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A STUDY ON ANTIMICROBIAL ACTIVITY OF EXTRACTS OF LUFFA ACUTANGULA VAR AMARA FRUITS

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ABSTRACT

The present study involves extraction, phytochemical investigation and *in-vitro* antimicrobial activity of *Luffa acutangula* var *amara* fruits known as Kadwi turai in Hindi. The phytochemical investigation revealed the presence of sterols and glycoside in chloroform extract. The chloroform extract showed significant antimicrobial activity than aqueous extract. Both extracts showed weak antifungal activities.

KEY WORDS: ***Luffa acutangula* var *amara* fruits, phytochemical investigation, antimicrobial activity, antibacterial activity, antifungal activity**

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INTRODUCTION

Since resistance of pathogenic bacteria towards available antibiotics is rapidly becoming a major worldwide problem, the design of new compounds to deal with resistant bacteria has become one of the most important areas of antibacterial research today. In addition, primary and opportunistic fungal infections continue to increase rapidly because of the increased number of immunocompromised patients. As known, not only biochemical similarity of the human cell and fungi forms a handicap for selective activity, but also the easily gained resistance is the main problem encountered in developing safe and efficient antifungals.[1] Thus, it is still necessary to search for new antimicrobial agents.

The World Health Organization estimates that some 80% of the people in developing world rely on traditional medicines and that, of these 85% use plants or their products as the remedies. India is endowed with an estimated 47,000 species of plant that include around 8000 plants which are known to have medicinal properties,[2] the plants have been a veritable source of drugs. Different extracts from traditional medicinal plants have been tested to identify the source of the therapeutic effects. The *Luffa acutangula* var *amara* (Family:Cucurbitaceae) is a fairly large climber found in Western, Central and Southern India. All parts of the plant are bitter in taste. Indigenous system of medicine are using the leaf or dried fruit powdered or fruit juice in the treatment of jaundice in the tribals of Madhya Pradesh of India.[3] The parts of plant were showed bronchitis, emetic, expectorant, demulcent ascites, uterine and vaginal tumours, cytotoxic activity, snake bite and CNS depressant activity in mice.[4-7] In view of these facts and continuous of our research work[8] present study was to explore composition of the *Luffa acutangula* var *amara* fruit extracts as antimicrobial agents.

MATERIALS AND METHODS

Chemicals

All research chemicals were purchased from Acros organics (NY, USA), Sigma-Aldrich (St.Loius, Missouri, USA), Lancaster Co. (Ward Hill, MA, USA), SD-fine (Mumbai). Solvents except laboratory reagent grade were dried and purified according to the literature whenever necessary. The solvent was removed under reduced pressure using rotary flash evaporator (Buchi, China).The extracts were allowed to dry in a desiccators and stored at 4°C.

Collection of Fruits

The fruits of *Luffa acutangula* var *amara* (LA) were collected from in and around Gadag, Karnataka (2010-11), was authenticated by Dr. B.D. Huddar, Professor, Department of Botany, HSK Institute of Science, Hubli, Karnataka, India.

Phytochemical investigations

The fruits were shade dried and powdered. The powdered fruits of LA were extracted with water (1% chloroform water) and chloroform solvents respectively. After extraction, the solvent was removed under reduced pressure (Buchi) using rotary flash evaporator then finally dried in desiccator. The dried respective, extracts were weighed and percentage yield calculated.

Preparation of aqueous and chloroform extract of Luffa acutangula var amara fruits

The aqueous (1% chloroform water) and chloroform extracts were obtained by cold maceration method. Approximately 30 g of the fruit powder was soaked in 200 ml of cold water (1% chloroform water)/chloroform for 48 hrs at room temperature with occasionally shaken. The extract was first filtered through Whatman no.1 filter paper to clarify and then

through a 0.45 μm membrane filter. The solvent was removed under reduced pressure using rotary flash evaporator. The extracts was evaporated to dryness at room temperature in a steady air current and the yield recorded as a percentage of the quantity of initial plant material used.[9]

Some part of the extracts (10.0 g) was reserved for preliminary phytochemical investigations and the rest (10.0 g) for antimicrobial activity. All the dried extracts were subjected to qualitative chemical tests to identify the presence of phytoconstituents.

Qualitative evaluation

The preliminary phytochemical tests[10] were performed on aqueous and chloroform extracts. The results were tabulated.

