ANTI-MICROBIAL, ANTI-OXIDANT & PHYTOCHEMICAL ANALYSIS oF *Citrus aurantium* (ORANGE) LEAF EXTRACT

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ABSTRACT

The present investigation was carried out to evaluate anti-microbial, anti-oxidant and phytochemical compound properties of *citrus aurantium* leaves. Plants have been the major original source of many drugs used in the treatment of diseases today. Antimicrobial, Anti-oxidative systems and the contents of phytochemical compounds were investigated in leaves of sour orange trees that are grown under atmospheric co2 concentrations. Phytochemical compounds which are extracted with methanol, ethanol, acetone and distilled water with different medicinal properties. The plant extracts were subjected to preliminary phytochemical screening for the presence of alkaloids, carbohydrates, glycosides, tannins, phenolic compounds, proteins, free amino acids, saponins, phytosterols, and flavonoids. The anti-microbial activity was carried out with two gram positive bacteria i.e., *Staphylococcus aureus, Bacillus subtilis* and two gram negative bacteria i.e., *Escherichia coli, Salmonella typhi* by agar well diffusion method. The anti-oxidant properties of these plant extracts of various concentrations (50,150,250 µg/ml) was determined by using methods like DPPH radical scavenging, hydroxyl radical scavenging, superoxide anion, hydrogen peroxide and nitrous oxide the results are compared with known standards. The plant extracts will inhibit free radicals at all concentrations tested. A systematic investigation is carried out to evaluate the total phenolics and flavonoid to explore relation with anti-oxidant properties.

Keywords: Citrus aurantium, anti-microbial activity,anti-oxidant activity, phytochemical analysis.

INTRODUCTION

The use of medicinal plants as a source for relief from illness can be traced back over five millennia (Thomson,W.A.R., (1978, stockwell, C., 1988). The main flavonoids found in citrus species are hesperidine, narirutin, naringin and eriocitrin (Mouly et al., 1994; Schieber et al., 2001). Epidemiological studies on dietary Citrus flavonoids improved a reduction in risk of coronary heart disease (Di Majo et al., 2005; Hertog et al., 1993) and are attracting more and more attention not only due to their antioxidant properties, but as anti-carcinogenic and anti-inflammatory agents because of their lipid anti-peroxidation effects (Stavric, 1993; Elangovan et al., 1994; Martın et al., 2002). The interest in these classes of compounds is due

to their pharmacological activity as radical scavengers (Cotelle et al., 1996). Several studies have demonstrated the antibacterial and/or antioxidant properties of these plants, mainly using in vitro assays. Moreover, some researchers reported that there is a relationship between the chemical structures of the most abundant compounds in the plants and their above mentioned functional properties (Dean and Svoboda, 1989; Farag et al., 1989). In addition, Citrus byproducts also represent a rich source of naturally occurring flavonoids (Horowitz, 1961).

MATERIALS AND METHODS

Collection of plant material

The plant material was collected from Botany Department, Osmania University and voucher specimen was deposited in the Herbarium of the Botanical Garden of Department of Botany (Osmania University).

Preparation of plant extracts

Fresh leaves of *citrus aurantium*, collected and washed thoroughly 2-3 times under running tap water and then with sterile water followed by shade-dried at room temperature for 1 week, grinded into uniform powder and used for extraction. 30gof the powder is mixed with 120ml of solvents in a 250ml conical flask and was kept at 25º c for 12h separately. The suspension was filtered through a Whatman no.4 filter paper. The organic solvents (Chloroform, ethanol, and methanol and petroleum ether) and aqueous extracts were concentrated by a rotary evaporator, while aqueous extract was dried using water bath. Finally, the extracted powder was resuspended in the respective solvents at a concentration of 1 mg/ml before it was tested for the phytochemical, anti-oxidant and antibacterial activity.

Test microorganisms

The four bacterial strains used in present study were collected. The bacteria used are *Bacillus subtilis*, *Escherichia coli*, *Salmonella typhi* and *Staphylococcus aureous***.**

Preparation of bacterial suspension

The screening of antimicrobial activity was carried out by Agar well diffusion method. The test organisms were sub cultured on LB broth. Take 1gm of LB broth and dissolve in 10ml of distilled water in a test tube and autoclaved. Then add 10µl of bacterial culture to the broth in laminar air flow and stored at 4˚C in refrigerator to maintain the stock.

