ANTIOXIDANT AND ANTIMICROBIAL ACTIVITY OF LUFFA ACUTANGULA FRUIT EXTRACT BASED ON TOTAL PHENOLIC CONTENT AS INDEX

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ABSTRACT

Luffa acutangula (L.) Roxb. (Cucurbitaceae) has been receiving ample of attention in Ayurveda, none of its therapeutic potentials were scientifically evaluated except few. Antioxidant activity of the extracts was evaluated by TPC estimation and DPPH free radical scavenging method. Antimicrobial potency of the extracts was evaluated by well diffusion method followed MIC & MMC. The obtained results were correlated with TPC of the extracts; hypothesizing that TPC may be the main active principle for its activity. Fruit aqueous extract was shown higher TPC and DPPH scavenging activity (243.17 ± 8.11mgGAE/g and IC-50: 163.42 ± 5.2, respectively) than methanolic extract (FME, 203.1 ± 9.22 mg GAE/g and IC-50: 253.8 ± 5.1, respectively). Plot between TPC of the extracts versus antioxidant activity showed a strong positive correlation indicating the role of TPC in the antioxidant activity of the extracts. In antimicrobial studies, FAE showed higher antimicrobial activity (IZD: 20.1 ± 2.1mm for *P. aeruginosa*) than the FME (IZD: 17.03 ± 1.9 mm for *B. cereus*). MIC and MMC were also observed to be very low for FAE than FME. TPC of the extract did not show good correlation with IZD indicating the TPC marginal role in antimicrobial activity of the extract.

KEY WORDS: Diphenylpicrylhydrazyl, Minimum Inhibitory Concentration (MIC), Minimum Microbicidal Concentration (MMC).

INTRODUCTION

Reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), organo peroxide, superoxide anion ($O_2^{\cdot-}$) and hydroxyl radical ($^{\cdot}OH$) etc. are generated by aerobic metabolism in biological system and also by exogenous sources such as drugs, ultra violet light, ionizing radiation and pollution. Under normal conditions, generated ROS are neutralized by inbuilt and default antioxidant enzymes present in the body such as catalase, superoxide dismutase, glutathione peroxidase etc. (Herbette,2007; McCord and Fridovich,1969). Non-enzymatic antioxidant molecules such as ascorbic acid, glutathione and uric acid also play a key role in detoxifying the free radicals (Marian Valko,2007). Increased oxygen flux conditions (i.e.exercise) or failure of antioxidant mechanism leads to over production of free radicals that may exceed system capacity to remove them. This situation ultimately culminates into damage of macromolecules such as proteins, lipids and nucleic acids followed by oxidative stress (Ji, 2007).

Oxidative stress, the consequence of imbalance of pro oxidants and antioxidants in the organism, is gaining recognition as a key phenomenon in the etiology of several age related and chronic diseases such as cancer, diabetes, neurodegenerative and cardiovascular diseases (Kourounakis,1999). On the other side, ROS also plays beneficial role at physiological concentrations in various metabolic processes (Allen and Tresini, 2000). Normal levels of antioxidant mechanisms are insufficient for the eradication of free radical induced injury. Therefore administration of antioxidants from natural/synthetic origin has a promising role to play. Many investigations indicate that phenolic constituents, such as flavonoids, phenolic acids, diterpenes and tannins are worthy of notice due to great value in preventing the onset and/or progression of many human diseases (Rice and Miller,1996; Srinivas Reddy,2008).

Infectious diseases remain the main cause of high mortality rates recorded in developing countries and widespread use of antimicrobials is associated with the emergence of antimicrobial resistant pathogens (Okusa,2007). This alarming situation has been leading to search for new compounds that can act either by direct antimicrobial activity or by inhibiting resistance mechanisms of microorganisms. Medicinal plants represent a valuable source for this kind of compounds. Though, *Luffa acutangula* has been receiving ample of attention in the Ayurveda medicine for the treatment of diabetes, wound healing and inflammation, none of its therapeutic potentials are scientifically evaluated except robisome inactivating activity (Wang,2002).

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Hence, the present study was undertaken to evaluate the antioxidant and antimicrobial properties of the *L. acutangula* fruit extract.

