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International Journal of Pharmacognosy and Chinese Medicine
ISSN: 2576-4772

Total Phenolic Content, Flavonoids Content, Antioxidant and Antimicrobial Activities of the Leaves, Peels and Fruits of Locally Available Citrus Plants Collected from Kavre District of Nepal

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Research Article

Volume 3 Issue 3

Received Date: September 16, 2019

Published Date: October 03, 2019

DOI: 10.23880/ipcm-16000181

Abstract

Aim of this study was to evaluate the total phenolic content (TPC), total flavonoids content (TFC), antioxidant and anti-bacterial activities of fruits, leaves and peels of locally available citrus species. Result revealed that TPC and TFC of the fruit, leaves and peels range from 6.1 to 34.22 mg GAE/ g DW and 1.71 to 28.96 mg QUE/g DW, respectively. Highest TPC and TFC were *Citrus medica* leaves. Furthermore, range of antioxidant activities of the fruits, leaves and peels range were analyzed using DPPH scavenging as well as reducing power assays. Both analysis revealed that Citrus maxima leaves, *Citrus aurantum* Peels and *Citrus medica* leaves have the highest antioxidant activities with IC_{50} values 54.86 ± 0.09, 56.69 ± 0.08 and 66.81 ± 0.03 µg/ml, respectively. Furthermore, analyzed plant extract revealed the range of antibacterial activities in both grams positive and gram negative bacteria. Our results revealed that not only fruits, and leaves but also peels showed significant biological activities.

Keywords: DPPH; Citrus; Flavonoids; Gallic Acid; Quercetin

Abbreviations: TPC: Total Flavonoids Content; CRD: Completely Randomized Design; TFC: Total Flavonoid Content.

Introduction

Edible fruits have played important role as a human nutritional supplements [1]. These days' practice to take fresh fruits subsequently after repast for vitamins,

minerals and other essential supplements in western and developing countries is immersing, adding the recovery of feeble health condition [2]. The importance of plant based products is valued not only in western culture, but also in eastern culture as well. Citrus fruits are one of the most popular, cheapest and affordable fruits widely available worldwide. In Nepal also, several different citrus species were available and are considered as an important sources of nutraceuticals [3].

Citrus plants are grown all over world around 140 nations, including Asia [4]. Citrus fruits is an important dietary supplements used in many countries around the worlds. It is taken in the form of fresh fruit, processed juice and beverages [5]. Citrus fruits are acidic, exotic fruits with juicy, bitter, fruit segments inside. [6]. These plants are a potent source of important bioactive secondary metabolites having anti-inflammatory, antioxidant, and lipid anti-peroxidation activities. The major phytonutrients reported in citrus plants are ascorbic acid, flavonoids and phenolic compounds [7-10]. The major flavonoids in citrus fruits are flavone-O-glycosides, naringenin, naringin, neohesperidin and poncirin, which are mostly responsible for the sensory quality of the citrus fruits [11,12]. Not only it is the source of flavonoids and phenolic compounds; it is also a huge source of vitamins, minerals including micronutrients and macronutrients [13,14]. Studies have reported that plant products act as antimicrobial agents against the bacteria and the fungus [15,16].

Fruits and vegetables are one of the important human diets in Asian countries. In Nepal several citrus fruits including *Citrus medica, Citrus aurantum, Citrus maxima* consumed mostly with the food. It is evident that consumption of the fruits and vegetables lower the risk of cardiovascular diseases, cancer and several other metabolic disorders [17,18]. Although some researcher has reported the health benefits of the fruits peels, mostly it is discarded. Present study aims to evaluate the biological significance of selected plant leaves, fruits and its peels collected from Kavre district of Nepal.

Materials and Methods

Collection of Plant Materials

The plant materials were collected from Dhulikhel Latitude and Longitude of 27.6167 and 85.55 respectively [19]. Dhulikhel is located in sub-locality, Dhulikhel locality, Bagmati District, Central Region State of Nepal Country 30.5 km away from the capital. Plants materials were collected in the polythene bags during morning hours and placed in the icebox for preventing any contamination and preservation.