Preliminary phytochemical analysis of Citrus aurantium leaves

Phytochemical screening of plant extracts was done following the standard procedures,

a) **Phytochemical screening:** In every phytochemical analysis, ethyl alcohol is mainly used as standard to identify various residues in the extract.

b) **Procedure for alkaloids:** To identify the presence of alkaloids in the extract 2ml of extract is taken and to that 2ml of wagner's reagent is added. A brownish precipitation formation is observed. Thus it indicates the presence of alkaloids.

c) **Cardiac glycosides:** To test the presence of glycosides, 2ml of extract is dissolved with 2ml of chloroform then carefully add concentrated sulphuric acid to form a layer. Deep reddish brown colour at the interface of steroid ring indicates the presence of cardiac glycosides.

d) **Flavonoids:** To know the presence of Flavonoids in the seeds, 2ml of extract is added to 2ml of 10% lead acetate. Yellowish green colour indicates the presence of flavonoids.

e) **Saponins:** For this, 2ml of extract is dissolved with 2ml of benedicts reagent. Blue black ppt indicates the presence of saponins.

f) **Tanins:** To know the presence of tanins, 2ml of extract is treated with 0.1% of Ferric chloride. Brownish green layer indicates the presence of tannins.

g) **Terpenoids: (salkowski test):** To identify the presence of terpenoids, 2ml of extract is dissolved with 2ml of chloroform and concentrated sulphuric acid is carefully added to form a layer. A reddish brown colour is observed which indicates the presence of terpenoids.

h) **Anthraquinones:** To test the presence of anthraquinones in fenugreek seed extract, 1ml of extract is boiled with 10%HCL for few minutes in boiling water bath.Then it is filtered and allowed to cool.Equal volume of CHCL3 is added to the filterate and few drops of 10% Ammonia is added to the mixture and heat.A rose pin colour is found which indicates the presence of anthraquinones.

i) **Reducing sugars**: The extract was shaken with distilled water and filtered. The filterate is boiled with Fehling's solution A and B for few minutes an orange red ppt indicates the presence of reducing sugars.

j) **Glycosides:** To identify this, extract is hydrolysed with HCL solution and neutralized with NAOH solution. Few drops of Fehling's solution A and B are added, Red ppt indicates the presence of glycosides.

k) **Phlobatanins**: The test the presence of Phlobatanins,the extract is dissolved in distilled water and filtered.The filterate is boiled with 2% HCL solution.Red precipitate shows the presence of phlobatanins.

Table 1, shows preliminary phytochemical screening of Citrus aurantium leaves

Sl. No	Phytochemicals	Distilled Water	Methanol	Acetone	Ethanol
1	Tanins	Positive	Positive	Positive	Positive
2	Anthraquinones	Positive	Positive	Negative	Positive
3	Flavanoides	Positive	Positive	Positive	Positive
4	Alkaloides	Positive	Positive	Positive	Positive
5	Terpenoids	Positive	Positive	Positive	Positive
6	Saponins	Negative	Negative	Positive	Positive

7	**Cardiac glycosides**	**Positive**	**Positive**	**Positive**	**Positive**
8	**Glycosides**	**Negative**	**Negative**	**Positive**	**Negative**
9	**Reducing Sugars**	**Positive**	**Positive**	**Positive**	**Positive**
10	**Phlobatanins**	**Negative**	**Negative**	**Negative**	**Negative**
11	**Steroids**	**Positive**	**Positive**	**Positive**	**Positive**
12	**Phenolic**	**Positive**	**Positive**	**Positive**	**Positive**
13	**Aminoacids**	**Negative**	**Negative**	**Negative**	**Negative**
14	**Proteins**	**Negative**	**Negative**	**Negative**	**Negative**
15	**Quinones**	**Positive**	**Positive**	**Positive**	**Positive**

