GC/MS analysis, antioxidant and antibacterial activities of essential oil extracted from Citrus paradisii macfad fruit peels.

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Abstract

The present study was concerned with analysis of the chemical constituents of essential oil of *Citrus paradisii* macfad fruit peels by GC-MS technique and also its antioxidant and antibacterial activities against some pathogenic bacteria. The essential oil was extracted from fruit peels by steam distillation using Clevenger's type apparatus. GC-MS analysis of essential oil showed presence of limonene (90%), Valenence (2.18%), Eicosene (2.01%), Tetrahydrolinalool (2.02%), α-Cadinene (1.425%), Nonanol (1.31%) and Dodecanol (1.06%) as the major constituents. The Antioxidant activity of essential oil was determined by using DPPH free radical scavenging and reducing power methods. The essential oil reduced the concentration of DPPH free radical with efficiency near to that of standard Gallic acid and the $IC_{50}$of essential oil was 1.15 mg/ml. In the reducing power assay the antioxidant activity increased with the increase in concentration and the activity of essential oil was more than that of standard antioxidant Gallic acid. The antibacterial activity was detected by agar well diffusion method against *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphyloceceus aureus*. The zones of inhibitions obtained were recorded and analyzed against standard control of Ampicillin. The essential oil showed higher antibacterial activity of 24.0 mm against *E. coli*and least antibacterial activity of 14 mm against *P. aeruginosa*. The MIC of the essential oil on the susceptible bacterial isolates was between 128-256µg/ml and MBC was between 256-512µg/ml. Present study concludes that essential oil from fruit peels of *Citrus paradisii*have potent antioxidant role and also have a broad spectrum antibacterial activity against human pathogens.

Keywords: Essential oil, GC/MS, Antioxidant, IC50, zone of inhibition, MIC, MBC.

Introduction

In our daily practice we see that fruits, flowers, leaves, stems, barks and roots of nearly all the plants have some pleasant smell. It has been observed that this pleasant smell of the fruits is actually due to the presence of certain steam volatile oils known as essentials oils. Essential oils are defined internationally as the products obtained by hydro distillation, steam distillation, dry distillation or by a suitable mechanical process without heating (for citrus fruits) of a plant or some part of it[1].

The essential oils are complex mixtures of hydrocarbons and their oxygenated derivatives, though some such as oil of bitter almond and oil of wintergreen consist mainly of one constituent, viz. benzaldehyde and methyl salicylate respectively [2].

The genus Citrus, belonging to the Rutaceae or Rue family, comprises about 140 *Citrus limon* (Lemon), *Citrus reticulate* (tangerine), *Citrus grandis* (shaddock), *Citrus aurantium* (sour orange), *Citrus medica* (Citron) and *Citrus aurantifolia* (lime) are some important fruits of genus Citrus [3]. Citrus fruits have numerous therapeutic properties like anticancer, antiviral, anti-tumor, anti- inflammatory activities and effects on capillary fragility as well as ability to inhibit platelet aggregation. *Citrus paradisii macfad*(*grapefruit*) is an important member of Citrus genus.

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It has been used as a folk medicine in many countries as antibacterial, antifungal, anti inflammatory, antimicrobial, antioxidant, antiviral agent and it act as astringent, and preservative also. It also has been used for cancer prevention, cellular regeneration, lowering cholesterol, cleansing, detoxification, heart health, and arthritis and weight loss[4].

Citrus peel essential oils are reported to be one of the rich sources of bioactive compounds namely coumarins, flavonoids, carotenes, terpenes and linalool etc [5]. Recently, Citrus peel essential oils have also been searched for their natural antioxidant and antimicrobial properties [6, 7]. It is widely accepted that biological activities of plant materials are strongly linked with their specific chemical composition, mainly the secondary metabolites such as plant phenolics and flavonoids [8]. The safety of synthetic antioxidants, such as butylatedhydroxyanisole (BHA) and butylatedhydroxytoluene (BHT) are now in doubted. Thus, attentions are now increasingly paid to the development and utilization of more effective and non-toxic antioxidants of natural's origin. A great number of natural medicinal plants have been tested for their antioxidant and results have shown that the raw extracts or isolated pure compounds from them were more effective antioxidants *in vitro* than BHT or vitamin E [9]. Increase in the emergence of new bacterial strains that are multi-resistant coupled with the non-availability and the high cost of new generation antibiotics have resulted in increase morbidity and mortality[10, 11]. Keeping in view the significance of Citrus essential oils, the present investigation aimed at study of chemical composition of essential oil of *Citrus*

