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# *In vitro* Evaluation of Biological Activities of Citrus Leaf Extracts

(Penilaian *in vitro* Aktiviti Biologi Ekstrak Daun Limau)

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ABSTRACT

*Leaves extracts of three different citrus species namely* Citrus sinensis *(Malta),* Citrus paradisi *(Grape fruit) and* Citrus jambhiri *(Khatti) were evaluated for their antioxidant, cytotoxic, antitumor, antimicrobial activities and finally the mineral contents were determined. Four types of extraction solvents (100, 80 and 50% methanol and distilled water heated at 50°C) were used for extraction of antioxidant compounds. Extracts yield increased with the elevated levels of aqueous component of organic solvent and our data showed the highest yield in aqueous extracts. All extraction regimes showed* in vitro *antioxidant activity.* Citrus paradisi *showed the highest total flavonoid content (TFC), total phenolic content (TPC), reducing power and 1,1-Diphenyl-2- picrylhydrazil free radical scavenging activity in all combinations of solvents followed by* Citrus sinensis *and* Citrus jambhiri. *Antioxidant activity was also increased with higher aqueous component of organic solvents in each case. While* Citrus sinensis *(in 100% methanolic solvent) and* Citrus paradisi *(in 50% methanolic extract) showed significant cytotoxicity ($LD_{50}$ values <1000). Antitumor activity was observed in all extracts, however* Citrus sinensis *and* Citrus paradisi *(in 50% aqueous methanolic) extracts had the highest antitumor activity from the selected citrus species whereas no antimicrobial activity was observed at higher concentrations (4000 $\mu gmL^{-1}$) against specified strains. We found high calcium contents in all three citrus species tested by atomic absorption method. The results showed that the leaves of selected Citrus species possess significant antioxidant, antitumor and cytotoxic activities. Citrus leaves extracts can be potentially helpful in antioxidant protection in food as well as in human body against lipid peroxidation and free radicals damage. It can further be evaluated after* in vivo *studies using animal models or identifications of high throughput methods for enhanced biological activities.*

*Keywords: Biological activities; citrus;* in vitro *evaluation; mineral micronutrients*

ABSTRAK

*Ekstrak daun tiga spesies limau berbeza iaitu* Citrus sinensis *(Malta),* Citrus paradisi *(Grape fruit) dan* Citrus jambhiri *(Khatti) telah dinilai untuk aktiviti antioksidan, sitotoksik, antitumor dan agen antimikrob serta menentukan kandungan mineral mereka. Empat jenis pelarut pengekstrakan (100, 80 dan 50% metanol dan air suling yang dipanaskan pada 50°C) telah digunakan untuk pengekstrakan sebatian antioksidan. Hasil ekstrak meningkat dengan peningkatan tahap komponen akueus pelarut organik dan data kami menunjukkan hasil tertinggi dalam ekstrak akueus. Semua rejim pengekstrakan menunjukkan aktiviti antioksidan* in vitro. Citrus paradisi *menunjukkan kandungan flavonoid keseluruhan (TFC), kandungan fenolik keseluruhan (TPC), kuasa penurunan dan aktiviti skaveng radikal bebas 1,1-difenil-2-pikrilhidrazil tertinggi dalam semua kombinasi pelarut diikuti oleh* Citrus sinensis *dan* Citrus jambhiri. *Aktiviti antioksidan juga ditingkatkan dengan peningkatan komponen akueus pelarut organik untuk setiap kes. Manakala* Citrus sinensis *(dalam pelarut metanol 100%) dan* Citrus paradisi *(dalam ekstrak metanol 50%) menunjukkan kesitotoksikan yang signifikan (nilai $LD_{50}$ < 1000). Aktiviti antitumor telah diperhatikan dalam semua ekstrak. Walau bagaimanapun, ekstrak* Citrus sinensis *dan* Citrus paradisi *(dalam akueus metanol 50%) didapati mempunyai aktiviti antitumor tertinggi daripada spesies limau terpilih manakala tiada aktiviti antimikrob telah diperhatikan pada kepekatan yang lebih tinggi (4000 $\mu gmL^{-1}$) terhadap strain khusus. Kami mendapati kandungan kalsium adalah tinggi dalam kesemua spesies limau yang diuji oleh kaedah penyerapan atom. Hasil menunjukkan bahawa daun spesies limau terpilih memiliki aktiviti antioksidan, antitumor dan sitotoksik yang signifikan. Ekstrak daun limau berpotensi membantu dalam perlindungan antioksidan dalam makanan serta dalam badan manusia terhadap peroksidasi lipid dan kerosakan oleh radikal bebas. Ia boleh dinilai selanjutnya berikutan kajian* in vivo *menggunakan model haiwan atau pengecaman kaedah berdaya pemprosesan tinggi untuk aktiviti biologi tertingkat.*

*Kata kunci: Aktiviti biologi; limau; mikronutrien mineral; penilaian* in vitro

## INTRODUCTION

Citrus is a perennial shrubs or tree, belongs to an important genus of plant family Rutaceae and is grown in many countries worldwide. Citrus ranks top in world production and trade among the fruit trees. Pakistan is one of the major producers of citrus in the world and during 2006-2007, it