ANTIMICROBIAL ACTIVITY

Antimicrobial activity of *C.aurantium* extracts were evaluated by the agar well diffusion method. It is a modified method of Murray et al. Antimicrobial susceptibility was tested on solid media in petri plates. For bacterial assay nutrient agar (NA) (40 gm/L) was used for developing surface colony growth. The minimum bactericidal concentration (MBC) values were determined by serial micro dilution assay. The suspension culture, for bacterial cells growth was done by preparing 2% Luria Broth (w/v), all the media prepared was then sterilized by autoclaving the media at (121°C) for 20 min. Wells (10mm diameter and about 2 cm a part) were made in each of these plates using sterile cork borer. Stock solution of each plant extract was prepared at a concentration of 1 mg/ml in different plant extracts viz. Methanol, Ethanol, Petroleum Ether, and Water. About 100 µl of different concentrations of plant solvent extracts were added by sterile pipette into the wells and allowed to diffuse at room temperature for 2hrs. Control experiments (Positive and negative) comprising inoculums without plant extract were set up. The plates were incubated at 37°C for 18-24 h for bacterial pathogens. The diameter of the inhibition zone (mm) was measured and the activity index was also calculated. Triplicates were maintained and the experiment was repeated thrice, for each replicates the readings were taken in three different fixed directions and the average values were recorded. The minimum inhibitory concentration is defined as the lowest.

In one set of petri plates, on first disc, methanol is added and it is called negative control. On second disc, *C.aurantium extract* sample is added and it is called test sample. On third disc, Ampicillin antibiotic is added and it is called positive control.

Table .2: Effect of plant extract on Bacterial culture

Bacterial culture		Negative control(DMSO)	Positive control(Antibiotic)	Plant extract
Organism	Concentration			
E.coli	10µl	0	21	11

	20µl	0	19	10
	30µl	0	18	13
Salmonella typhi	10 µl	0	19	12
	20 µl	0	17	11
	30 µl	0	17	14
Bacillus subtilis	10 µl	0	19	10
	20 µl	0	18	14
	30 µl	0	19	14
Staphylococcus aureus	10 µl	0	18	11
	20 µl	0	20	12
	30 µl	0	20	13

ANTI-OXIDANT ACTIVITY

DPPH method

Plants were measured by 1, 1- diphenyl-2-picryl hydroxyl (DPPH). In brief, 0.1 mm solution of DPPH in methanol was prepared. The plant extract at various concentrations (10-100) to each test tube with 3ml of DPPH solution was added and wrap the tubes with aluminium foil. The mixture was shaken vigorously and allowed to stand at room temp for 30 min. Incubate 15 minutes in dark room. UV absorbance was recorded at 517nm.

TBA method

Extracts (2 ml) and standard solutions were added to 1 ml of 20% aqueous trichloroacetic acid and 2 ml of 0.67% aqueous thiobarbituric acid. After boiling for 10 min, the samples were cooled. The tubes were centrifuged at 3,000 rpm for 30 min. Absorbance of the supernatant was evaluated at 532 nm in a spectrophotometer. The antioxidant activity was calculated by percentage of inhibition in this method as follows: % Inhibition = 100 – [(A1 – A0) ×100] Where A0 is the absorbance of the control and A1 is the absorbance of the sample extracts (Elmastas et al, 2007)8. The plants extracts at various concentrations (10-100) to each test tube and add 1ml of plant extract to each test tube. To this add 2ml of TBA and 1ml

of distilled water and incubate in bath water bath for 30 minutes, Absorbance was recorded at 760nm.

TCA method

The plant extract at various concentrations (10-100) to each test tube and Add plant extract methanol to each tube, Both TBA and TCA were added to each flask. Then the tubes are kept in water bath for 10 minutes at 100˚C and cool. Centrifuge at 10000 rpm for 20 minutes and supernatant in fresh tubes Absorbance was recorded at 775nm.

FRAP method

This method measures the ability of antioxidants to reduce ferric iron. It is based on the reduction of the complex of ferric iron and 2, 3, 5-triphenyl-1, 3, 4-triaza-2-azoniacyclopenta-1, 4- diene chloride (TPTZ) to the ferrous form at low PH. This reduction is monitored by measuring the change in absorption at 593 nm, using a diode-array spectrophotometer. Three millilitre of prepared FRAP reagent is mixed with 100 ml of diluted sample; the absorbance at 593 nm is recorded after a 30 min incubation at 37 C. FRAP values can be obtained by comparing the absorption change in the test mixture with those obtained from increasing concentrations of Fe3+ and expressed as mM of Fe2+ equivalents per kg (solid food) or per L (beverages) of sample. The plant extract at various concentrations (10-100) to each test tube 2.5ml of 0.2M phosphate buffer was taken in an each test tube. To this add 2.5ml of 1%potassium Ferro cyanide. Then 1ml of plant extract was added, reaction mixture was incubated for 20 minutes at 50˚C.Then 2.5ml of TCA was added and centrifuge at 3000 RPM for 10 minutes. Collect 2.5ml of supernatant, 2.5ml of distilled water and 0.5ml of Fecl3 was added. UV absorbance was recorded at 770nm.