paradisii macfad fruit peels and evaluation of its antioxidant and antibacterial activities against some pathogenic bacteria

Materials and Methods

Plant Material:

The fruits of *Citrus paradisii*macfadwere collected from the Horticulture fields of SHIATS, Allahabad in the month of January, 2014. The plant material was identified and authenticated in the post graduate department of Horticulture, SHIATS, Allahabad.

Extraction of essential oil:

The sample of fresh and ambient dried Citrus peels was subjected to hydro distillation for 3h using a Clevenger type apparatus. Distillates of essential oil were dried over anhydrous sodium sulphate, filtered and stored at -40C until analyzed [12].

GC-MS analysis of essential oil:

The essential oils were analyzed by GC/MS according to [13]. GC/MS analysis was performed on a Thermo quest Finnegan Trace. GC/MS was equipped with column (60mx0.25mm) with film thickness 0.25µm. The injections temperature was 2000C and oven temperature was raised from 600C. (5min hold) to 2600C (10 min hold) at a rate of 40C/min. transfer line temperature was 2600C. 1µml of sample was injected and helium was used as the carrier gas at a rate of 1.0 ml/min. the mass spectrometer was scanned over the 40 to 60 m/z with an ionizing voltage of 70eV and identification was based on standard mass to detected possibilities of essential oils components.

Antioxidant Assay

DPPH Free Radical Scavenging Method;

The DPPHradical scavenging activity assay elucidated by [14]was followed with slight alterations. The hydrogen atom or electron donating ability of the corresponding essential oil was measured from the bleaching of the purple colored methanol solution of DPPH. This spectrophotometric assay uses the stable radical, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), as a reagent. The different concentrations of extracted essential oil were used. BHT and gallic acid were used as standard in 0.2-1.0 mg/ml solution. DPPH (0.002%) was prepared in methanol and to 1 ml of DPPH solution; 1 ml of sample (essential oil) solution was added. The solution mixture was kept in dark for 30 min and absorbance was measured at 517 nm. DPPH solution (1 ml) was used as blank. The absorbance was recorded and inhibition was calculated using the formula given below.

Calculation:

$$\% \text{ scavenging activity} = \frac{A - B}{A} \times 100$$

Where; A= absorbance of the control and

B= absorbance of the sample

IC_{50}= value, which is represented as the concentration of sample that caused 50% scavenging as calculated from the plot of inhibition percentage vs concentration.

Reducing power method[15, 16]

Different concentrations each of essential oil were taken separately (in different test tubes) in 1 ml distilled water and added in 0.2 M phosphate buffer (2.5 ml, pH 6.6) and 1% potassium ferricyanide (2.5 ml). The mixtures were incubated at 50^0C for 20 min and 10% TCA (2.5 ml) was added to each mixtures. All the whole mixtures were centrifuged for 10 min and then 2.5 ml of the supernatant was transferred into the different test tubes containing distilled water (2.5 ml) and 0.1% ferric chloride (0.5 ml) was added to each test tube. After shaking, mixtures were left at room temperature for 5 min and then absorbance was read at 700 nm. Methanol was used as blank. Increased absorbance of the reaction mixtures indicates increase in reducing powder. The reducing power of different samples was compared with their concentration vs absorption plot.

Antibacterial activity assay:

Test organisms:

The organisms used comprises of two gram-positive bacteria (*S. aureus* and *B. subtilis*) and two gram-negative bacteria (*E. coli* and *P. aeruginosa*). The test organisms were obtained from the Research Laboratory of Microbiology and Fermentation Technology SHIATS, Allahabad, India.