Extraction

The shade dried peels and leaves were grounded using high ability grinding machine in powder form. In-room temperature the powdered plant material (20gm) was extracted successively using 200 ml of methanol. Filtration of individual extracts was done through Whatman No. 1 filter paper and Vacuum Evaporator (P201502902-1) used to evaporate the liquid solvents from the extract to acquire dehydrated extracts. Subsequently, next to drying, crude extracts were weighed. Stock vials were used to store and kept in the refrigerator (0- 4°C) for further use.

Phytochemical Analysis and Determination of Total Phenol Content

The phytochemical analysis of Alkaloids, Flavonoids, Phenolic content, Saponin, Quinone, Sterols, Cardiac glycoside, Tannin, Terpenoid and reducing compound was performed with slight modification [20,21]. Total phenolic content estimation was done using Folin Ciocalteu's technique with a slight alteration. Aliquots of 1 ml and standard gallic acid (10, 20, 40, 60, 80, 100 µg/ml) were placed into the test tubes and 0.5 ml of Folin Ciocalteu's reagent and 4.5ml of distilled water was mixed and further shaken. 4 ml of 7 % sodium carbonate was added after 5 minutes. Then the blue color mixture was shaken and incubated at 40ºC in the water bath. UV Visible instrument spectrophotometer was used to measure absorbance at 760 nm. The experiment was performed in triplicates. The blank was made using reagent blank with solvent. As standard gallic acid was used. Standard gallic acid was used for calibration curve plotting.

Free Radical Scavenging Activity Determination

DPPH (2,2-diphenyl 1-picrylhydrazyl) radical was used to determine the free radical scavenging capacity of the extracts as described by Barros, et al. [22]. With slight modification with 95% methanol each sample stock solution (1.0 mg/mL) was diluted to ultimate concentrations of 20 –100 µg/mL. Various concentrations of extracts (0.3 mL) were assorted with freshly primed methanolic solution comprising DPPH radicals (0.004% (w/v), 2.7 mL). Vigorous shaking was done and endorsed to stand for 60 min in the dark (until stable absorption values were obtained). The range of reduction of the DPPH radical was dogged by determining the absorption at 517 nm. For reference standard ascorbic acid was used and the DPPH solution was used as the control.

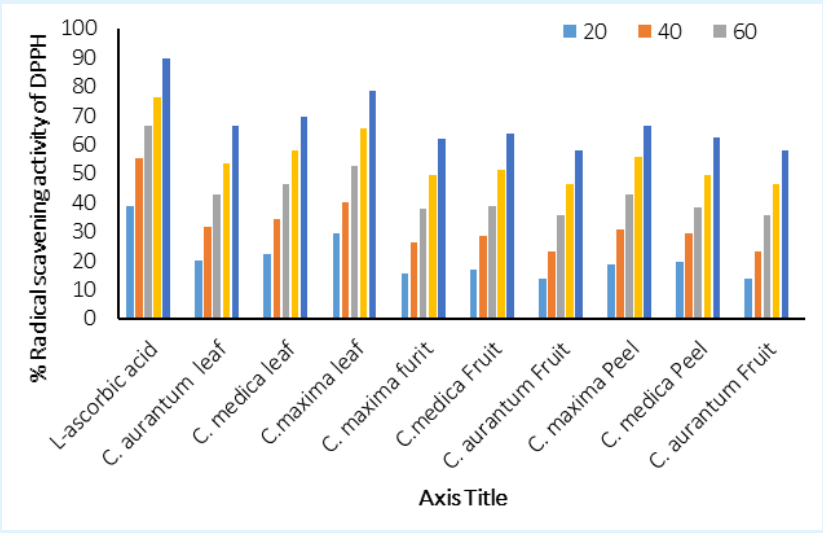

Figure 1: DPPH radical scavenging activity of the at concentration 20,40,60,80 and 100 µg/mL.

Determination of Total Flavonoid Content

Aluminum chloride colorimetric assay was used for total flavonoid content. The 1ml standard quercetin solution (100, 200, 400, 600, 800, 1000 µg/ml) and 1ml of aliquots were positioned into test tubes and 0.3 ml of 5 % sodium nitrite solution and 4ml of distilled water was added into each. 5 minutes later, 0.3 ml of 10 % aluminum chloride was added. At the 6th minute, 2 ml of 1 M sodium hydroxide was added. Finally, 10 ml volume was made up with distilled water and mix well yellowish color was developed. The absorbance was taken at a 510 nm spectrophotometer using a UV-visible instrument. Distilled water was used to perform blank. For standard Quercetin was used. The experiment was carried out in triplicates. The standard quercetin was used to plot the calibration curve. The total flavonoids of polyherbal formulation were expressed as mg of quercetin equivalents / 100 g of dry mass [23].