was cultivated over an area of 192.7 thousand hectares and the production remained at 2459.5 thousand tones (Faostat 2007) in Pakistan. The genus derives its commercial importance from its fruit, which is of great economic and health value. It can be consumed fresh or processed to obtain juice (Talon & Gmitter 2008). Majority of citrus fruits are preferably eaten fresh e.g. oranges, mandarins, grapefruits, clementines and tangerines. Orange and grapefruit produce very palatable juice and hence are used to make nutritious and popular breakfast (Duyn & Pivonka 2000). Bulk of the total production of orange and mandarins is used for juice making. Lemons and limes can be used to make lemonades and pickles and their juices can be added to various food preparations as flavoring agents. Delicious marmalades are made from oranges. Citrus plants have great medicinal values and are useful remedy for lot of diseases e.g. toothache, constipation, diarrhea and vomiting (Singh & Rajam 2009). Citrus fruits are rich source of active compounds and beneficial for human health e.g. vitamin C, carotenoids, flavonoids, limonoids, essential oils, acridone alkaloids, minerals and vitamin B complex. Flavonoids especially polymethoxyflavones, flavanone glycosides and limonoids are natural secondary metabolite compounds of citrus (Ladaniya 2008). Flavonoids and phenolic acids are especially common in leaves, flowering tissues and woody plant parts (Larson 1988). Flavonoids have several health promoting activities including antioxidant, heart protection and anti-allergic, anti-cancerous antiviral, antibacterial and antifungal. Lemon juice has been used in treating common cold and as diuretic, astringent, antiscorbutic and in reducing fever (Manners 2007). Essential oils obtained from citrus leaves are insecticidal in nature (Singh & Rajam 2009). Citrus byproducts has been checked for antioxidant activity (Federica et al. 2011; Khizar et al. 2009). Antioxidants have been investigated in vegetables, fruits, leaves, cereal crops, roots, barks, spices and herbs (Ramarathnam et al. 1997). Antioxidants are the substances that inhibit oxidation of the substrate even when present in low concentration as compared to oxidisable substrate (Ani et al. 2006). These can defend human body from free radicals and postpone the development of several ailments such as cancer, liver injury and several cardiovascular diseases (Zengin et al. 2011). Most of the antioxidants are synthetic, for example butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). These are prohibited by governmental policy because they are carcinogenic when given *in vivo* (Sonia & Mohamed 2008). Plants are the potential source of naturally occurring antioxidants ascorbic acid, benzoic acids, carotenoids, cinnamic acids, flavonoids, folic acid, tocotrienols and tocopherols (Saha et al. 2008). Therefore, a significant attention is paid to find newest and secure antioxidants from natural sources which can substitute these artificial antioxidants (Sonia & Mohamed 2008).

Plants contain thousands of biologically active molecules. For their investigation, it is important to have the necessary tools like suitable biological assays and chemical screening methods (Bijen et al. 2002). Brine shrimp cytotoxicity assay is a rapid general bioassay technique for the natural and synthetic products that tell us about cytotoxicity and wide range of pharmacological activities e.g. anticancer, antiviral and pesticidal activity (Rahman et al. 2006). It is an evident that brine shrimp assay is predictive of cytotoxixity and pesticidal activity (Krishnaraju et al. 2005). Ferrigni et al. (1982) proposed the idea to use antitumor potato disc-assay, the same procedure was modified by Galsky et al. (1980). This assay is very easy, cheap and quick method used to evaluate natural as well as synthetic antitumor agents (Wedge & Camper 1999). The development of possible strategy is required to screen potent plant extracts and products for anticancer activity. The present study aimed at the evaluation of biological activities of citrus leaves due to unavailability of such information of this important plant. This study will provide a cheaper source of biologically active compounds from the unused parts of the citrus plant.

## Materials and Methods

### Collection of plant material and extraction procedure

Fresh leaves of three different species of Rutaceae family *Citrus jambhiri* (Khatti), *Citrus paradisi* (grape fruit) and *Citrus sinensis* (Malta) free from any insect infestation, infection or damage were collected from different locations in Islamabad followed by frequent washes with distilled water. Four different extraction regimes based on types of extraction solvents were used to grind 10 g of leaves sample in 100 mL of extraction solvents. They were 100% methanol (Me 1), methanol 80% (Me 2), methanol 50% (Me 3) and distilled water heated at 50°C in water bath (Dw). After shaking in water bath for 4 h at 100 rpm at room temperature, only samples in distilled water were heated at 50°C while no heat treatment was given to Me 1, Me 2 and Me 3 samples. The leaves extracts were filtered with cheese cloth followed by centrifugation at 4000 rpm for 10 min and then filtered by Whatman filter paper. Filtrates stored in refrigerator were labeled as CLF's (Citrus leave filtrates) for analysis of antioxidant assays.

### Total flavonoid contents (TFC)

Total flavonoid contents of citrus leaves filtrates were determined according to method reported by Biglari et al. (2008) with slight modifications. Fifteen mg/mL sample of each of the filtrate was used with the different dilutions (0.05, 0.1, 0.15 and 0.25 mg/mL) of Rutin (standard). Then 0.75 mL of Sodium nitrite solution and Aluminum (III) chloride solutions (10%) were added, respectively and incubated for 5 min at room temperatures followed by addition of 5 mL of 1 molar sodium hydroxide solution and vigorous shaking until pink colorations. Spectrophotometer was used to measure absorbance at 510 nm.

### TOTAL PHENOLIC CONTENT (TPC)

Total phenolic contents of the citrus leaves filtrates were checked calorimetrically by using Folin-Ciocalteu reagent (FCR) as reported by Singleton et al. (1999). Fifty mg/mL of each sample filtrate was used with different dilutions of Gallic acid (standard) e.g. 0.15, 0.25, 0.75 and 1 mg/mL. Appropriate amount of Folin-Ciocalteu reagent was added and incubated for 1 min at room temperature followed by addition of 5% sodium carbonate solution. The whole reaction mixture was placed at laboratory conditions for 30 min until blue colorations. Spectrophotometer was used to measure absorbance at 760 nm.

