Antioxidant activity from the leaves of different citrus (*Citrus species*) under sub-tropical conditions of Punjab

ABSTRACT

The lab experiment was conducted during 2019-2021 to assess the antioxidant activity from the leaves of different citrus (*Citrus species*) in the laboratories of Guru Nanak Dev University (GNDU). The leaves of different citrus species were procured from the orchard of Department of Agriculture, Sri Guru Granth Sahib world University, Fatehgarh Sahib, Punjab. The antioxidant activity of leaves of different citrus species (lime, lemon, pummelo, mandarin, grapefruit and sweet orange) were measured using the 2, 2-diphenyl-1-picrylhydrazyl radicals (DPPH) and ferric reducing antioxidant power (FRAP) assays. For anti-oxidant properties studies, about 35 leaves/species were collected from the orchard of citrus. They were cleaned with running water, freed of dust and then shadow dried in the shade and then transferred to a hot air oven for 24 hours at $40°c$. Until a stable weight. Drying leaves will be formed into fine powder by using grinder method. After it, the powder was closed in a tight container and stored in a cool and dry place. The results of lab analysis revealed that the antioxidant activity of leaves extract of citrus species in both assays *viz.* DPPH scavenging activity and FRAP assay showed that the highest activity was displayed by grapefruit with an IC_{50} = 2.492 mg/ml and EC_{50}=0.835 mg/ml whereas, the least activity was revealed by sweet orange species with an IC_{50}= 4.113 mg/ml and EC_{50} = 2.240 mg/ml. Thus, it lead to a better understanding of volatile compounds present in leaves of six different citrus species. This knowledge could be utilized subsequently by food ingredients industries for various applications and innovation, specifically related to flavour compounds.

Keywords: Antioxidant, DPPH, FRAP, Flavour, Volatile Compounds.

1. Introduction:

Citrus belonging to genus citrus, subtribe *Citrineae*, the tribe *Citreae* within the subfamily *Aurantioideae* of the *Rutaceae* family having chromosome no. 2n=18 (Kahn

et al., 2001) grown in both tropical and subtropical regions of the world (Wu *et al* 2018). In India, citrus fruit is cultivated in an area of 1,034 thousand hectares with an annual production of 13,200 thousand tons and in Punjab, it is cultivated in an area of 59,980 hectares with an annual harvest of 13, 49,523 tonnes (NHB, 2018-2019). Citrus is a long- lived perennial crop and is cultivated in more than 100 countries around the world (Saunt, 1990). This genus may be further divided into two subgenera (Citrus and Papeda), based on leaf, flower and fruit properties (Kumar *et al*., 2013). Citrus have a potent source of significant bioactive secondary metabolites having antioxidant, lipid anti-peroxidation activities and anti-inflammatory activities. The main phytonutrients described in citrus plants are flavonoids, ascorbic acid and phenolic compounds (Mohammadian *et al.,* 2011; Ramful *et al*., 2010; Arora and Kaur, 2013; Sah *et al.*, 2011). The main flavonoids in citrus fruits which are commonly responsible for the sensory quality of the citrus fruits are naringenin, flavone-O-glycosides, naringin, poncirin and neohesperidin (Hasan, 2018). Citrus is not only the source of phenolic compounds and flavonoids but it is also a vast source of minerals, vitamins including macronutrients and micronutrients (Buachan *et al.,* 2014; Dureja and Dhiman, 2012 and Pandey *et al.,* 2019).

Citrus plant products (i.e. leaves, oil, by products) have been and will continue to be important candidate of new pharmaceutical compound (Juan *et al.,* 2016). It has antibacterial, anti-inflammatory, antiparasitic, antiproliferative, antifungal activity and antioxidant properties (Juan *et al.,* 2016). Citrus flavonoids can prevent cancer through selective apoptosis, cytotoxicity and antiproliferative actions. Acetone and hexane extracts of *C. sinensis* leaf exhibited inhibition zones of 27mm towards Helicobacter pylori (Juan *et al* 2016). Aqueous, petroleum and ethanol ether extracts of *C. sinensis* L. indicated activity against Candida albicans (Trovato *et al* 2000). The leaf extract of *C. sinensis* shows activity against Pseudomonas aeruginosa, klebsiella pneumonia and Staphylococcus aureus (Nada *et al.,* 2014).

