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Original Article

Phytochemical Screening, Phenolic Content and Antioxidant Activity of *Citrus aurantifolia* L. Leaves Grown in Two Regions of Oman

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Abstract

Lime or *Citrus aurantifolia* L., is a small citrus fruit that belongs to the family Rutaceae. The fruit and leaves of *C. aurantifolia* have been used traditionally for weight loss, skin care, relief from constipation and treatment of scurvy, etc. The purpose of this study was to determine the flavonoid, total phenolic content, and investigation of anti-oxidant potential of *Citrus aurantifolia* leaves grown in Oman. Lime leaves were collected from Batinah (Nakhal) and Ad-dakhiliya (Nizwa) region of Oman. The leaves were dried, powdered, and extracted with ethanol using a hot extraction method. Standard chemical tests were used to check the presence of secondary plant metabolites in the lime leaves extract. Quantitative analysis of the phenolics and flavonoids were done by colorimeteric methods. The antioxidant activity was assessed by DPPH and phosphomolybdenum in vitro assay methods respectively. Chemical analysis of the lime leaves extract showed the presence of major secondary plant metabolites. The obtained results revealed variation in the content of flavonoid (41.38-64.2 µg of QE/mg of dry extract) and phenolic compounds (96.55-322.57 µg of GAE/mg of dry extract). Both the lime leaves extracts from Nakhal and Nizwa showed concentration dependent moderate anti-oxidant activity (11.79-56.89 and 10.11-51.91%). Lime leaves from Batinah region also exhibited better total antioxidant capacity calculated with reference to the standard gallic acid. The aerial part of C. *aurantifolia* seems to be a rich source of flavonoids. Further studies are recommended to isolate and quantify the pure phytoconstituents from lime leaves grown in the Batinah (Nakhal) region which might serve as natural antioxidants in food and drug industry.

Keywords: Antixidants, *Citrus aurantifolia*, DPPH, Flavonoids, Omani lime, Total phenol.

1. Introduction

Medicinal plants have been the source of therapeutic agents since time immemorial. These plants have played an important role in drug discovery and still majority of people in developing countries rely on herbal medicines for their primary health care [1]. *Citrus* fruit crops such as oranges, grapefruit, lemons, limes, etc., belong to the family Rutaceae. Undoubtedly, citrus fruits are of great economic

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importance, but these are widely used by herbalists worldwide as traditional medicines for skin care, weight loss, improved digestion, nausea, urinary disorders, and for gum diseases. Scientific studies have shown them to contain many useful flavonoids and other phytochemicals that are effective antimicrobials and chemotherapeutic medicinal agents [2].

Omani lime (*Citrus aurantifolia* L) is used in beverages, food additives, and cosmetic industries and is regarded as the fourth most important fruit crop in terms of cultivated area and production in the Sultanate of Oman [3]. According to USDA database, limes contain lower amounts of Vitamin C but higher content of vitamin A than lemons [4]. Citrus fruits are well known for their antioxidant actions due to high content of ascorbic acid and other secondary plant metabolites such as flavonoids, terpenoids, etc. The bioactive phytochemicals present in the lime leaves include flavonoids such as Rutin, quercetin, kaempferol, nobiletin, and essential oils. These chemicals are capable of functioning as antioxidant and thus can play an important role in prevention of degenerative diseases such as cancer, Alzheimer, and Parkinson's disease caused by oxidative stress [5, 6].

The current study was undertaken to quantify the contents of phenolics, flavonoids and to assess the *in vitro* anti-oxidant potential of *C. aurantifolia leaves* grown in Batinah and Dakhliya region of Oman.

2. Materials and Methods

2.1. Plant Material

The lime leaves (250 gm) were collected from Nakhal (Al Batinah region) and Nizwa (Al-Dakhiliya region) cities of Oman in the month of May 2015. The collected plant material was identified by a faculty member of Oman Medical College and a voucher specimen was preserved in Department of Pharmacy for future reference. The leaves after washing under running water were dried under the sun and then powdered using a kitchen grinder (Moulinex, France).