### REDUCING POWER

Reducing capacity of citrus leaves filtrates were checked by using method previously reported by Oyaizu (1986). Different dilutions of ascorbic acid (standard) and each filtrate e.g. 10, 20, 40 and 60 mg/mL (Table 1) were mixed with 2.5 mL of 0.2 M sodium phosphate buffer (pH6.6) and 2.5 mL of 1% Potassium-ferricyanide solution and placed in water bath at 50°C for about 20 min. Then 2.5 mL of 10% Tri-chloroacetic acid solution was added followed by centrifugation for 10 min at 3000 rpm. Supernatant was mixed with 0.5 mL 0.1% $FeCl_3$ solution for absorbance of blue-green solution at 700 nm with the help of spectrophotometer.

### DIPHENYL PICRYL HYDRAZIL (DPPH) ASSAY

The effect of filtrates of citrus leaves on DPPH radicals were determined as described by Yi et al. (2008) with slight modifications. Different dilutions of ascorbic acid (standard) and each filtrate e.g. 0.5, 1.0, 1.5 and 2.0 mg/mL (Table 2) were mixed with equal amounts of 0.2 mM DPPH solution in methanol. Reaction mixtures were incubated in darkness for about 30 min at room temperatures to measure absorbance at 517 nm using 0.2 mM DPPH solution as control. Four different blanks were used that contained respective solvents instead of leave extracts. DPPH free radical scavenging ability of citrus leaves filtrates was measured.

### ANTIBACTERIAL ASSAY

Disc diffusion method was assessed to determine antibacterial activity as described by Mahesh and Satish (2008) with slight modifications. Stock solution of concentration 40000 ppm was prepared by dissolving 40 mg of filtrates in 1 mL Dimethylsulfoxide (DMSO). Further dilutions (4000, 400 and 40 ppm) were made by serial dilution. Kanamycin (2 mg/mL in DMSO) was used as positive control and pure DMSO as negative control. Six bacterial strains were used, two were Gram positive that included *Staphylococcus aureus* (ATCC 6538) *and Micrococcus luteus* (ATCC 10240) and four was Gram negative that included *Escherichia coli* (ATCC 15224), *Bordetella bronchiseptica* (ATCC 4617), *Salmonella typhimurium* (ATCC 14028) and *Enterobacteraerogens* (ATCC 13048). Plates containing these bacterial strains were incubated at 37°C overnight and zones of inhibitions were recorded.

### ANTIFUNGAL ASSAY

Disc diffusion method was assessed to determine antifungal activity as described by Mahesh and Satish (2008) with slight modifications. Terbinafine was used as positive control and Dimethylsulfoxide was used as a negative control. Five different fungal strains were used (Mucor species (FCBP 0300), *Aspergillus niger* (FCBP 0198), *Aspergillus flavus* (FCBP 0064), *Aspergillus fumigatus* (FCBP 66) and *Fusarium solani* (FCBP 0291)). Seven days old fungal strains culture was inoculated in

TABLE 1. Dilutions of samples and standard for reducing power assay

| Filtrate volume (mL) | Respective solvents volume (mL) | Total sample volume (mL) | Standard dilutions (µg/mL) |
|---|---|---|---|
| 2.5 | 22.5 | 25 | 50 |
| 5.0 | 20 | 25 | 100 |
| 10 | 15 | 25 | 200 |
| 15 | 10 | 25 | 300 |

TABLE 2. Dilutions of samples and standard for DPPH assay

| Filtrate volume (mL) | Respective solvents volume (mL) | Total sample volume (mL) | Standard dilutions (µg/mL) |
|---|---|---|---|
| 0.125 mL | 24.875 | 25 | 5 |
| 0.25 mL | 24.75 | 25 | 10 |
| 0.375 mL | 24.625 | 25 | 15 |
| 0.5 mL | 24.5 | 25 | 20 |

pre-autoclaved SDA media and poured in separate petri plates and left for solidification. After solidification, filter paper discs were placed on test fungal strain and 5 µL of different concentrations of filtrate were applied and incubated overnight at 28°C for evaluations of zone of inhibition after 72 h of incubation.

### BRINE SHRIMP CYTOTOXICITY ASSAY

The toxicity of dried citrus leave filtrates were determined by brine shrimp cytotoxicity assay as reported by Mclaughlin and Rogers (1998). Different concentrations of Brine shrimp (Artemiasalina) eggs (Ocean Star Inc., USA) solutions including 1000, 100 and 10 ppm were made. A plastic divider of 2 mm with several holes was used to divide the dish to make two unequal size compartments. Eggs (about 25 mg) were placed in large portion that was covered with aluminum foil, while the smaller compartment was placed in front of light. After 24 h hatching of eggs started and phototropic nauplii (brine shrimp larvae) were collected by pipette from the lightened portion. Twenty five µL of each stock solution was taken in the glass vials and solubilized in 2 mL of seawater. Ten shrimps were transferred in each glass vial using pipette and volume was raised up to 5 mL. Three replicates were prepared for each concentration. The nauplii were added by counting visually in pipette stem against a light background. The vials were incubated under light at 25-28°C for 24 h. Survived nauplii were examined with 3× magnification glass. Percentage death was calculated by using Abbott's formula, then $LD_{50}$ calculation by using Finny (1971) software.