There is an increasing interest in the antioxidant compounds derived from plants, which are very valuable for humans (Al- Alanbari and Hasan, 2015). Phenolic compounds are usually found in citrus plants and they have been reported to have numerous biological effects, included antioxidant and antibacterial activity (Mokbel and Hashinaga,2005; Win *et al.*, 2011; liu *et al.*, 2012). Previous study by Xu and chang 2008; Khatua *et al.,* 2013 showing that flavonoid and phenolic content could be

correlated to their antioxidant activities. Plant include citrus contain flavonoid and phenolic compounds (Souri *et al.,* 2008). DPPH (2, 2-diphenyl-1-picrylhydrazyl radicals) and FRAP (ferric reducing antioxidant power) can be used to predict antioxidant activity of vegetables, fruits, and food (Zielinski *et al.,* 2014 and Fidrianny *et al.,* 2014). Thus, keeping the above fact in view, an experiment was conducted to assess the about antioxidant activity from the leaves of different citrus species.

2. Materials and Method

The present studies was conducted during 2019-2021 in the laboratories of Guru Nanak Dev University (GNDU), Amritsar-143005. Leaves of different citrus species were procured from the orchard of Department of Agriculture, Sri Guru Granth Sahib world University, Fatehgarh Sahib, Punjab. For anti-oxidant properties studies, about 35 leaves/species were collected from the orchard of citrus. They were cleaned with running water, freed of dust and then shadow dried in the shade and then transferred to a hot air oven for 24 hours at 40°c. Until a stable weight. Drying leaves will be formed into fine powder by using grinder method. (Ma YQ *et al.,* 2009). After it, the powder was closed in a tight container and stored in a cool and dry place. The methodology for the analysis of anti-oxidant properties are:

Antioxidant activity of citrus leaves

The antioxidant activity of leaves of different citrus species (lime, lemon, pummelo, mandarin, grapefruit and sweet orange) were measured using the 2, 2-diphenyl-1-picrylhydrazyl radicals (DPPH) and ferric reducing antioxidant power (FRAP) assays.

DPPH Assay

The DPPH free radical scavenging activity of plant extracts was conducted according to the method described by Blois MS, (1958) using a spectrophotometric method with modifications. Extracts were assayed within the concentration range of 0.625–10 mg/ml. Leaves extracts (10mg) were incubated with DPPH solution (Add 20 µl of leaf extract sample in triplicate and then 200 µl of DPPH solution in it.) at room temperature (37°C) in dark for 30 min and absorbance was measured at 517 nm. The DPPH free radical scavenging activity was calculated using the following equation and IC_{50} values were determined. Routen Hydrate was used as the reference standard.

$$\textbf{DPPH activity}\ (\%) = [(\mathrm{Ac} - \mathrm{As})/\mathrm{Ac}] \times 100$$

Where, Ac = absorbance of control

As = absorbance of sample

FRAP Assay

The ferric reducing power assay was performed using the method given by Oyaizu, (1986). In this protocol, different concentrations (0.625, 1.25, 2.5, 5 and 10 mg/ml) of extracts (3µl) and purified compounds were added in a test tube. To this test tube, phosphate buffer (added 25µl from 2.5ml solution, pH 7.3) was added along with 1% potassium ferricyanide solution (25 µL from 2.5 ml solution) and the mixture was incubated for 20min at 50° C. Following incubation, 10% trichloroacetic acid (TCA) was added. The reaction mixture (25µL) was added to another tube after 20min. The final volume was raised to 50 µl using distilled water. Finally, 5µl of 0.1 % ferric chloride (freshly prepared) was added to the mixture and absorbance was taken at 700 nm. The absorbance was recorded at 700 nm using a multi-well plate reader (BioTek Synergy HT, Winooski, USA) against the blank. Results were expressed in percentage reducing activity given as:

$$\textbf{Reducing activity}\ (\%) = [1 - (1 - \mathrm{As/Ac}) \times 100]$$

Where, As = absorbance of sample

Ac = absorbance of the standard (blank) at the highest tested concentration

Statistical analysis

For antioxidant activity assays, all analyses were done in triplicate (n=3). Results were reported as Mean± SE. Statistical analysis was carried out using the software SPSS v 16.0 and comparison of averages was based on the analysis of variance (TWO-WAY ANOVA) at significance level p-value< 0.05.

3. Results and discussion

3.1 Antioxidant activity of different citrus species

The antioxidant activities of leaves extracts of six different citrus species (lime, lemon, pummelo, mandarin, grapefruit and sweet orange) were measured by using different *in vitro* assays *viz.* 2, 2-diphenyl-1-picrylhydrazyl radicals (DPPH) and Ferric reducing antioxidant power (FRAP) assays.