1. Test for proteins and amino acids:

a. Millon's test: 2 m1 of Millon's reagent was added to the small quantity of extract; the appearance of white precipitate which turned red upon gentle heating indicated the presence of proteins.

b. Ninhydrin test: 2 ml of Ninhydrin reagent was added to 1 ml of extract; the appearance of purple/ violet colour showed the presence of amino acids.

2. Tests for carbohydrates

Small quantities of extracts were dissolved in little quantity of distilled water and filtered separately. The filtrates were used to test the presence of carbohydrates.

a. ***Molisch's test****:* The filtrate was treated with Molisch reagent and concentrated sulphuric acid was added through the sides of the test tube and formed a layer. A reddish violet ring showed the presence of carbohydrates.

b. ***Fehling's test***: The filtrates were hydrolysed with dilute hydrochloric acid, neutralized with alkali and heated with Fehling's A and B solution. Formation of red precipitate indicated the presence of reducing sugars.

3. ***Tests for phenolic compounds/ flavanoids***

a. Shinoda test: A few fragments of magnesium ribbon 1.0 ml of concentrated hydrochloric acid were added to the alcoholic solution of extract, the appearance of magenta colour after few minutes indicated the presence of flavonoids.

b. Ferric chloride test: A few drops of neutral ferric chloride solution were added to the little quantity of alcoholic extract, the formation of blackish green colour indicated the presence of phenolic nucleus.

c. Lead acetate test: A few drops of lead acetate solution (10%) were added to the alcoholic extract, the formation of yellow precipitate indicated the presence of flavonoids.

d. NaOH test*:* A few drops of NaOH were added to alcoholic solution, intense yellow colour which disappeared after adding dilute HCl indicated presence of flavonoids.

4. Tests for tannins:

a. Ferric Chloride test: A few drops of 1% neutral ferric chloride solution was added to the extract, the formation of blue, green or brownish green colour indicated the presence of tannins.

b. Match stick test*:* Dipped the match stick in test solution and air dried. A drop of concentrated hydrochloric acid was added on matchstick, held near the flame. Match stick took pink to purple and red colour indicated presence of tannins.

5. Test for glycosides

a. General test for the presence of glycosides

Test I: 200 mg of the extract was warmed in a test tube with 5 m1 of dilute (10%) sulphuric acid on a water bath at 100 °C for two minutes, centrifuged, pipetted supernatant, then neutralized the acid extract with 5% solution of sodium hydroxide (noting the volume of NaOH added). To this, 1.0 m1 of each Fehling's solutions A and B were added until alkaline (test with pH paper) and heated on a water bath for 2 min. noted the quantity of red

precipitate formed and compared with that formed in Test II.

Test II: 200 mg of the extract was heated in a test tube with 5 m1 of water instead of sulphuric acid. After boiling to this added volume of water equal to the volume of NaOH used in the above test. Added1.0 mlof Fehling's solutions A and B until alkaline and heated on water bath for two minutes. The quantity of the red precipitate formed was noted and compared the precipitate formed in Test II with that formed in Test I. If the precipitate in Test I is greater than in Test II then glycoside may be present. Since Test II represents the amount of free reducing sugar already present in the crude drug, whereas Test I represents the free reducing sugar plus those released on acid hydrolysis of any glycosides in the crude drug.

***6. Test the extract for free sugar*:** After complete removal of free sugars, the extract was hydrolyzed with mineral acid and then tested for the glycone and agylcone moieties.

***Raymond's test*:** The test solution when treated with dinitrobenzene in hot methanolic alkali gave violet color.

Legal's test: The extract was treated with pyridine and added alkaline sodium nitroprusside solution, the blood red color appeared.

***Bromine water test*:** Test solution when treated with bromine water gave yellow precipitate.

7. Tests for alkaloids

The extracts were basified with ammonia and extracted with chloroform. The chloroform solution was acidified with dilute hydrochloric acid. The acid layer was used for testing alkaloids.

a. Wagner's test: The acid layer was treated with a few drops of Wagner's reagent; the appearance of reddish brown precipitate indicated the presence of alkaloids.

b. Mayer's test: The acid layer was treated with a few drops of Mayer's reagent; the appearance of creamy white precipitate indicated the presence of alkaloids.

***c. Dragendroff's test**:* The acid layer was treated with a few drops of Dragendroff's reagent; the appearance of reddish brown precipitate indicated the presence of alkaloids.

8. Tests for sterols

The water extract freed from chlorophyll was dissolved in chloroform, filtered and the filtrate was tested for sterols and triterpenes.