### POTATO DISC ANTITUMOR ASSAY

Antitumor assay was performed according to procedure described by Mclaughlin and Rogers (1998). *A. tumefaciens* strains were cultured in LB medium for 48 h and inoculum containing three concentrations of the extracts (40, 400 and 4000 ppm), *Agrobacterium* culture and DMSO was used for the assay. Under aseptic conditions, fresh potatoes were surface sterilized with 1% $HgCl_2$ solution for 4 - 5 min and rinsed three times with distilled autoclaved water. Potato discs of approximately 2 mm height and 8 mm in diameter were cut with the help of cork borer. Ten discs were placed on petri plates containing autoclaved agar medium (1.5%). Fifty µL of above said inoculum was added on the top surface of each potato disc, sealed with parafilm and incubated at 28°C in dark for 21 days. Potato discs were stained with Lugol's solution (10% KI + 5% I2) and timorous discs were examined under dissecting microscope (Figure 4).

### MINERAL MICRONUTRIENTS

Availability of different mineral micronutrients in citrus leaves was determined by perchloric acid digestion method (Allen et al. 1974). Dried powdered leaves (0.5 g) were taken in flasks. Acid solution (6.5 mL) mixed in the following ratio, perchloric acid, sulfuric acid and nitric acid (0.1:1:5) were added, boiled in fume hood on hot plate for complete digestion indicated by white fumes. Few drops of distilled water were added and the mixture was allowed to cool down, then volume raised up to 50 mL with distilled water. Diluted extracts were passed through Whatmann filter paper. Filtrates were collected in labeled plastic bottles and concentrations of the nutrients were determined by Shimadzu AA-670 Atomic absorption spectrophotometer.

## RESULTS AND DISCUSSION

The amount of extracted antioxidant compounds from plants mainly depends upon the efficiency of solvent and methods used for extraction. To check which solvent is more effective for antioxidant extraction from three different citrus species, we used four different extraction solvents and the total yield was calculated (Table 3). Our data has shown a gradual increase in the antioxidants in all three selected citrus species extracts with increase in aqueous component of organic solvent. Maximum yield was observed in distilled water heated at 50°C. This showed that pre-heated distilled water (50°C) can extract maximum number of compounds that are involved in different biological activities of citrus species. This maximum yield in distilled water might be due to combined effect of solvent as well as the elevated temperature.

Flavonoids gained much attention recently because of the high antioxidants and their ability to scavenge free radicals with less toxicity than synthetic antioxidants. TFCs of the filtrate of citrus leaves were determined in 'Me 1', 'Me 2', 'Me 3' and 'Dw' in the comparisons with rutin (standard) as shown in Figure 1. TFCs for *C. paradisi* were 250.5 ± 3.18, 271.6 ± 5.09, 309.4 ± 3.4 and 393.6 ± 5 in 'Me 1', 'Me 2', 'Me 3' and 'Dw' filtrates, respectively. TFC for *C. sinensis* were 214.9 ± 1.27, 227.35 ± 1.62, 270.9 ± 2.68 and 381.8 ± 4 in 'Me 1', 'Me 2', 'Me 3' and 'Dw' filtrates, respectively. TFCs for *C. jambhiri* were 190.9± 0.49, 196.4 ± 0.56, 276.7 ± 4.94 and 323.9 ± 3.74 for 'Me 1', 'Me 2', 'Me 3' and 'Dw' filtrates, respectively.

Polyphenolic compounds are responsible for antioxidant activity of plant materials and are highly effective radical scavengers (Pan et al. 2008). The antioxidant activities of phenolics are due to their redox properties. The phenol moiety (hydroxyl group on aromatic ring) helps them to work as reducing agents, hydrogen donors and singlet oxygen quenchers (Chua et al. 2008). Considering the heterogeneity of natural phenols and the possibility of interference from other readily oxidized substances, several methods including Folin-Ciocalteu, permanganate titration, colorimetric with iron salts and ultraviolet absorbance have been used for total phenol determination. But in most direct comparisons, the Folin-Ciocalteu method has been found preferable and is being used by many researchers. In this method, phenols form a blue colored phosphomolybdic–phosphotungstic–phenol

TABLE 3. Effects of extraction solvents on citrus leaves extracts yield

| Solvent/Combination of solvent | *Citrus jambhiri* | *Citrus sinensis* | *Citrus paradisi* |
|---|---|---|---|
| Methanol | 8% | 7% | 8% |
| 80% Methanol | 11% | 11% | 11.5% |
| 50% Methanol | 13.5% | 13% | 13.6% |
| Distilled water (50°C) | 16% | 15% | 15.9% |