2.2. Preparation of Ethanolic Leaves Extract

Approximately 50 g of powdered lime leaves was suspended in 500 mL of 95% ethanol in a round bottom flask and gently boiled on a water bath for 2 hrs. The ethanolic extracts were removed by vacuum filtration and then concentrated under reduced pressure to obtain crude viscous extracts. The yield of the green viscous masses was determined and was stored at 4 $^{\circ}$C until further use in experiments.

2.3. Preliminary Phytochemical Testing

The prepared alcoholic extracts of *C. aurantifolia* leaves were subjected to chemical identification tests to detect the presence of major classes of phytochemicals [7].

2.4. Quantification of Total Phenolic Contents

Total phenolic content (TPC) in the leaf extracts were determined with the help of Folin-Ciocalteu reagent according to the previously reported method [8]. Standard Gallic acid (GA) in a concentration of 5-80 µg/mL was used to construct the external calibration curve in order to determine the TPC as µg/mg Gallic acid equivalent (GAE) of dry leaf powder.

2.5. Quantification of Total Flavonoids Content

Total flavonoids content (TFC) was estimated by using Aluminum chloride ($AlCl_3$) colorimetric method following a standard procedure [9]. Quercetin (20, 40, 60, 80 and 100 µg/mL) was used to construct the standard plot. The results of TFC are reported as µg of quercetin equivalents (QE) in each mg of the dry leaf extract.

2.6. Evaluation of Antioxidant Activity

In vitro antioxidant activity of alcoholic extracts of lime leaves was assessed by two methods namely DPPH free radical scavenging assay [10] and total antioxidant capacity method [11].

2.6.1. DPPH Radical Scavenging Assay

1, 1, diphenylpicryl hydrazyl (DPPH) was dissolved in methanol to prepare a 100 µM working standard solution. To 1 mL of various concentrations of ethanolic extract (25, 50, 100 and 200 µg/mL) 2 mL of DPPH solution was added and the resulting mixture was mixed well. After incubation in dark at room temperature for 45 min, the absorbance of these solutions was measured at 517nm [10]. A control was prepared in a similar manner by replacing the amount of sample with methanol. % Inhibition of DPPH radical was calculated using the following formula:

% inhibition of DPPH = $[(A_{control} - A_{sample})/ A_{control}] \times 100$

Where; $A_{control}$ and A_{sample} are absorbance of control and sample, respectively.

2.6.2. Determination of Total Antioxidant Capacity

Total antioxidant capacity was determined by using phosphomolybdenum reagent as per the reported procedure [11]. Gallic acid was used as a positive control to compute the results. Total antioxidant capacity of the lime leaves extracts is expressed as Gallic acid equivalent (mg/g of dry extract).

2.7. Statistical Analysis

All samples were analyzed in triplicate and the results are presented as the Mean ± SD. Statistical significance between groups were analyzed by applying student's *t*-test. Values of *P* less than 0.001 were considered statistically significant.

3. Results and Discussion

3.1. % Yield and Phytochemical Screening of Leaves Extract

Approximately, 7.1 g (14.2% w/w) and 9.4 g (18.8% w/w) of viscous masses were obtained from Nakhal and Nizwa lime leaves, respectively by a hot extraction method using ethanol as a solvent. Phytochemical analysis of lime leaf extracts showed the presence of major

Table 1. Results of phytochemical screening of ethanolic extracts

Phytochemical class	Test	Nakhal lime leaves	Nizwa lime leaves
Tannins	$FeCl_3$ test	+	+
Steroids	Salkowski test	+	+
Flavonoids	Ammonia test	+	+
Proteins & amino acids	Ninhydrin test	-	-
Alkaloids	Wagner's and Meyer's test	+	+
Carbohydrates	Molisch's test	+	+
Saponins	Froth test	-	-

classes of phytochemicals such as alkaloids, carbohydrates, tannins, flavonoids, and steroids (Table 1). Proteins and saponins were not detected in the lime leaves. The biological activity of medicinal plants is attributed to the secondary plant metabolites. Polyphenolic compounds are known to exhibit a wide array of biological actions including anti-oxidant activity. Phenolic compounds primarily neutralize or scavenge the free radicals by donating the electrons [12].