The results obtained from this study helps food industry researchers in understanding the relationship between natural phytochemicals and their roles as antioxidants and antimicrobials, which plays key role in preserving the food materials. This study also provides a scientific data for herbal medicine practitioners to prove the therapeutic potentials of *Luffa acutangula.*

2. MATERIALS AND METHODS

2.1 Fruit material

Fruit material was collected from the forest at the foot of seven hills, Tirupati, Andhra Pradesh, India and was authenticated by Prof. Madhava Chetty, Department of Botany, Sri Venkateswara University, India.

2.2 Chemicals and reagents

Methanol was obtained from Merck Ltd. (Mumbai, India.). Gallic acid, Ascorbic acid, Folin-ciocalteu reagent (FCR), phenol red, diphenylpicrylhydrazyl (DPPH) and Tris buffer were procured from Sigma Chemicals and Nutrient agar was obtained from Himedia (Mumbai, India).

2.3 Microorganisms

Bacterial strains (*Bacillus cereus, Bacillus cerculences, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pnemoneae,)* and fungal cultures (*Candida albicans, Saccharomyces cerviceae, Aspergillus fumigatus, Candida tropicana, Candida krusei*) procured from Microbial Type Culture Collection (MTCC), India were used.

2.4 Extraction procedure

Around 300 g of fresh fruit material was washed with tap water, air dried and chopped into small peaces and subjected to drying under shade. The dried material was coarse powdered with mortar and pestle. The powdered material was successively extracted three times with hexane, to remove wax, methanol and water at room temperature in a cycle of 48 hours each. The combined methanolic extracts were then concentrated in a rotavapour (Heidolf, Germany) at reduced pressure below 40°C. Aqueous extract was dried by lyophilization.

2.5 HPTLC Fingerprinting

HPTLC Fingerprinting: chromatography was performed on 5X10cm HPTLC plates coated with 0.25mm layer of silica gel 60F254 (Merck, Darmstadt, Germany). Before using, the plates were washed with methanol and activated at 110°C for 5min. Samples were applied as 4mm wide bands and 6mm apart by using a Camag (Muttenz, Switzerland) Linomat IV sample applicator equipped with a 100µl syringe. A constant application rate of 6µl/sec was used. Mobile phase for stem bark methanolic extract was chloroform:methanol:water (5:2:0.5) and chromatograms were monitored at 366nm.

2.6 Total phenolic content (TPC)

Total phenolic content (TPC) of the fruit extract was analyzed by the Folin–Ciocalteu colorimetric method using gallic acid as standard (Srinivas Reddy,2007). Each reaction mixture contained 100 µl of standard gallic acid solution or 100 µl sample solution, 6 ml distilled-deionized water, 500 µl FCR and 1.5 ml of Na_2CO_3 (20 g/100 ml). The reagent blank was performed by replacing the gallic acid solution with 100 µl of water. After 2 h of reaction at ambient temperature, the absorbance of each reaction mixture was measured at 765 nm by a UV-Vis spectrophotometer. TPC was expressed as mg gallic acid equivalents (mg GAE) per gram of dry mass of fruit extract.

2.7 DPPH free radical scavenging activity

The DPPH free radical scavenging activity was measured using a method described previously (Blois,1958). All the stock extracts were prepared in DMSO at a concentration of 1 mg/ml and were serially two-fold diluted including the standard (ascorbic acid). DPPH was prepared freshly in absolute alcohol at a concentration of 4.9 mg/25 ml. The reaction mixture consisted of 125 µl of DPPH, 100 µl of freshly prepared 0.5 mM tris buffer (pH 7.2) and 25 µl test extract in plate (96 wells). The plate was incubated at room temperature for 10 min and absorbance was measured at 517 nm. The percentage of free radical scavenging activity was determined as depicted in Equation 1.

Radical Scavenging (%) = [(Control " Sample)/ Control] × 100 .... Eq 1

2.8 TPC versus antioxidant activity

Ranges of gallic acid equivalents (GAE) of the fruit extract from 2.34 to 150 µg GAE were subjected to DPPH free radical scavenging activity. Results were plotted against TPC versus antioxidant activity and their correlation was evaluated.