3.1.1. DPPH Assay for antioxidant activity of different citrus species

During the lab experimentation, DPPH assay was carried out to evaluate antioxidant properties of extract of leaves of different citrus species (Table 1 and Fig. 1) at various different concentrations *viz.* 10, 5, 2.5, 1.25, 0.625 mg/ml. The DPPH radical scavenging activity of leaves extract was observed highest in grapefruit (IC_{50} = 2.492 mg/ml) followed by, lemon (IC_{50} =3.100 mg/ml) and lowest activity displayed by sweet orange (IC_{50} = 4.113 mg/ml). The increasing order of radical scavenging activity among leaf extracts of different citrus species were observed in the order:

grapefruit > lemon > pummelo > lime > mandarin > sweet orange

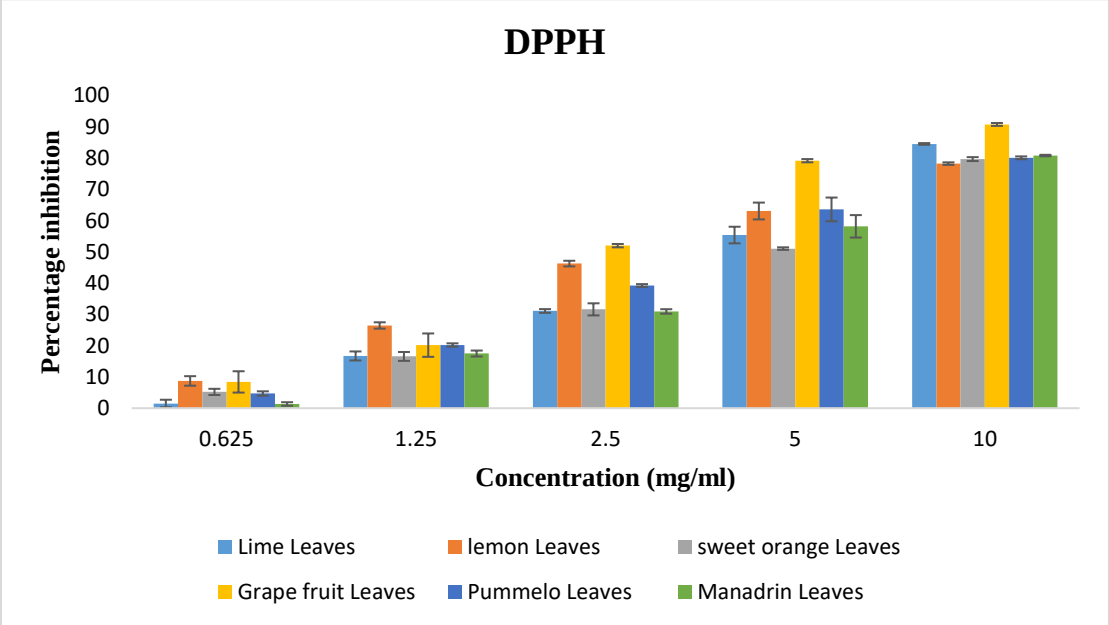

Fig. 1: Antioxidant activity of leaf extract of six selected citrus species (lime, lemon, pummelo, mandarin, grapefruit and sweet orange) by using DPPH radical scavenging activity at different concentrations viz. 10, 5, 2.5, 1.25, 0.625mg/ml.

The observed radical scavenging activity in terms of inhibition percentage and IC_{50} values. These findings were similar to results of Maisuthisakul *et al* (2007) who measured antioxidant activity of aromatic and medicinal plants IC_{50} is a widely used parameter and it showed that, Smaller the value of IC_{50}, higher will be the antioxidant

activity of the sample. Moreover, Dulay *et al* (2016) observed antioxidant properties of the leaves extracts of three citrus species and concluded that the *C. microcarpa* considerably recorded the maximum scavenging activity with 48.67%, followed by *C. maxima* having 43.51% *C. aurantium* had the minimum activity. Furthermore, Loizzo *et al.*, (2012) observed the antioxidant activity of *C. aurantifolia* by DPPH assays from the methanol extracts and n-Hexane fractions, which were consequences in strong antioxidant activity of methanol extract by L1 and L2 with IC_{50} values of 75.4 and 76.9µg/ml whereas, n-Hexane fraction displays a minor radical scavenging activity with an IC_{50} value ranging from 131.5 to 162.3 and 161.9µg/ml. Similar results were reported by Deng *et al.,* (2020) and Rekha *et al.*, 2012).

Table 1: Antioxidant activity of leaf extracts of different citrus species through DPPH radical scavenging assay.

