungal drugs cause side effects such as liver, kidney and gastro intestinal tract toxicity. Severe hepato toxicity had also been reported in patients undergoing antifungal drug therapy. However, herbal remedies often do not produce any side effects. Therefore, alternative medicine became popular remedy to various types of ailments.

In conclusion, *Madhuca indica* extracts have revealed significant antifungal activities against test organism (*Rhizopus stolonifer*) used for the study. Further investigations detailing about the chemical constituents responsible for the activity in the hydro and alcoholic extracts are anticipated and the plant may be further explored for its potential in treatment of fungal infection.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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