2.9 Antimicrobial activity

Sensitivity test was performed on *Bacillus cereus, Bacillus cerculences, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pnemoneae, Candida albicans, Saccharomyces cerviceae, Aspergillus fumigatus, Candida tropicana* and *Candida krusei* by the method described elsewhere (Reddy,2008). The dried fruit extract was dissolved in both solvents (methanol and water) to a final concentration of 30 mg/ml and filtered by 0.45 ìm Millipore membrane filters (Bedford, USA). Culture media was prepared by dissolving 40 g of nutrient agar in one liter of distilled-deionized water and autoclaved at 121°C (15 psi) for 15 min. 20 ml of agar was dispersed on a petridish and was allowed to solidify. Each petridish was divided into 4 sectors and in each sector a 4 mm bore was made. From the stock solution (30 mg/ml), 50 µl of different test extract or standards was loaded (30 µg/bore) in a separate bore. Thereafter, 10 µl of inoculam was applied on to the top of petridish and incubated for 24 h at 37°C for bacteria and 28°C for fungi. The results of the antimicrobial sensitivity assay of fruit extract in different solvents were expressed by measuring their corresponding inhibitory diameter zones (IZD). Chloramphenicol and Amphotericin B were used as standards for bacteria and fungus, respectively, at a concentration of 30 ìg/bore.

2.10 Minimal Inhibitory Concentration (MIC) assay

Minimal Inhibitory Concentration (MIC) assay was performed in nutrient broth supplemented with 10% glucose containing 0.05% of phenol red (NBGP) as per method described by Zgoda and co-workers (2001). All the test extracts including standard antibiotics were initially dissolved in DMSO (5 mg/200 µl). The solution obtained was added to NBGP to a final concentration of 5000 µg/ml for each crude extract which were serially diluted by two fold to obtain a concentration range of 5000 to1.22 µg/ml. 100 µl of each concentration was added to a well (96-wells microplate) containing 95 µl of NBGP and 5 µl of standard inoculums (the appropriate inoculum size for standard MIC is $2x10^4$ to 10^5 CFU/ml). The final concentration of DMSO in the well was less than 1%. Negative control wells consisted of 193 µl of NBGP, 2 µl of DMSO and 5 µl of the standard inoculum. The plates were covered with a sterile plate sealer, and agitated to mix the content of the wells using a plate shaker and incubated at 37^0C for 24 h. The assay was repeated twice and the microbial growth was determined by observing the change in colour in the wells (red when there is no growth and yellow when there is growth). Lowest concentration showing no colour change was considered as the MIC.

2.11 Minimum microbicidal concentration (MMC)

For the determination of minimum microbicidal concentration (MMC), a portion of liquid (5 µl) from each well that had shown no change in colour was plated on the agar plate and incubated at 37^0C for 24 h. The lowest concentration that yielded no growth after this sub-culture was taken as the MMC.

2.12 TPC versus antimicrobial activity

A range of total phenolic content (15-500 µg) of the extract was subjected to antimicrobial activity. The results obtained were plotted with TPC versus antimicrobial activity and their correlation was monitored.

2.13 Statistical Analysis

All experimental values were expressed as mean ± S.D. Statistical analysis were performed by Student's t-test at a probability of $p<0.05$ using MS Excel Software.

3. Results

3.1 Extraction yield

The percentage extraction yield of methanol and aqueous extract of *L. acutangula* fruit was 15.02 % and 9.98 %, respectively.

3.2 HPTLC Fingerprinting

Fruit methanolic extract (FME) gave 4 spots at the following Rf values: 0.05, 0.18, 0.41, and 0.80. The corresponding HPTLC chromatograms are presented in Fig.1.

3.3 Total phenolic content (TPC)

Total phenolic content of the extracts was expressed as mg GAE/g dry weight of fruit extract (gallic acid equivalents) in which, FAE showed greater amount of GAE (243.17±8.11mg GAE/g) compared to FME (203.1±9.22 mg GAE/g).