Agar well diffusion method:

Agar well diffusion method elucidated by [17, 18] with modifications was followed. The antibacterial activity of essential oil of *Citrus pardisii*macfadfruit peels against four pathogenic bacteria was evaluated by using agar well diffusion method. The Nutrient agar plates were prepared by pouring 15 ml of molten media into sterile petri-plates. About (108-109) colony- forming unit per ml were used. Wells or cups of 5mm size were made with sterile borer into agar plates containing the bacterial inoculum. 10µL of microbial broth was spread on the surface of nutrient agar plates; 20µL volume of the essential oil of density (2.0mg/ml) measured by weight: volume ratio was poured into a well of inoculated plates. Ampicillin (2mg/ml) was used as a positive control which was introduced into the well instead of essential oil.

Solvent DMSO was used as a negative control which was introduced into well instead of essential oil. The plates thus prepared were kept at room temperature for ten minutes allowing the diffusion of the extract into the agar. After incubation for 24 hrs at 37^oC, the plates were observed. The antibacterial activity was present on the plates; it was indicated by an inhibition zone surrounding the well containing the plant extract. The zone of inhibition was measured and expressed in millimeters. Antibacterial activity was recorded when radius of zone of inhibition was greater than 4 mm [19]. The antibacterial activity results was considered as inactive if < 4.5 mm; 4.5-6 mm as

partially active ; while 6.5-9 mm as active and greater than 9mm as very active [20].

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

MIC and MBC of essential oil from fruit peels was also determined [21, 22]. Dilution of the essential oil of *Citrus paradisii* fruit peels was prepared in sterile nutrient broth to achieve a decreasing concentration ranging from 1000µg/ml to 12µg/ml in sterile tubes labeled 1 to 5. Each dilution was seeded with 10µl of the standardized bacterial visible growth when compared with the control was considered as the MBC. Each trial was repeated thrice and compared with control tubes.

inoculums (108-109) CFU/ml. The inoculated culture tubes were incubated at 37°C for 24hrs. A set of tubes containing only seed broth (*i.e.* without essential oil) was kept as control. The lowest dilution of the essential oil that retained its inhibitory effect resulting in no growth (absence of turbidity) of a microorganism was recorded as the MIC value of the extract. After incubation, 10µl of content of each test tube was transferred with a loop on to nutrient agar media. Agar plates were incubated for 24 hours at 37°C. The lower concentration that did not permit any

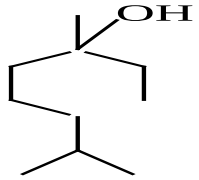
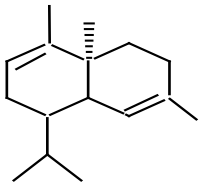
Statistical analysis

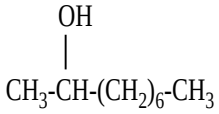
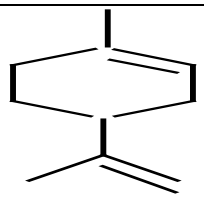
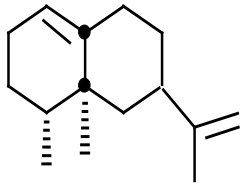
The data recorded during the course of investigation was subjected to statistical analysis by "Analysis of variance technique" [23]. The significant and non-significant treatment effects were judged with the help of 'F' (variance ratio) table. The significant differences between the means were tested against the critical difference at 5% probability level.

Results & Discussion

The components present in the essential oil were analyzed by GC/MS using the retention time of the components. The GC/MS analysis of the essential oil shows the following compounds (**Table 1**) along with their structure and percentage. It shows that there are seven major components present in the essential oil viz; limonene (90%), Valenence (2.18%), Eicosene (2.01%), Tetrahydrolinalool (2.02%), α-Cadinene (1.425%), Nonanol (1.31%) and Dodecanol (1.06%).