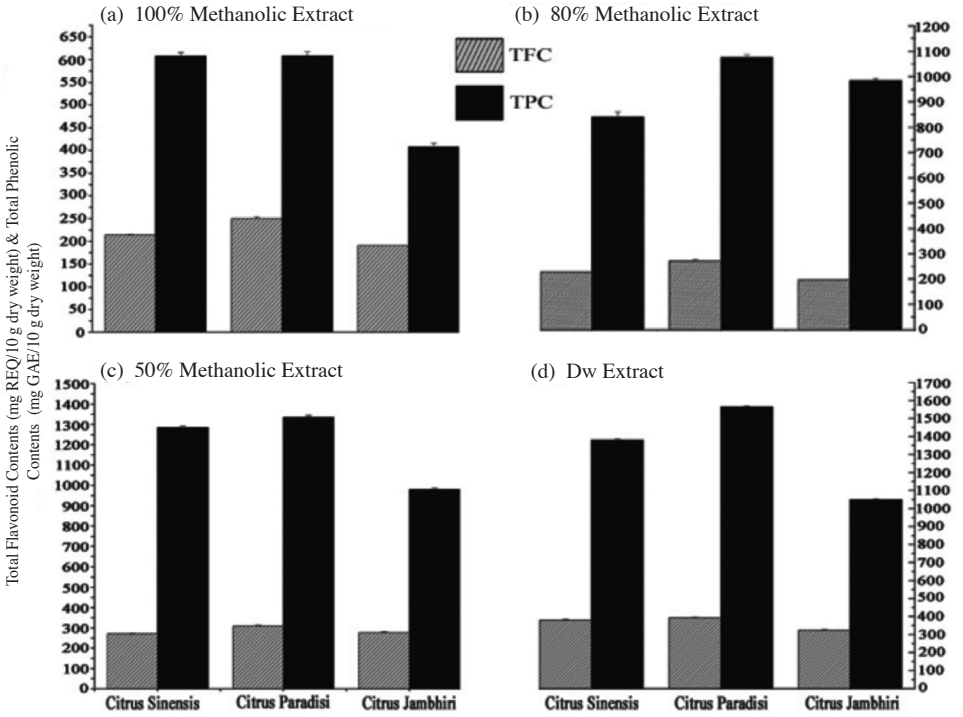


FIGURE 1. Total flavonoid (TFC) and phenolic (TPC) contents of citrus leaves extracts obtained with different extraction solvents. (a) 100% methanolic extract (Me 1), (b) 80% methanolic extract (Me 2), (c) 50% methanolic extract (Me 3) and (d) Distilled water extract (Dw). (REQ; Rutin equivalent; GAE; Gallic acid equivalent)

complex in alkaline solution (Singleton et al. 1999). In this research, the TPC was determined in comparison with standard gallic acid and the results were shown in terms of mg GAE/g dry extract. TPC of *C. paradisi* were 608.4 ± 9.05, 1075.6 ± 8.9, 1335.8 ± 8.83 and 1564.4 ± 6.92 in 'Me 1', 'Me 2', 'Me 3' and 'Dw' filtrates, respectively (Figure 1). TPCs of *C. sinensis* were 608 ± 7.77, 985 ± 7.7 1284.8 ± 7 and 1379.3 ± 7.42 in 'Me 1', 'Me 2', 'Me 3' and 'Dw' filtrates, respectively. TPCs of *C. jambhiri* were 408 ± 7.77, 841.2 ± 9, 981.6 ± 6.54 and 1049.25 ± 3.88 for, 'Me 1', 'Me 2', 'Me 3' and 'Dw' filtrates, respectively. Conclusively the comparisons of total flavonoid contents and total phenolic contents of three citrus species showed that *C. paradisi* possess highest TFC and TPC followed by *C. sinensis* and *C. jambhiri*.

Reducing capacity of several compounds helps to indicate their antioxidant potential. Antioxidant compounds (reductants) caused reduction of ferric complex to ferrous form, i.e. reducing power of sample could be checked by measuring the formation of blue colored complex at 700 nm wavelength. Samples that possess higher reducing powers have much better ability for donating electrons. Free radicals by accepting donated electrons form stable substances result in ending of radical chain reactions. Reducing power of the citrus leave filtrates were determined in four different types solvents, 'Me 1', 'Me 2', 'Me 3' and 'Dw' in comparisons with ascorbic acid as a standard (Figure 2). At highest concentration (0.3 mg/mL) reducing power of *C. paradisi* were 1.032 ± 0.031, 1.352 ± 0.035, 1.7099 ± 0.021 and 1.7807 ± 0.024 in 'Me 1', 'Me 2', 'Me 3' and 'Dw' filtrates, respectively. Reducing power of *C. sinensis* were 0.752 ± 0.032, 1.5991 ± 0.031 and 1.695 ± 0.028 1.734 ± 0.027 in 'Me 1', 'Me 2', 'Me 3' and 'Dw' filtrates, respectively. Reducing powers of *C. jambhiri* were 0.7058 ± 0.027, 1.2904 ± 0.029, 1.4157 ± 0.024 and 1.4217 ± 0.02 in 'Me 1', 'Me 2', 'Me 3' and 'Dw' filtrates, respectively.

DPPH assay is routinely used for screening antioxidant activity of natural compounds. Basic principle is reducing DPPH free radical in antioxidant presence that donates hydrogen. It is very sensitive method with ability to detect scavenging compounds even in low concentrations and can