3.4 DPPH free radical scavenging activity

FAE showed higher DPPH free radical scavenging activity than the FME. Both the extracts were initially tested at their stock concentration (3 mg/ml) for antioxidant potency and the extracts were further serial diluted to derive their IC-50 values. The IC-50 values for FAE, FME and ascorbic were found to be 163.42 ± 5.23, 253.84 ± 10.32 and 36.02 ± 2.03 µg/ml, respectively (Fig 2).

3.5 TPC versus antioxidant activity

The concentration of the TPC that required scavenging 50% (IC-50) of the DPPH free radical for FAE and FME was found to be 30.89±3.01 µg GAE and 33.6±2.3 µg GAE, respectively (Fig 3).

3.6 Antimicrobial activity

In microbial growth inhibitory studies, aqueous extract (FAE) showed higher antimicrobial activity than the methanolic extract (FME) indicated by forming clear inhibitory zones. FAE was found to be more effective towards the microbes *P. aeruginosa* and *C. Tropicana,* FME was found to be more effective towards the microbe *B. cereus.* Both the extracts were less active than the standard drugs Chloramphenicol and Amphotericin B (Table1).

3.7 MIC and MMC determination

MIC and MMC values for different microbes including standard drugs are depicted in Table 2. The MIC values for different microbes ranged from 78 to 625 µg/ml and 156 to 1250 µg/ml for FAE and FME respectively. MMC values for different microbes ranged from 78-1250 and 156-5000 for FAE and FME respectively. *P. aeruginosa* strain was found to be more sensitive for FAE and B. *cereus* strain was found to be more sensitive for FME .

3.8 Antimicrobial activity versus TPC

Since P. aeruginosa and B. cereus were the most sensitive bacteria for FAE and FME respectively, these bacterial strains were chosen to evaluate the correlation between the TPC of the extracts and its antimicrobial activity. Ranges of TPC (15-500 µg/ml) of both the extracts were subjected to antimicrobial activity in agar plates and the inhibitory zones were calculated. The correlation between the TPC and antimicrobial activity was calculated and presented in the Figure 4. At 500 µg GAE, FAE and FME have shown 22.3 ± 2.13 and 18.2 ± 2.01 mm IZD respectively.

DISCUSSION

The present study was designed to evaluate TPC, antioxidant activity and antimicrobial activity of the *L. acutangula* fruit extract. Both the extracts of *L. acutangula* fruit (FAE and FME) showed significant TPC. It was reported in the previous investigations that the antioxidant properties of phenolic and related compounds were due to their capacity to scavenge free radicals generated in the aqueous phase and to increase the resistance of lipids against peroxidation (Rice and Miller,1996). Hence the present study was undertaken to evaluate the antioxidant activity. Assay for scavenging of DPPH radical is commonly used to determine the antioxidant activities for a relative convenience compared to other methods. Many reports showed that effect of antioxidants on DPPH radical scavenging is due to their hydrogen donating ability (Kim,2003). In the present study, FAE and FME showed significant DPPH free radical scavenging activity, indicating that *L. acutangula* is a potent free radical terminator which may be through hydrogen donating property.

Based on the results obtained it can be hypothesized that TPC of the extract may be the main reason for its observed antioxidant activity. In order to prove this hypothesis, a range of the TPC equivalents from the both extracts were subjected to antioxidant activity. As shown in Figure 3, IC-50 values for both the extracts were similar though their antioxidant potencies differed based on the dry weight of the extracts. A correlation was drawn between the TPC of the extracts and its antioxidant activity (Fig 3). It was found that there was a significant correlation between antioxidant activity and TPC of the extracts (FAE:R^2= 0.976 and FME: R^2 = 0.984). Hence, it is clear that antioxidant activity of the *L. acutangula* could be mainly due to its TPC.

In addition to antioxidant activity, an attempt was made to evaluate the antimicrobial activity of the FAE and FME in which FAE showed strong antimicrobial activity than the FME. Initially, FAE and FME were tested at fixed concentrations for antimicrobial activity and both showed significant antimicrobial activity but the real extent of their inhibitory activities against the test organisms could be well understood only by comparing the MIC and MMC values obtained. FAE showed lower MIC and MMC values than the FME indicating that FAE might be more potent antimicrobial agent than FME.