Antibacterial Activity

The antibacterial screening of the plant extract was carried out against four pathogenic strains, viz. Enterococcus spp, *Staphylococcus aureus* ATCC 12600, *Bacillus subtilis* ATCC 6051 and *Klebsiella pneumonia* by the disk-diffusion method [24]. The Mueller-Hinton agar plate dried surface was inoculated over the entire sterile agar surface by streaking the swab. Methanol was used as a negative control and Ampicillin was used as a positive control. The experiment was performed in triplicates under aseptic conditions. Plates were incubated for 18 h at 37ºC. The antibacterial activity was evaluated by measuring the zones of inhibition. The mean value of the diameter of the inhibition zone of the triplicates sets was taken as the final value.

Statistical Analysis

Completely randomized design (CRD) approach was used with three replicates and data so generated for different attributes were analyzed using Origin.

Results and Discussion

The methanol extract of leaves was green in color with the herbal aroma whereas the fruit extract of *C. maxima* was red in color compared to that of *C. medica* which was pinkish white in color with drupe like aroma. The peel extract was dark green with light brownish appearance with sour like aroma. The phytochemical screening of the extract is summarized in Table 1. The results indicated the presence of large amounts of Alkaloids, Protein, Quinones and Sterols was observed in *C. maxima* leaves extract whereas the moderate amount of Saponin, Terpenoid and Reducing sugar in *C. medica* leaves compared to moderate amount of Alkaloids, Proteins, Saponin, Terpenoid, Reducing sugar, Quinones and Sterols in *C. maxima* Leaf extract. A similar type of result is seen in the fruits and peels extract concluding the fact that *C. medica* is much rich in phytochemicals compared to that of *C. maximus* to

every extent. These phytochemical presences indicate the active activity of the extract concluding the result that *C. medica* shows higher activity compared to that of *C. maxima*. The moderate amount of saponin may act as a potent antiulcer drug along with gastro-protective properties. The defensive happenings of these active saponins are probably due to the activation of mucous membrane protective factors rather than due to inhibition of gastric acid secretion [25].

Phytonutrients	*Citrus Medica*			*Citrus Maxima*			*Citrus Aurantium*		
	Leaves	Fruits	Peels	Leaves	Fruits	Peels	Fruits	Leaves	Peels
Alkaloid	+	+	+	+	+	+	+	+	+
Saponin	+	-	+	+	-	+	+	-	+
Protein	+	-	+	+	-	+	+	+	+
Quinone	+	+	+	+	+	+	+	+	+
Sterol	+	+	+	+	+	+	+	+	+
Cardiac Glycoside	-	+	+	+	+	+	+	-	+
Tanin	-	-	-	-	-	-	-	-	-
Terpenoid	+	-	-	+	-	-	-	+	-
Reducing Sugar	+	+	-	+	-	-	+	+	-

Table 1: Phytochemical Analysis of the Citrus fruits peels and leaves.

Furthermore, the total phenolic content (TPC) and total flavonoids content (TFC) of the peels, leaves and fruit were analyzed using the standard protocol described in material and methods. Our results revealed that the TPC and TFC range from 5.8 to 34.22 mg GAE/g DW and 1.71 to 28.96 mg QUE/g DW, respectively. Results revealed TPC of the C. maxima and C. medica leaves methanol extract contain 22.24±0.11 mg GAE / g DW and 34.22±0.09 mg GAE/g DW respectively. Whereas, fruits and peels of *C. maxima* and *C. medica* contain 11.8±0.04, 9.33±0.06 mg GAE /g DW and 16.09±0.07, 8.3±0.06 mg GAE/ g DW, respectively. Similarly, TFC content of methanol extract of *C. maxima* and *C. medica* leaves was found to be 23.08±0.03, 28.96±0.02 mg QUE/g DW respectively. Furthermore, fruits were found to be 3.76±0.02, 2.58±0.04 QUE/g DW and peels was found to be 4.65±0.02, 2.23±0.02 QUE/g DW. The results were summarized in Table 2. For the natural products, especially fruits with their various parts leaves, fruits, and peels are recognized as nutritious based on their TPC and TFC profile which are further related to the medicinal value index. The phenolic and flavonoids have high yields especially in the moderate polar to non-polar behaving solvent supporting the use of methanol as extracting solvent [26]. Next we evaluate the antibacterial activities of methanol extract various parts plants materials and results were summarized in Table 3. It was analyzed extract revealed least inhibitory activities against *S. aureus* with zone of inhibition range from 9.5 mm and 10 mm in peels extract of *C. medica* and *C. maxima* respectively while the highest zone of inhibition was measured as 11.5 mm in both *Enterococcus sp.* and *Klebsiella pneumoniae* with *Citrus maxima* leaves extract. On the other hand, *Bacillus*