***a. Salkaowski test**:* A few drops of concentrated sulphuric acid were added to the chloroform solution, shaken and allowed to stand, the appearance of red colour in lower layer indicated the presence of sterols.

b. Liebermann-Burchardt test: To the chloroform solution (2 mg in 1ml), added a few drops of acetic anhydride and mixed well. To this was added 1ml of concentrated sulphuric acid through the sides of the test tube, appearance of first red, then blue and finally green colour indicated the presence of sterols.

BIOLOGICAL EVALUATION

Test microorganisms

Standard strains were procured from the American Type Culture Collection (ATCC) and Gene Bank, Institute of Microbial Technology, Chandigarh, India. Two Gram-positive bacterial strains *Streptococcus aureus* (ATCC 9144) and *Bacillus subtilis* (ATCC 6633) and two Gram-negative bacterial strains *Pseudomonas aeruginosa* (ATCC 25668), *Escherichia coli* (ATCC 2091) were used for antibacterial activity and fungal strains *Candida albicans* (ATCC 2091), *Aspergillus niger* (ATCC 6275) and *Aspergillus fumigatus* (ATCC 13073) were used for antifungal activity.

Cup-diffusion method

Disc-diffusion method[11] was used for the evaluation of microbial sensitivity of the extracts using readymade Hi media, SDA (Sabouraud Dextrose Agar) media by boiling to dissolve 37 gms of Hi media SDA in 100 ml of water for antifungal activity. The Muller-Hinton Agar (MHA) was used for antibacterial activity

The medium is then transferred to conical flask and was plugged with non-absorbent cotton and then sterilized by autoclaving. This liquid medium when hot was poured into Petri dishes and solidified; culture broth was added and spread evenly with the help of sterile cotton swabs. Now filter paper discs (8mm) were dipped in specific amount (25, 50, 75, 100mg/mL) of extracts and then incubated. After incubation the petri dishes were observed for growth inhibition zone around the discs. The diameter of zone of inhibition is directly proportional to antimicrobial activity of the compound. The diameter of zone of inhibition was compared with that of standard drugs.

Minimum inhibitory concentration (MIC) [12]

The i*n-vitro* antibacterial and antifungal activity for extracts was evaluated using the conventional serial dilution method. Twofold serial dilutions of the extracts and reference drugs (Fluconazole and Ceftriaxone) were prepared in SDA and MHA media respectively. Drugs were dissolved in Dimethyl formamide (DMF) used as solvent and the solution was diluted with water. Further progressive double dilution with melted SDA/MHA was performed to obtain the required concentrations in µg/mL. The bacterial and fungal innocula were prepared by suspending 24 h old bacterial and fungal colonies from MHA/SDA media in 0.85 % saline. The innocula was adjusted to 0.5 Mc-Farland Standard (1.5×10^8 CFU/mL).[13] The suspensions were then diluted in 0.85% saline to give 10^7 CFU/mL. The tubes were spot-inoculated with 1 µg of each of the prepared bacterial and fungal suspensions (10^4 CFU /spot) and incubated at 37^0C for 24h. At the end of the incubation period, the MIC was determined, which is the lowest concentration of the test compound that resulted in no visible growth in the tube. A control test was also performed with test medium supplemented with solvent at the same dilutions as used in the experiment in order to ensure that the solvent had no influence on bacterial and fungal growth.

RESULTS AND DISCUSSION

Morphological characters of the fruits, percentage yield and preliminary phytochemical investigations of aqueous and chloroform extracts of LA fruits are as shown in Table 1 and 2 respectively.

The aqueous extract of LA fruits was revealed that the presence of carbohydrates due to violet ring formation of furfural derivatives, this condenses with α-napthol to form coloured products. From the analysis it suggests that carbohydrates are reducing in nature, aldehydes of sugars reduce copper sulphate in alkaline solution to cuprous oxide (Cu_2O) and in the process get oxidized to acids. Further glycon and aglycon portion of glycoside in extracts was hydrolysed with 10% sulphuric acid, which resulted in the formation of red colour ppt with Fehling’s test thus revealing the presence of glycosides. Both aqueous and chloroform extracts of LA fruits showed red colour with acetic anhydride indicates sterols presence due to probably esterification of the hydroxyl group of sterols in the extracts as well as other rearrangements in the molecule by the addition of acetic anhydride. Whereas the both aqueous and chloroform extracts of LA revealed negative results for proteins and amino acids, phenolic compounds/flavonoids, alkaloids tannins and carbohydrates negative in chloroform extract.