Table 1: GC/MS analysis of the essential oil of *Citrus paradisii* macfad

S.No.	COMPOUNDS	RETENTION TIME (min.)	MAIN BASE PEAKS (%)	STRUCTURES
1	Dodecanol	29.1	114,111,97,83,70,56(BP),43	CH_3-$(CH_2)_{11}$-OH
2	Eicosene (E)	31.089	282,280,221,207,168,140,125,111,97,83(BP),69,55,43	CH_3-$(CH_2)_{18}$-CH_3
3	Tetrahydro Linalool	36.469	158,146,96,73(BP),57,44,	
4	α-cadinene	29.444	204,161,119,105(BP),91,81,55,41	

5	Nanonaol	24.143	144,140,111,97,83,70,56,45(BP)	
6	Limonene	26.223	136,121,107,93,79,68(BP),53,44	
7	Valenence	32.099	204,189,161,148,119,105(BP),91,79,69,45	

Antioxidant Activity

The DPPH free radical scavenging activity of essential oil of *Citrus paradisii* macfad fruit peelsat various concentrations was determined and compared with that of the standard BHT and Gallic acid **(Table 2 and Fig. 1).** Five different working solutions of essential oil were used having concentration (1.0, 0.8, 0.6, 0.4 and 0.2 mg/ml). All the tested samples showed lower DPPH radical scavenging activities (59.36%, 43.31%, 31.46%, 18.21% and 13.12%) respectively when compared with the standards. The essential oil reduced the concentration of DPPH free radical with efficiency near to that of Gallic acid but less than BHT. The IC_{50} value for essential oil was 1.15mg/ml.

Table 2: DPPH free radical scavenging (%) ofessential oil of *Citrus paradisii* macfad, gallic acid and BHT.

Conc. (mg/ml)	Absorbance of essential oil at 517nm	% Scavenging activity	Absorbance of Gallic acid at 517nm	% Scavenging activity	Absorbance of BHT at 517nm	% Scavenging activity
1.0	0.347±0.011	56.07	0.319±0.007	59.36	0.234±0.018	66.42
0.8	0.445±0.017	43.31	0.470±0.007	49.02	0.265±0.006	61.97
0.6	0.538±0.027	31.46	0.532±0.028	42.29	0.332±0.011	52.36
0.4	0.642±0.044	18.21	0.554±0.037	39.91	0.365±0.007	47.63
0.2	0.682±0.034	13.12	0.445±0.042	36.15	0.578±0.029	37.31

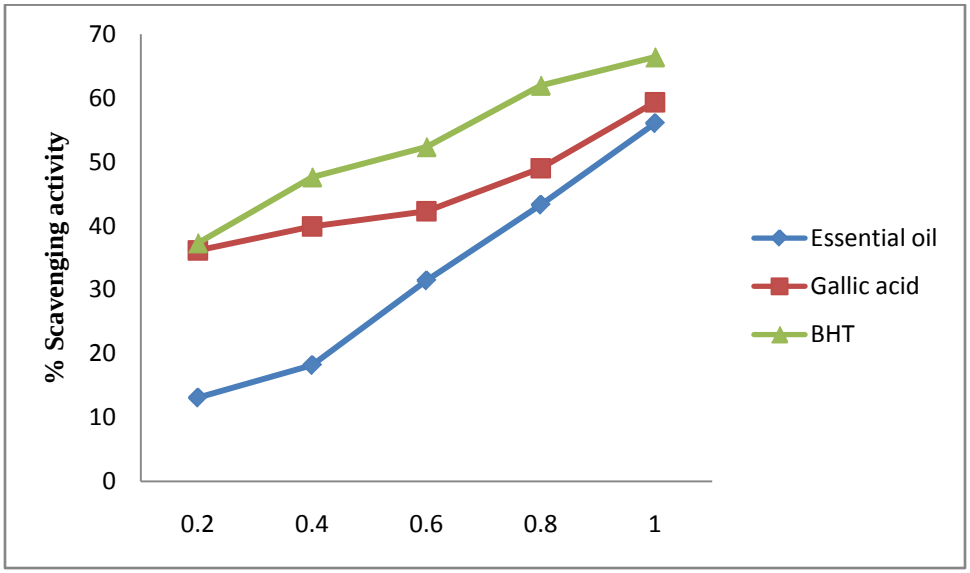


Fig.1: DPPH scavenging activity for BHT, gallic acid and essential oil of *Citrus paradisii* macfad.