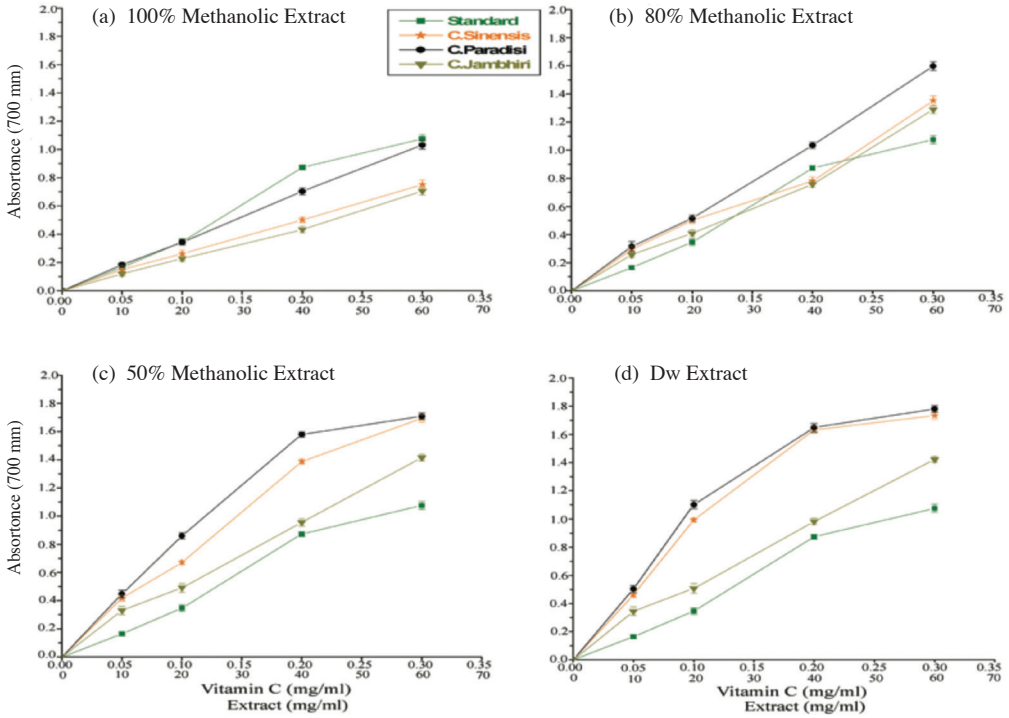


FIGURE 2. Comparison of reducing powers of Vitamin C and citrus leaves extracts obtained with different extraction solvents. (a) 100% methanolic extract (Me 1), (b) 80% methanolic extract (Me 2), (c) 50% methanolic extract (Me 3) and (d) Distilled water extract (Dw)

accommodate many samples in short time duration. DPPH is stable violet colored free radical with nitrogen atom present in centre that when reduced is converted to yellow color by hydrogen or electron donating ability of antioxidant compounds found in extracts. Radical scavenging activity of citrus leave filtrates was checked in 'Me 1', 'Me 2', 'Me 3' and 'Dw' in comparison with ascorbic acid as a standard and the results are shown graphically with concentration on x-axis and scavenging activity percentage on y-axis. At highest concentration (2 mg/mL), scavenging activity for *C. paradisi* were 83 ± 2.36, 88.4 ± 2.01, 93 ± 1.95 and 94 ± 2.1 in 'Me 1', 'Me 2', 'Me 3' and 'Dw' filtrates. Scavenging percentage for *C. sinensis* were 81.5 ± 2.3, 85.7 ± 1.95, 91 ± 1.99 and 92 ± 1.99 in 'Me 1', 'Me 2', 'Me 3' and 'Dw' filtrates. Scavenging activity of *C. jambhiri* were 80.1± 2.38, 82.5± 1.99, 85.6 ± 1.7 and 87.6 ± 1.76 in 'Me 1', 'Me 2', 'Me 3' and 'Dw' filtrates. Collectively, our results showed almost the same trend as in TFC, TPC and Reducing powers i.e. *C. paradisi* showed the highest DPPH radical scavenging activity in water extracts (Figure 3). $IC_{50}$ values were less for *C. paradisi* that showed extracts had higher radical scavenging activity (Table 4).

Medicinal plants are usually good source of antimicrobial compounds too. Majority of medicinal plant parts are used for raw drugs extraction and possess variations in their medicinal properties. But unfortunately no antimicrobial activity was observed in the citrus leaf extracts against the selected antibacterial and antifungal strains. However, the fruit peels of *C. bergamia* Risso has been shown to be effective against gram negative bacteria (Mandalari et al. 2007) and similar antifungal activity in the essential oils of *C. maxima* Burm. and *C. sinensis* Osbeck was also reported (Singh et al. 2010). Leaf extracts might show antimicrobial activity against other bacterial and fungal strains or with further modifications in the extraction protocols.

Brine shrimp assay is suggested to be a convenient probe for the pharmacological activities in plant extract (Mayer et al. 1982). Our results showed increased mortality rate with the increase in concentration. CSM and CPM50 showed significant lethality against brine shrimps. Brine shrimps (50-56.6%) were killed by 1000 µg/mL concentration, respectively (LD 50 <1000) (Table 5). Khan et al. (2011) have reported brine shrimps lethality of ethanolic extracts of *Zanthoxylum budrunga* leaves that belongs to the same family as citrus species i.e. Rutaceae. Our results are consistent with their findings. All brine shrimps (100%) were killed at 1000 µg/mL (LD 50 = 72.64). Significant lethality of citrus plant leaves extracts to brine shrimp is indicative of the presence of potent cytotoxic and probably insecticidal compounds which warrant further investigation.

*A. tumefaciens* was used as the tumor causing agent in potato disc because of its unique capacity for trans-kingdom sex i.e. transfer of genetic material between prokaryotic and eukaryotic cells (Stachel & Zambryski 1989). Our data has shown the evidences of antitumor activity of all the leaf extracts; however CSM50 and CPM50 showed significant antitumor activity and percentages of inhibitions, 58.6 and 52.5%, respectively (Table 6). Similar