Scientific Name	Parts used	TPC (mg GAE / g DW)	TFC mg QUE / g DW	DPPH IC50(µg/mL)	Reducing power EC_{50} (µg/mL)
Citrus medica	Leaves	34.22±0.09	28.96±0.02	66.81±0.03	480.43±0.86
Citrus maxima		22.24±0.11	23.08±0.03	54.86±0.09	430.54±0.63
Citrus aurantum		32.07±0.77	21.32±0.18	72.59±0.31	663.49±0.97
Citrus medica	Fruits	9.33±0.06	2.58±0.04	77.41±0.38	500.2±1.15
Citrus maxima		11.8±0.04	3.71±0.02	80.43±0.17	464.03±0.4
Citrus aurantum		6.1±0.06	1.71±0.02	86.29±0.31	557.81±1.45
Citrus medica	Peels	8.3±0.06	2.23±0.02	79.32±0.4	771±1.51
Citrus maxima		16.09±0.07	4.65±0.02	71.93±0.36	762.8±1.69
Citrus aurantum		6.03±0.05	5.88±0.047	56.69±0.08	632.9±1.6

Table 2: Total polyphenol content (TPC), total flavonoid content (TFC) and Antioxidant activities of Citrus Fruits, Peels and leaves.

Organism	Gram reaction	Zone of Inhibition (mm)					
		Citrus medica		Citrus maxima		Citrus aurantinum	+ ve control
		Peels	Leaves	Peels	Leaves	Leaf	Ampr
Staphylococcus aureus ATCC 12600	+ve	9.5	10.5	10	11	10	12
Bacillus subtilis ATCC 6051	+ve	10	11	10	10.5	10.5	13
Klebsiella pneumoniae	-ve	10	10.5	9.5	11.5	10	14
Enterococcus sp.	+ve	9	11	9.5	11.5	10.5	13.5

Table 3: Antimicrobial activity of Citrus Fruits, Peels and Leaves.

The occurrence of alkaloids, flavonoids, and saponin as summarized in Table 1 may be responsible for good antibacterial activity. The solvent methanol did not show any activity while the positive control showed the highest zone of inhibition in all microbes. The curtailed bacterial growth resistance may be the result of the absence of structural interaction amongst solvent, extracted composites, and microbes. As verified in the literature the difference in the interaction between the compounds of the same class with different bacterial strains such as quinolone-based antibiotics happenstance with different efficiency and also face different modes of resistance. This clarifies the ultimate reason for the difference in phenolic compounds and biologically active compounds extracted from natural arsenal performing differently in different biological containment.

DPPH and reducing power assays were used to evaluate the antioxidant power of the leaves, peels and fruits of the selected citrus species. The IC50 value of the different part of the citrus species examined range from 35.05 to 86.29 µg/mL. The highest antioxidant activities were measured with the peel extract of *C. medica* in both DPPH and ABST assays with IC50 and EC50 values (35.05 ± 0. 11) and (255.38±1.74) µg/mL, respectively. All the citrus species in this study have revealed significant antioxidant activities.

Conclusion

The results revealed that citrus leaves and peels have shown significant biological activities. It is evident that not only fruits but leaves and peels of the citrus plant species have the comparable antioxidant and antimicrobial activities with that of fruits. Our results open up the possibilities in future to identify the potent antimicrobial agents from these species.

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