Table 3: Anti-oxidant activity of methanolic extract *Citrus aurantium*

Concentration(µl)	*O.D/(770nm)*
10µl	0.060
20µl	0.110
30µl	0.106
40µl	0.140
50µl	0.100

Table 4: Anti-oxidant activity methanolic extract of *Citrus aurantium*

Concentration(µl)	*O.D/(775nm)*
20µl	0.010
40µl	0.060
60µl	0.80
80µl	0.120
100µl	0.140

RESULTS AND DISCUSSION

Phytochemical screening

By the results of phytochemical screening we can conclude that *Citrus aurantium* leaf extract gives positive result in compounds with methanol, ethanol, acetone and distilled water but there is absence of amino acids, proteins and phlobatanins.

Antimicrobial Activity

The petri plates were observed for zones of inhibition after incubating over night with plant compound. *Invitro* antimicrobial activity of methanol extracts of *citrus aurantium* leaves were showed significant zone of inhibition was evaluated against Gram positive (*Bacillus subtilis*) and Gram negative (*Escherichia coli, Salmonella typhi*) bacteria. The antibacterial activities of the extracts increased linearly with increase in concentration of extracts (µg/ml). As compared with standard drugs, the results revealed that in the extracts for antibacterial activity the growth inhibition zone measured ranged from 11 to 20 mm for all the sensitive bacteria.

Antioxidant Activity

Four different methods to evaluate the antioxidant activity (DPPH, FRAP, TBA,TCA) *Acalypha indica* leaves extract can be considered good sources of natural compounds with significant antioxidant activity, It is very difficult to assess the antioxidant activity of a product on the basis of a single method. Antioxidant activity assessment may require a combination of different methods, and the results obtained in this study confirm the difficulty of comparing the results of the many different methods used to test antioxidant activities. The correct estimation of the antioxidant activity of a given essential oil requires the evaluation of its optimal concentration. On the other hand, the differences found in the different methodologies may, to a certain extent, be explained by the relative amounts of minor compounds in the oils, which may play a major role in the final oil antioxidant effect. The

antioxidant power measured depends on the chosen method, the concentration and the nature and physicochemical properties of the studied antioxidant.

DISCUSSION

The powdered whole plant of *Citrus aurantium* was individually extracted with different solvents. The colours of the extracts were noted. The preliminary phytochemical screening of the extracts *C.aurantium* showed the presence of alkaloids, glycosides, phenols, tannins, saponins and Steroids. Preliminary organic analysis of drugs helps to undertake further studies on the isolation and identification of specific chemical constituents. Due to the presence of different phytochemical compounds of *C.aurantium* leaf extracts has efficient anti-microbial activity against different micro-organisms (E. coli, Streptococcus, *Salmonella typhi, Bacillus subtilis).*Antimicrobial properties of medicinal plants are being increasingly reported from different parts of the world. The World Health Organization estimates that plant extract or their active constituents are used as folk medicine in traditional therapies of 80% of the world's population. In the present work, the extracts obtained from *sour orange* show strong activity against most of the tested bacterial strains. The results were compared with standard antibiotic drugs. The leaves methanolic extract of this plant showed better antioxidant potential when compare standard ascorbic acid by DPPH scavenging assay method. The absorbance at 517nm by UV visible spectrophotometer. It means methanol extract of plant at higher concentration captured more free radicals formed by DPPH resulting into decrease in absorbance and increase in IC 50 value. The above results show that the activity of methanol extracts of *citrus aurantium* shows significant antibacterial activities. This study also shows the presence of different phytochemicals with biological activity that can be of valuable therapeutic index. The result of phytochemicals in the present investigation showed that the plant contains more or less same components like saponins, triterpenoids, steroids, glycosides, anthraquinone, flavonoids. Results show that plant rich in tannin and Phenolic compounds have been shown to possess antimicrobial activities against a number of microorganisms.

CONCLUSION

The present results will form the basis for selection of plant species for further investigation in the potential discovery of new natural bioactive compounds. Further studies which aimed at the isolation and structure elucidation of antibacterial active constituents from the plant have been initiated.The results obtained using Four different methods to evaluate the antioxidant activity (DPPH, FRAP, TBA,TCA) leaves extract can be considered good sources of natural compounds with significant antioxidant activity.

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