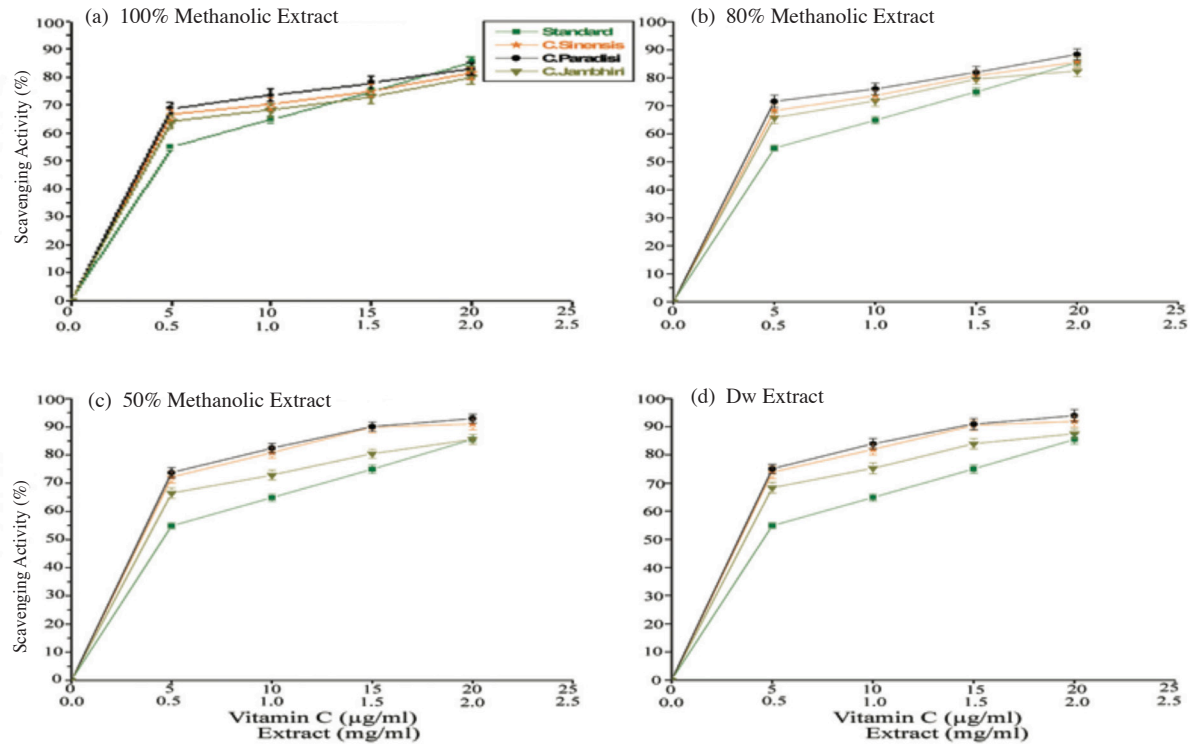


FIGURE 3. Comparison of DPPH radical scavenging activities of Vitamin C and Citrus leaves extracts. (a) 100% methanolic extract (Me 1), (b) 80% methanolic extract (Me 2), (c) 50% methanolic extract (Me 3) and (d) Distilled water extract (Dw)

TABLE 4. $IC_{50}$ values for DPPH assay in all solvents/combination of solvents

| Solvent/Combination of solvent | *Citrus sinensis* IC 50 (µg/mL) | *Citrus paradisi* IC 50 (µg/mL) | *Citrus jambhiri* IC 50 (µg/mL) |
|---|---|---|---|
| Methanol | 113 ± 2.2 | 81.34 ± 2.37 | 155 ± 2.4 |
| 80% Methanol | 126.7 ± 1.99 | 88.28 ± 2.11 | 148.6 ± 2.01 |
| 50% Methanol | 108.3 ± 1.96 | 95.9 ± 1.68 | 162.7 ± 1.7 |
| Distilled water (50°C) | 90 ± 2 | 83.6 ± 1.88 | 145.5 ± 1.84 |

$IC_{50}$ =50% Inhibitory Concentration

TABLE 5. Results of brine shrimp cytotoxicity assay

| No. | Extracts | Percentage mortality after 24 h | | | $LD_{50}$ ppm |
|---|---|---|---|---|---|
| | | 1000 ppm | 100 ppm | 10 ppm | |
| 1 | *Citrus jambhiri* methanolic extract | 26.7 | 20 | 10 | >1000 |
| 2 | *Citrus jambhiri* 80% methanolic extract | 26.7 | 13.3 | 6.6 | >1000 |
| 3 | *Citrus jambhiri* 50% methanolic extract | 20 | 10 | 3.3 | >1000 |
| 4 | *Citrus jambhiri* water extract | 33.3 | 23.3 | 13.3 | >1000 |
| 5 | *Citrus sinensis* methanolic extract | 50 | 40 | 23.3 | 799.62 |
| 6 | *Citrus sinensis* 80% methanolic extract | 40 | 20 | 10 | >1000 |
| 7 | *Citrus sinensis* 50% methanolic extract | 50 | 23.3 | 13.3 | >1000 |
| 8 | *Citrus sinensis* water extract | 23.3 | 16.6 | 6.6 | >1000 |
| 9 | *Citrus paradisi* methanolic extract | 46.6 | 40 | 33.3 | >1000 |
| 10 | *Citrus paradisi* 80% methanolic extract | 33.3 | 26.6 | 20 | >1000 |
| 11 | *Citrus paradisi* 50% methanolic extract | 56.6 | 43.3 | 36.6 | 375.27 |
| 12 | *Citrus paradisi* water extract | 16.6 | 13.3 | 6.6 | >1000 |

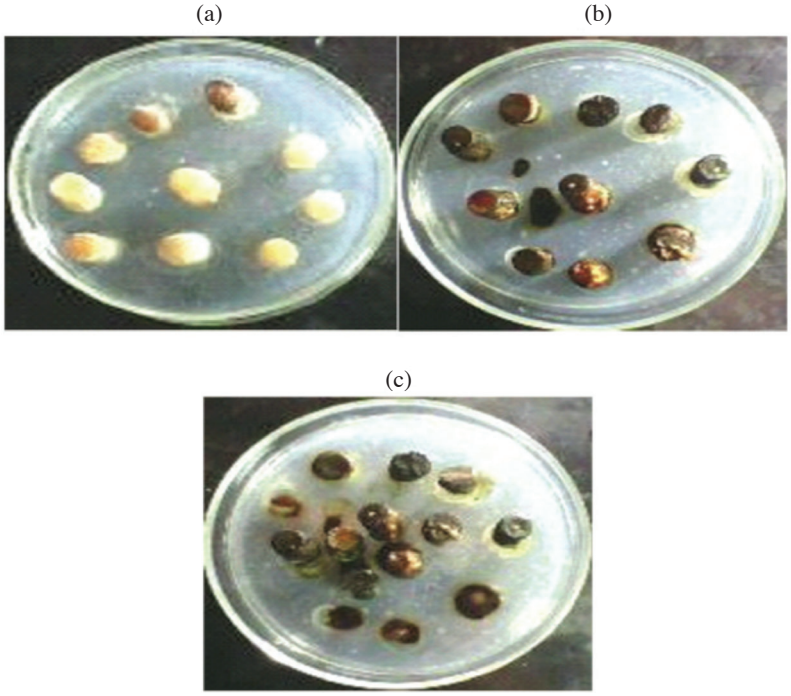


FIGURE 4. Antitumor assays of *Citrus sinensis'* 50% methanolic extract after staining with Lugol's reagent that helps tumors to glow in light to be seen with naked eye. (a) Negative control (b) *Citrus sinensis'* 50% methanolic extract and (c) Positive control