A correlation was drawn between the TPC and antimicrobial activity of the extract, expecting that TPC may be the constitute for its antimicrobial activity. But the results showed very low correlation ($p<0.05$) between them. Though, equal amount of TPC equivalents were taken from both the extracts to evaluate their antimicrobial activity, they showed different potencies. Hence, it can be concluded that TPC is not the key request for the antimicrobial activity of the extract.

4. CONCLUSION

Aqueous and methanolic extract *of L. acutangula* fruit showed to possesses rich concentration of total phenol. Both the extract has shown good antioxidant activity which may be mainly attributed to its phenolic content. The total phenolic content of the both extract alone was not influences the antimicrobial activity of *L. acutangula*. Since, extracts were showing significant antioxidant and antimicrobial activity, their role in food industry as preservators can be tested.

Fig.1. HPTLC chromatogram of Fruit methanolic extract was evaluated on mobile phase chloroform: methanol:water at 5:2:0.5 ratio and the chromatogram was monitored at 366 nm.

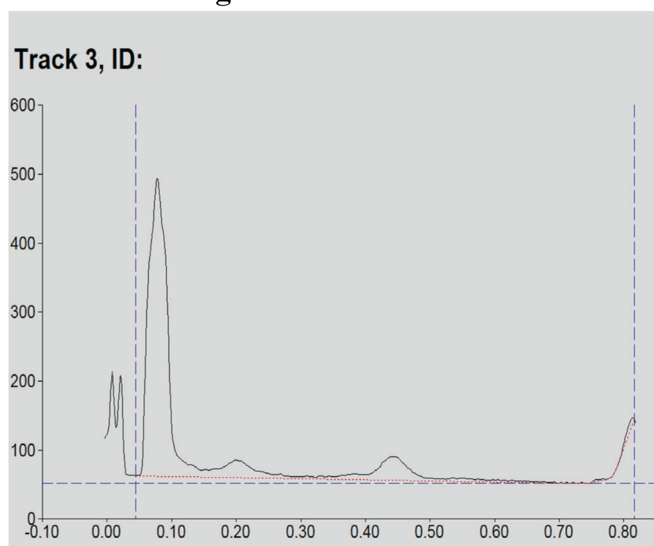

Fig.2. Antioxidant activity of the *L. acutangula* fruit methanolic and aqueous extracts (FME and FAE, respectively) were evaluated by DPPH free radical scavenging activity. Values are Mean ± SD. Ascorbic acid was used as positive control.

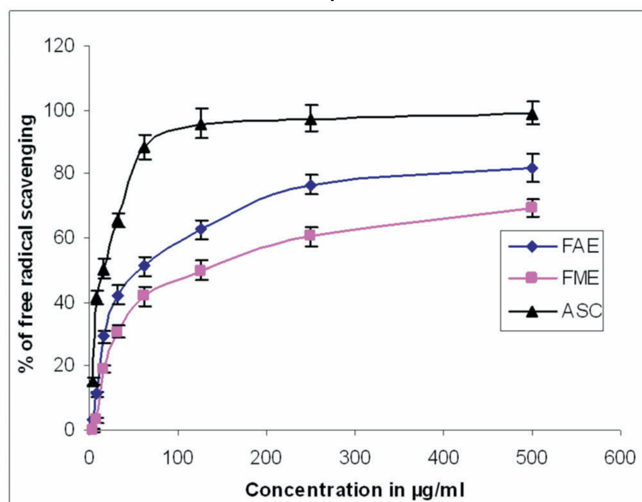

Fig.3. A range of TPC equivalents from the *L. acutangula* fruit methanolic and aqueous extracts (FME and FAE, respectively) were subjected to DPPH free radical scavenging activity. A correlation (R^2) was drawn between TPC and antioxidant activity