Another method to evaluate antioxidant ability was based on the reduction of Fe^{3+} to Fe^{2+} in which the yellow colour of the test solution changes to various shades of green and blue, depending on the reducing power of each sample. The presence of reducing agent causes the conversion of ferricyanide complex to the ferrous form that may be followed at 700 nm due to the formation of Perl's Prussian blue $Fe[Fe_4(CN)_6]_3$. Increasing absorbance at 700 nm indicates an increase in reductive ability.

The reducing power of the essential oil was determined and the results obtained are presented in **table 3**. Reducing power of test samples was found more significant than the standard gallic acid but less as compared to the BHT. It was observed that as the concentration essential oil increases (0.125, 0.25, 0.5 and 1.0mg/ml) the absorbance of samples increased gradually **(Fig. 2)**.

Table 3:Reducing Power Activity of Essential oil of *Citrus paradisii* macfad, Gallic Acid and BHT

Conc (mg/ml)	Absorbance of Essential oil at 700nm	Absorbance of Gallic Acid at 700nm	Absorbance of BHT at 700nm
0.125	0.686±0.005	0.480±0.017	1.080±0.128
0.25	0.703±0.012	0.528±0.003	1.190±0.065
0.50	0.768±0.038	0.588±0.017	1.274±0.051
1.0	0.811±0.026	0.687±0.009	1.365±0.190

According to[24] antioxidant action of reductants was based on breaking of the free radical chain by donating a hydrogen atom; the presence of reductants that was antioxidants in *Citrus paradisii* macfad essential oil makes possible the reduction of Fe^{3+} ferricyanide complex to ferrous form. Therefore, Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm.

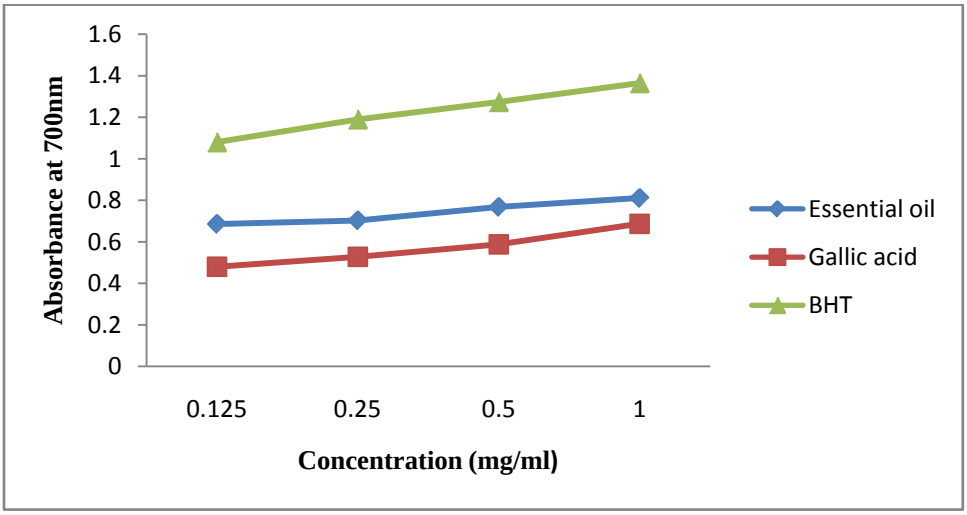
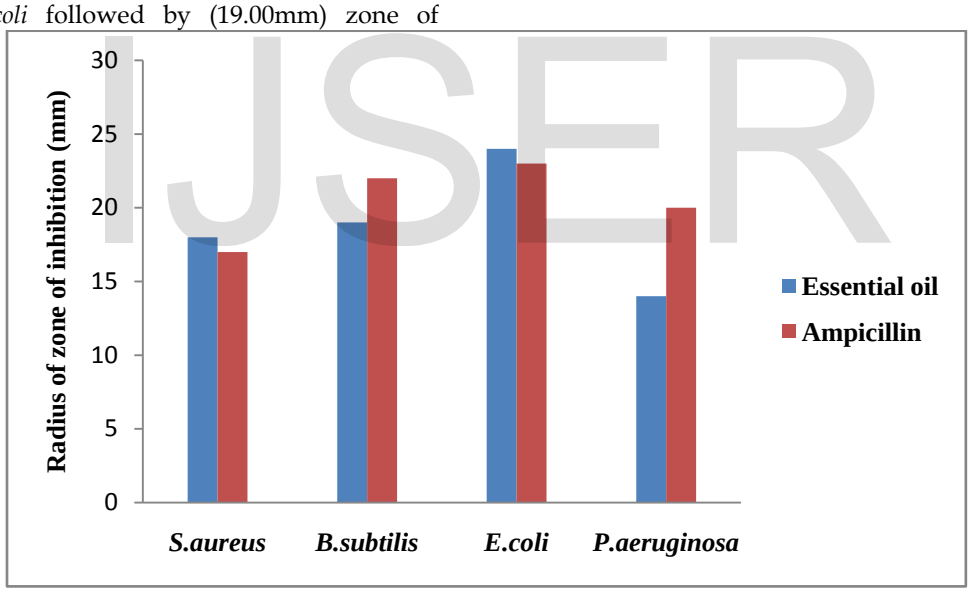