TABLE 6. Results of potato disk antitumor assay

| No. | Extracts | Percentage inhibition ± SD | | | $IC_{50}$ ppm |
|---|---|---|---|---|---|
| | | 40 ppm | 400 ppm | 4000 ppm | |
| 1 | *Citrus jambhiri* methanolic extract | 9.5±1.8 | 18.4±2.1 | 26.7±1.1 | >4000 |
| 2 | *Citrus jambhiri* 80% methanolic extract | 7.1±1.4 | 13.9±1.9 | 20.4±1.9 | >4000 |
| 3 | *Citrus jambhiri* 50% methanolic extract | 10.5±1.6 | 19.4±2.3 | 30.5±2.4 | >4000 |
| 4 | *Citrus jambhiri* water extract | 9.2±2.0 | 17.9±1.7 | 24.3±2.0 | >4000 |
| 5 | *Citrus sinensis* methanolic extract | 12.4±1.2 | 24.2±1.1 | 35.1±2.1 | >4000 |
| 6 | *Citrus sinensis* 80% methanolic extract | 13.5±1.9 | 26.4±1.2 | 40.8±1.9 | >4000 |
| 7 | *Citrus sinensis* 50% methanolic extract | 20.5±1.1 | 40.2±1.5 | 58.6±1.5 | 1207.7 |
| 8 | *Citrus sinensis* water extract | 17.3±0.9 | 32.9±2.1 | 47.5±1.7 | >4000 |
| 9 | *Citrus paradisi* methanolic extract | 12.9±1.8 | 24.2±2.5 | 34.5±1.2 | >4000 |
| 10 | *Citrus paradisi* 80% methanolic extract | 12.2±2.1 | 25.5±1.2 | 38.5±1.9 | >4000 |
| 11 | *Citrus paradisi* 50% methanolic extract | 19.1±1.5 | 37.4±1.8 | 52.5±1.8 | 2484.1 |
| 12 | *Citrus paradisi* water extract | 16.9±1.2 | 31.4±1.7 | 45.8±2.0 | >4000 |

findings were seen with anti-tumor activity of methanolic extract of *C. maxima* (Burm.) Merr. leaves that showed antitumor activity in Ehrlich's Ascites carcinoma cell in swiss albino mice (Kundusen et al. 2011). The presence of flavonoids and limonoids in the citrus plants are postulated to be the cause of their antitumor and anti-inflammatory effects (Middleton et al. 2000).

Mineral micronutrients were assessed to determine mineral contents of citrus species. This is the first report on atomic absorption analysis of citrus leaf extracts of selected species. Our results showed high calcium components in all the selected citrus species e.g. 22.62, 23.99 and 4.59 mg/kg followed by magnesium contents e.g. 2.65, 2.67, 1.305 mg/ kg in *C. paradisi*, *C. sinensis* and *C. jambhiri*, respectively (Table 7). Again our results are consistent with similar findings on *C. reticulata* Blanco and *C. aurantium*, regarding mineral composition i.e. higher calcium (35 mg/g dry weight) followed by magnesium contents (9 mg/g dry weight) (Darier 1991).

## CONCLUSION

The results presented in this paper are the first information on antioxidant, antitumor and cytotoxic potential of

TABLE 7. Mineral micronutrients expressed as mg/kg

| No. | Minerals | *Citrus paradise* mg/kg | *Citrus sinensis* mg/kg | *Citrus jambhiri* mg/kg |
|---|---|---|---|---|
| 1 | Calcium | 22.62 | 23.99 | 4.5933 |
| 2 | Cadmium | 0.0449 | 0.0523 | ND |
| 3 | Cobalt | 0.0474 | 0.0479 | 0.0007 |
| 4 | Copper | 0.0327 | 0.0124 | 0.01 |
| 5 | Iron | 0.2816 | 0.2118 | 0.1761 |
| 6 | Lead | 0.121 | 0.09 | 0.113 |
| 7 | Magnesium | 2.651 | 2.677 | 1.305 |
| 8 | Manganese | 0.01711 | 0.0167 | 0.0255 |
| 9 | Nickel | 0.1459 | 0.174 | 0.0135 |
| 10 | Potassium | 4.437 | 0.793 | 0.0215 |
| 11 | Sodium | 0.4091 | 0.2776 | 0.145 |
| 12 | Zinc | 0.113 | 0.1141 | 0.056 |

ND: Not determined

selected citrus species leaves. The results indicated that Citrus species leaves possess significantly antioxidant activity as it can be attributed to its high phenolic, flavonoid and phenolic acid content. All species exhibited significant reducing power and scavenging activity against DPPH free radicals. So it is concluded that citrus fruit leaves can be a good source of phenolic compounds and can be utilized for beneficial purposes. Based on recent results obtained, citrus leaves extracts may be helpful in antioxidant protection in food as well as in human body against lipid peroxidation and free radicals damage.

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