3.2. Determination of Total Phenolic Content (TPC) by Folin Ciocalteu Reagent (FCR)

The TPC of the lime leaves extract was determined by using Folin ciocalteu reagent (FCR) and is expressed in terms of gallic acid equivalent (GAE) per mg of the dried extract. Phenols on reaction with an oxidizing agent phosphomolybdate in FCR under alkaline conditions, lead to the formation of a molybdenum blue colored complex, the intensity of which can be measured at 765 nm colorimetrically. The TPC was calculated using the following linear regression equation obtained from the standard plot of gallic acid.

$$Y=0.0025x + 0.0007$$

$$r^2 = 0.999$$

Where, Y is absorbance and x is amount of gallic acid in µg.

Table 2. TPC and TFC in ethanolic extracts of *C. aurantifolia* leaves

Lime leaves	Total phenolics (µg of GAE/mg of dry extract)	Total flavonoids (µg of QE/mg of dry extract)
Nakhal	96.55 ±7.5	64.2±2.8
Nizwa	322.57±15.4	41.38 ±5.5
p- value	<0.001*	<0.001*

Total phenol and flavonoid contents are expressed as Mean±SD, n=3, *Significant values, $p<0.001$ determined using Student t test.

3.3. Estimation of Total Flavonoids Content (TFC) by Aluminum chloride colorimetric method

Flavanoid is an important class of plant and fungus secondary metabolites. These are widely distributed in plants and exhibit a large number of biological functions. The TFC of the various leaves extract is expressed in terms of quercetin equivalent (µg/mg of the extract). The total flavonoid contents were calculated using the following linear regression equation obtained from the standard plot of quercetin.

$$Y=0.0084x + 0.0076$$

$$r^2 =0.998$$

Where, Y is absorbance and x is amount of quercetin.

TFC in lime leaves extracts differed significantly from one region to another as indicated by Student 't' test ($p<0.001$) and contrary to TPC, Nizwa leaves showed a slightly better level of flavonoids in comparison to Nakhal variety (Table 2). This suggests that Nizwa lime leaves contain some other phenolic compounds which do not belong to flavonoid class of compounds.

3.4. In- vitro Antioxidant Activity and Total Antioxidant Capacity

DPPH free radical scavenging method is a widely used and a reliable method to evaluate the *in vitro* antioxidant activity of natural products and plant extracts. The DPPH radical is a stable organic, free radical with the absorption maximum band around 515 to 528 nm. The natural or synthetic antioxidants such as ascorbic acid, tocopherol, cysteine, glutathione, gallic acid etc., have the ability to reduce the DPPH radial (purple color) to a yellow colored compound. The extent of color change depends on the hydrogen donating ability of the antioxidants. Therefore, in the current study, we have used DPPH method to evaluate the antioxidant activity of the lime leaves extracts.

The ethanolic extracts of both *C. aurantifolia leaves* exhibited moderate antioxidant activity, but scavenged the free

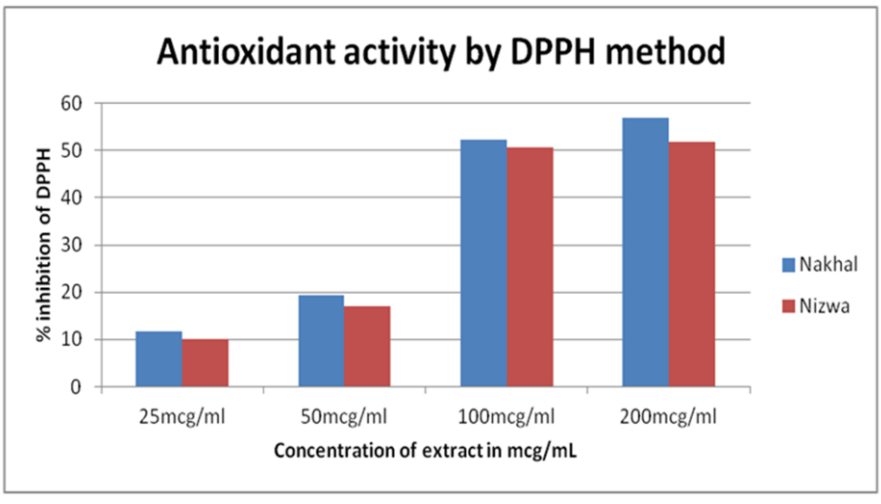

Figure 1. DPPH radical scavenging activity of ethanolic extract of lime leaves.