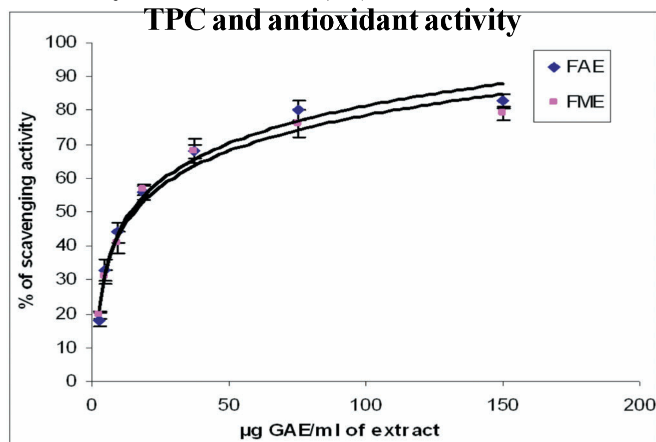

Fig.4 A range of TPC equivalents from the *L. acutangula* fruit methanolic and aqueous extracts (FME and FAE, respectively) were subjected to antimicrobial activity and a correlation was drawn between them.

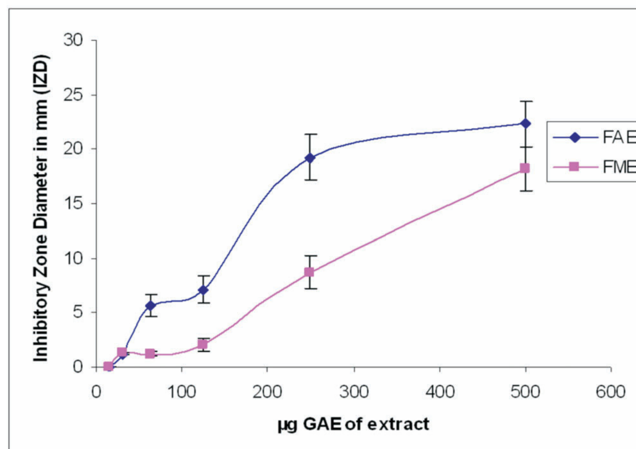

Table.1 Methanolic and aqueous extracts of *L. acutangula* were evaluated for antimicrobial activity by agar hole diffusion method. Values are Mean ± SD.

S. No	Microbe	Inhibitory Zone Diameter (mm)		
		FAE	FME	Standard
1	*B. cerculences*	11.2 ± 2.3	10.1 ± 2.1	19.2 ± 1.56
2	*P. aeruginosa*	20.1 ± 2.1	14.13 ± 2.2	15.23 ± 3.12
3	*E. coli*	13.8 ± 2.0	-	16.53 ± 3.21
4	*B. cereus*	12.6 ± 2.2	17.03 ± 1.9	20.15 ± 2.13
5	*K. pnemoneae*	18.2 ± 2.9	13.03 ± 2.1	21.56 ± 3.22
6	*S. areues*	10.3 ± 3.0	8.16 ± 2.3	18.02 ± 1.09
7	*A. fumigatus*	12.3 ± 2.2	10.2 ± 2.23	16.23 ± 2.10
8	*S. cerviceae*	16.5 ± 1.6	-	15.22 ± 2.06
9	*C. krusei*	15.8 ± 2.7	11.32 ± 1.8	17.02 ± 3.12
10	*C. albicans*	12.5 ± 2.1	9.2 ± 1.09	17.22 ± 2.03
11	*C. tropicana*	19.0 ± 3.0	6.19 ± 1.5	16.21 ± 2.2

Table 2. MIC and MMC values for different microbes against fruit extracts were evaluated in 96 well plate. Values are Mean ± SD.

S. No	Microbe	MIC (µg/ml)			MMC (µg/ml)		
		FAE	FME	Stan.	FAE	FME	Stan.
1	*B. cerculences*	312	1250	19	312	1250	39
2	*P. aeruginosa*	78	312	39	78	312	78
3	*E. coli*	156	-	39	156	-	39
4	*B. cereus*	312	156	19	312	156	39
5	*Klebsiella pnemoneae*	156	312	19	312	625	19
6	*S. areues*	625	1250	39	1250	5000	78
7	*A. fumigatus*	625	1250	78	625	1250	78
8	*S. cerviceae*	312	-	78	625	-	78
9	*C. krusei*	156	625	39	312	1250	78
10	*C. albicans*	625	1250	78	625	1250	78
11	*C. tropicana*	156	1250	39	312	> 5000	39

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