Fig. 2: Reducing power activity of *Citrus paradisii* essential oil and standards (gallic acid and BHT).

Antibacterial activity:

The antibacterial activity reveals that theessential oil of *Citrus paradisii* macfad is highly active against both Gram positive and Gram negative bacteria. The essential oil of *Citrus paradisii* macfad shows highest zone of inhibition (24mm) against *E.coli* followed by (19.00mm) zone of inhibition against *B.subtilis*, (18mm**)** against *S.aureus* and lowest (14mm) zone of inhibition against *P.aeruginosa.* Graph 1 summarizes the microbial growth of methanolic extract and ampicillin used as a positive control.



Graph 1: Antibacterial activity (in mm) of essential oil of *Citrus paradisii* macfad and ampicillin.

Ampicillin used as a positive control showed wide zones of inhibition against all the test organisms while dimethyl sulphoxide (DMSO) negative control shows no zones of inhibition. It was completely resistant to all the test organisms. The MIC which is the lowest concentration of a essential oil that still retained an inhibitory effect against the growth of a microorganism and MBC which is the lower concentration that did not permit any visible growth was both assessed by using broth dilution method. The MIC values of essential oil of *Citrus paradisii* macfadfruit peels for different pathogenic bacteria were ranged from 128-256µg/ml and MBC was between 256-512µg/ml (**Table 4**).

Table 4: MIC and MBC of *Citrus paradisii* macfadessential oil against different bacteria

Conc (µg/ml)	*E.coli*	*S.aureus*	*P.aeruginosa*	*B.subtilis*
1024	**-**	**-**	**-**	**-**

512	**-**	**-**	**-**	**#**
256	**#**	**#**	**#**	*****
128	*****	*****	*****	**+**
64	**+**	**+**	**+**	**+**
32	**+**	**+**	**+**	**+**

(+) Growth, (-) No growth, (*) MIC and (#) MBC

Conclusion:

On the basis of results obtained it can be concluded that essential oil of *Citrus paradisii* macfad possess terpenoids and flavonoids mostly polyphenols and have potent antioxidant and antibacterial activities. Further the potential of this plant can be explored more and more, in order to develop an alternative therapy for treatment of various diseases. The present study also suggests that the use of this medicinal plant may be exploited for health supplements. Thus justifying its traditional use.

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