Table 1
Physical characteristics of L. acutangula var amara fruits

Source	Value (%)			Yield (%)	
	Loss on Drying	Ash values		Extracts	
		Total ash	Acid insoluble ash	Aqueous extract	Chloroform extract
L. acutangula var *amara* fruits	12.10	10.30	2.10	15	5

Table 2
Phytochemical analysis of L. acutangula var amara fruit extracts

Test	Aqueous extract	Chloroform extract
Carbohydrates	+	_
Proteins & amino acids	–	–
Phenolic compounds/ Flavonoids	_	_
Tannins	_	_
Glycosides	+	_
Alkaloids	_	_
Sterols	+	+

+ indicates presence, - indicates absence

Antibacterial sensitivity
The results of antibacterial sensitivity of solvent extracts of *L. acutangula* var *amara* fruits by disc diffusion method are as in depicted in Table 2. The results reveal that extracts are potent antimicrobials against all the bacterial organisms studied. The antibacterial activity was screened from the zone of inhibition and minimum inhibitory concentration.

Among extracts studied, chloroform extract showed higher degree of inhibition followed by the aqueous extract. The diameter of inhibition zones for each of the samples were compared with (positive control) standard antibiotic (ceftriaxone 1 mg/mL). In negative control has not shown any inhibitory effect. Highest antibacterial activity (15mm) was against *Streptococcus aureus*, *Bacillus subtilis* (gram negative) and *Pseudomonas aeruginosa*, *Escherichia coli* (gram negative) in chloroform extract at concentration of 25 mg/ml, when compared with standard drug ceftriaxone 1 mg/mL. An aqueous extract found to be moderate antibacterial activity compared with chloroform extract. We observed that as the concentration increases of both extracts, the antibacterial activity is also increases. The chloroform extract showed moderate at antifungal activity (10 mm) against *Candida albicans* at concentration of 50 and 75 mg/mL, when compared with standard drug fluconazole 1 mg/mL and the aqueous extract was showed moderate to weak antifungal activity.

In MIC study the chloroform extracts showed potent antimicrobial activity against *Streptococcus aureus, Bacillus subtilis* at concentration of 64 μg/mL and *Pseudomonas aeruginosa*, *Escherichia coli* at concentration of 32 μg/mL, it revealed that chloroform extract showed more antibacterial against gram negative bacteria when compared with

standard drug ceftriaxone 0.5 µg/mL. In case of aqueous extracts the antibacterial showed against gram negative bacteria *Pseudomonas aeruginosa*, *Escherichia coli* at concentration of 64 µg/mL. Form the results we predict that chloroform extract showed more antibacterial activity than aqueous extract. The results are tabulated in Table 3.

Table 3
Zone of inhibition of L. acutangula var amara fruit extracts

Name of extracts	Concen tration (mg/mL)	Microorganism and average zone of inhibition(mm)						
		Sa[a]	Bs[a]	Pa[b]	Ec[b]	Ca[c]	An[c]	Af[c]
Chloroform	25	15	15	15	15	08	08	08
	50	15	18	18	18	10	08	08
	75	18	20	20	18	10	08	08
Aqueous	25	10	10	15	10	08	(-)	(-)
	50	12	15	15	10	08	08	08
	75	15	15	15	10	08	08	08
Ceftriaxone	1	25	25	25	25	ND	ND	ND
Fluconazole	1	ND	ND	ND	ND	30	30	30

(-): Resistance; Disc diameter=8mm.
[a]The screening organisms. Gram-positive bacteria: Streptococcus aureus (Sa), Bacillus subtilis (Bs).
[b]The screening organisms. Gram-nagative bacteria: Pseudomonas aeruginosa (Pa), Escherichia coli (Ec).
[c]The screening fungal organisms: Candida albicans (Ca), Aspergillus niger (An), Aspergillus fumigatus (Af), ND: Not determined.

Table 4.
Minimum inhibitory concentration of L. acutangula var amara fruit extracts

Name of extracts	MIC values µg/mL			
	Streptococcus aureus	*Bacillus subtilis*	*Pseudomonas aeruginosa*	*Escherichia coli*
Chloroform	64	64	32	32
Aqueous	(-)	(-)	64	64
Ceftriaxone	0.5	0.5	0.5	0.5

(-): Resistance

CONCLUSION

The chloroform and aqueous extracts of *Luffa acutangula* var *amara* fruits showed good yield and chloroform extracts was found to possess antimicrobial activity. Further phytochemical investigation documented the rich source of sterols compounds, glycosides and other important secondary metabolites which attract any investigator to exploit the plants for screening for pharmacological activities.

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