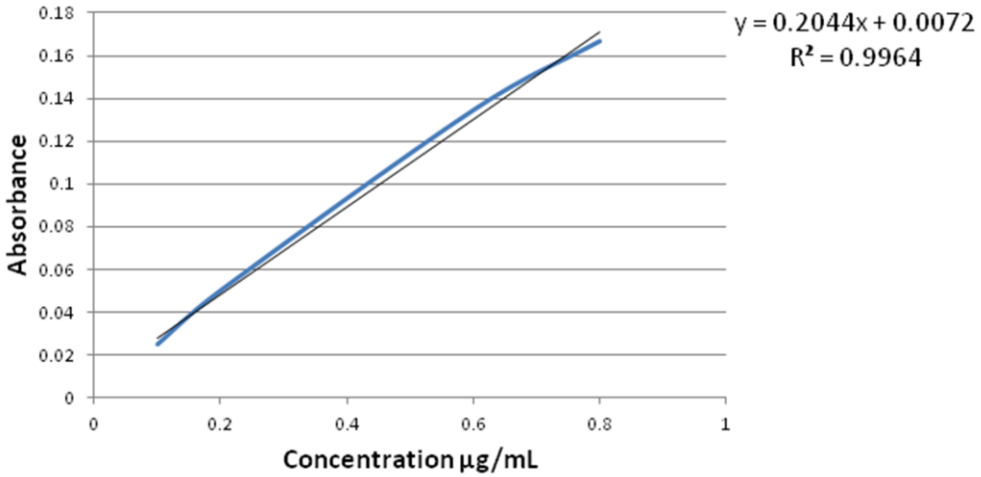

Figure 2. Standard curve of Gallic acid for the estimation of TAC.

radicals of DPPH in a concentration dependent manner (Figure 1). The antioxidant activity of lime leaves could be due to the presence of ascorbic acid content and biologically active secondary plant metabolites such as alkaloids, tannins, steroids etc, which have reducing property and thus known to exhibit significant antioxidant activity [13]. The % inhibition of DPPH radicals was found to be in the range of 11.79-56.89 and 10.11-51.91% for Nakhal and Nizwa lime leaves respectively. The antioxidant activity was observed to increase with increasing the concentration and the highest activity (56.1% and 51.91%) was observed at 200 ug/mL. No statistical difference in antioxidant activity was observed by student t test. Surprisingly, no correlation was observed between total phenolic content and antioxidant activity.

The (total antioxidant capacity) TAC of lime leaves was investigated by using the Phosphomolybdenum reagent method. Gallic acid, a poly phenolic acid, was used as a standard to construct an external calibration curve to obtain the line of the equation. Total antioxidant activity was calculated as mg of Gallic acid equivalent (GAE) / gm of the dried extract. The absorbance of the extract/standard was measured at 695 nm and the total antioxidant capacity was computed from the following linear regression equation;

$$Y=0.2044x +0.0072$$

Table 3. Results of total antioxidant capacity of the extracts of *C. aurantifolia* leaves.

Lime leaves extract	Antioxidant capacity (mg of GAE/g of dry extract)
Nakhal	0.381±0.01
Nizwa	0.291±0.01
p- value	0.00038*

Results are expressed as mean±SD, n=3, * $p<0.001$ by Student‘t’ test

$r^2 = 0.996$

Where, Y= absorbance and x= total antioxidant capacity in GAE.

A significant statistical difference was observed in the total antioxidant of *C. aurantifolia (*leaves). The antioxidant capacity of Nakhal lime leaves was found to be better than the Nizwa leaves (Table 3). A direct relationship was observed between the TAC, antioxidant assay by DPPH and TFC.

4. Conclusion

In this study, phenolic content and antioxidant activity of *Citrus aurantifolia* leaves grown in Nizwa and Nakhal region of Oman have been examined. The result showed that Nizwa lime leaves extract had a higher content of total phenol while Nakhal lime extract showed higher content of total flavonoid, antioxidant capacity and antioxidant activity. The results of our study showed the presence of major classes of phytochemicals in both varieties and a direct relationship between antioxidant capacities and total flavonoid content in the lime leaves. Further studies are recommended to isolate and quantify the pure phytoconstituents from lime leaves which might serve as natural antioxidants in food and drug industry.

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