Concentration (mg/ml)	Lemon	Grapefruit	Mandarin	Pummelo	Lime	Sweet orange
0.625	8.716 ± 1.526	8.406 ± 3.414	1.3409 ± 0.574	4.693 ± 0.698	1.496 ± 1.213	5.209 ± 0.984
1.25	26.457 ± 1.016	20.165 ± 3.740	17.483 ± 0.955	20.217 ± 0.523	16.709 ± 1.444	16.55 ± 1.419
2.5	46.261 ± 0.899	51.985 ± 0.538	30.943 ± 0.716	39.247 ± 0.441	31.098 ± 0.595	31.614 ± 1.939
5	63.074 ± 2.692	79.164 ± 0.516	58.174 ± 3.591	63.589 ± 3.791	55.389 ± 2.659	51.057 ± 0.403
10	78.236 ± 0.403	90.768 ± 0.458	80.815 ± 0.236	80.093 ± 0.449	84.528 ± 0.268	79.680 ± 0.627
IC_{50}	3.100	2.492	3.825	3.378	3.773	4.113

Source of variation	Df	F-ratio	HSD
Variety	5	45.90*	10.850
Concentration	4	2.028E3*	
Variety × concentration	20	7.686*	

3.1.2 FRAP Assay for antioxidant activity of different citrus species:

The lab experiment results for anti-oxidant activities analysis using FRAP assay revealed that the reducing power of leaves extracts was determined and the reducing power varied among all six citrus species (Table 2) at different concentrations *viz.* 10, 5, 2.5, 1.25, 0.625 mg/ml. grapefruit (EC_{50}= 0.835 mg/ml) possess highest reducing power followed by lemon (EC_{50}= 0.928 mg/ml), mandarin (EC_{50}=1.324 mg/ml), pummelo (EC_{50}= 1.502mg/ml), lime (EC_{50}= 1.525 mg/ml) and sweet orange (EC_{50}=2.240 mg/ml) exhibited lowest reducing power. The increasing reducing power in different citrus species was in order:

grapefruit > lemon > mandarin> pummelo > lime > sweet orange.

The observed reducing power activity in terms of effective percentage and EC_{50} values In a similar study, Gursoy *et al* (2010) reported that the essential oil of *Citrus nobilis* (lour) shown a great antioxidant property and the concentration increase, antioxidant property also increases. Similarly, Fidrianny *et al* (2016) worked on leaves extracts of five citrus species using FRAP methods which resulted in very solid antioxidant. Phenolic compounds in *C. aurantifolia* leaves extracts were the major contributor in EC_{50} of FRAP capacity. Leaves extracts of *C. hystrix* and C *aurantifolia* had linear result in FRAP assays. Similar results were reported by Al-Alnbari and Hasan (2015), Ahmed *et al* (2019) and Pandey *et al* (2019) who analyzed the antioxidant activities of citrus leaves by using reducing power assays and revealed that the *Citrus maxima* leaves and *Citrus medica* leaves have the highest antioxidant activities with IC_{50} values 54.86 ± 0.09 and 66.81 ± 0.03 µg/ml, respectively. The results also showed that the differences in the antioxidant activity of citrus species might be due to the existence of

large amounts of alkaloids, sterols, protein and quinones. These phytochemical presences show the vigorous activity of the extract.

Table 2: Antioxidant activity of extract of leaves of six different citrus species by using FRAP reducing assays.

Concentration (mg/ml)	Grapefruit	Lemon	Mandarin	Pummelo	Lime	Sweet orange
0.625	39.969 ± 0.473	40.588 ± 0.893	35.895 ± 0.806	28.468 ± 2.094	26.869 ± 1.396	11.088 ± 0.646
1.25	56.059 ± 0.893	59.825 ± 1.252	49.355 ± 1.385	49.510 ± 1.877	45.178 ± 0.744	32.439 ± 1.117
2.5	79.886 ± 0.893	65.033 ± 0.794	60.753 ± 0.608	60.443 ± 0.810	66.116 ± 0.497	60.186 ± 0.849
5	86.952 ± 0.186	79.268 ± 1.030	75.967 ± 4.061	76.740 ± 0.458	78.803 ± 0.389	72.769 ± 0.983
10	88.782 ± 0.223	90.407 ± 1.404	87.674 ± 0.868	90.304 ± 0.361	90.253 ± 0.409	89.066 ± 0.338
EC_{50}	0.835	0.928	1.324	1.502	1.525	2.240
Source of variation	**Df**	**F-ratio**	**HSD**			
Variety	5	119.444*	7.695			
Concentration	4	2.275E3*				
Variety × concentration	20	20.941*				

Conclusion

On the basis of one year experiment, it can be concluded that the antioxidant activity of leaves extract of citrus species in both assays *viz*. DPPH scavenging activity and FRAP assay showed that the highest activity was displayed by grapefruit with an IC_{50} = 2.492 mg/ml and EC_{50}=0.835 mg/ml whereas, the least activity was revealed by sweet orange species with an IC_{50}= 4.113 mg/ml and EC_{50} = 2.240 mg/ml. The results of this study also lead to a better understanding of volatile compounds present in leaves of six different citrus species. This knowledge could be utilized subsequently by food ingredients industries for various applications and innovation, specifically related to flavour compounds.

Disclaimer (Artificial intelligence)

Option 1:

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

5. References:

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