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A COMPARATIVE STUDY OF *IN VITRO* TOTAL ANTIOXIDANT, *IN VIVO* ANTIDIABETIC AND ANTIMICROBIAL ACTIVITIES OF ESSENTIAL OILS FROM LEAVES AND RIND OF *CITRUS RETICULATA* BLANCO cv. MURCOT (HONEY)

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Abstract

Citrus reticulata Blanco cv. Murcot (honey) is one of the most important fruit tree crops in Pakistan. A detailed study regarding total antioxidant capacity (TAC), radical scavenging, antidiabetic and antimicrobial effects of the essential oils from leaves (CRL) and rind (CRR) of this plant were investigated. Essential oils obtained from steam distillation process were subjected to 2,2'-azinobis(3-ethylbenzothiazoline-6-sulpohonic acid) (ABTS) radical cation decolourization, ferric reducing antioxidant power (FRAP), 2.2'-Diphenyl-1-picrylhydrazil (DPPH) radical decolourization, lipid peroxide inhibition using linoleic acid emulsion, superoxide anion radical scavenging and iron chelation activity assays. A linear correlation was observed between the percent inhibition of ABTS radical cation and the amount of essential oils from leaves (R^2=0.9978) and rind (R^2 = 0.985). The percent superoxide anion radical scavenging activity was found to be 15.25 and 48.75 for CRL and CRR, respectively. The reducing power in terms of FRAP values were found to be 1.2 and 0.173 mM $FeSO_4$ for CRL and CRR, respectively. Both CRL and CRR exhibited good metal chelating activities, 56.10 and 61.82 for CRL and CRR, respectively. Best results regarding antidiabetic activity were shown by essential oils from leaves of *Citrus reticulata* with low as well as high concentrations. The oils also showed antibacterial and antifungal activities comparable to chloramphanicol. The data obtained from oils demonstrate the powerful antioxidative, radical scavenging, antidiabetic and antimicrobial properties of the plant.

Introduction

The genus *Citrus* includes various species of limes, lemons, oranges, grapefruit and mandarins. Cultivated widely, *Citrus* fruits are the most popular fruits in the world. In Pakistan, citrus fruit is one of the most important tree crops. In spite of the high diversity of the 'citrus fruit', only a small number of cultivars are exploited commercially. After processing citrus fruits, peel and membranous residue, amount to approx. 40-50% of 'wet fruit mass'. The peels and leaves of citrus fruits are a potential source of essential oils (Baddock, 1999) and yield essential oil in the range of 0.5-3.0 kg/ton (Sattar & Mahmud, 1986). The essential oils thus obtained are a mixture of volatile compounds such as monoterpene hydrocarbons (70-95%), which give fresh, light and fruity smell.

Citrus essential oils have been used as flavouring agents in foods, beverages, liquors and confectionaries and as aromatic agents in perfumery, soaps and other household products (Matsuura *et al.,* 2006). In some cases, the composition of the flavouring agents can play an active role in the microbiological stability of the products. Moreover, various products made from essential oils have been used in aroma therapy and may relax some physical and psychological conditions (Susan, 1996). It is well known that essential oils have various functional properties such as attractive aroma, a repellent against insects and animals and inhibitory effects against microorganisms. Moreover citrus essential oils possess physiological activities such as antioxidative action against linoleic acid oxidation (Song *et al.,* 2001; Malhotra *et al.,* 2009; Fahad *et al*., 2013), DPPH radical scavenging activity and tyrosinase inhibitory activity (Choi *et al.,* 2000).

There is increasing interest in the radical scavenging activities of some natural antioxidants, especially those found in edible plants, which may play a significant role in preventing various diseases.

In recent years the scientists are in search of natural alternatives for food additives. Plants and plant products are a source of natural alternatives to improve the shelf life and the safety of food. Recently the interest in the application of essential oils to control plant and post harvest pathogens has increased and their potential role in food preservation has been exploited (Vazquez *et al.,* 2001; Lanceotti *et al.,* 2004).

Citrus essential oils have a very pronounced antimicrobial activity (Caccioni *et al.,* 1998; Belletti *et al.,* 2004; Baik *et al.,* 2008; Kim *et al.,* 2008).

Keeping in view the significance of Citrus essential oils, the present investigation aimed at study composition of essential oils of *Citrus reticulata* Blanco cv. Murcot (honey). Moreover antioxidant and anti microbial activities of essential oils were also analyzed.

Materials and Methods

All the chemicals used were of analytical reagent grade. The solvents used were obtained from E. Merck (Germany). Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid), linoleic acid, sodium acetate, potassium dihydrogen phosphate (KH_2PO_4), Dipotassium hydrogen phosphate (K_2HPO_4), ABTS (2, 2'-azinobis-(3-ethylbenzo-thiazoline)-6-sulphonic acid diammonium salt), gallic acid, quercetin, tween 20 (polyoxyethylenesorbitan monolaurate), iron(II) sulphate, potassium thiocyanate, 1, 1-diphenyl-2-picrylhydrazil (DPPH), 2, 4, 6-tripyridyl-*s*-triazine (TPTZ), Folin-Ciocalteau's reagent (FC reagent) were purchased from Fluka (Switzerland).

Plant material: The plant materials i.e., *Citrus reticulata* Blanco cv. Murcot (honey) obtained from their natural habitat in Pakistan, identified by botanist, were water-cleaned and shade-dried and their parts i.e. Rind and leaves were subjected to hydro distillation with varied yields (Table 1). The samples obtained were used either neat or in diluted form in various antioxidant and radical scavenging assays.

Total antioxidant capacity: FRAP (Ferric reducing antioxidant power) values were determined following the method of Benzie and Strain (1996). Final results were expressed as FRAP values (mM $FeSO_4$. $7H_2O$). ABTS scavenging activity was determined by following the method of Re *et al.,* (1999) with minor changes. DPPH free radical scavenging activity of the plant oils was measured by using the method of Shimada *et al.,* (1992). Total antioxidant activity of the essential oils was determined according to the method employed by Mitsuda *et al.,* (1996) Superoxide anion radical scavenging activity was determined using the method of Nikishimi *et al.,* (1972) Metal (Ferrous ion) chelation by plant samples was estimated according to the method employed by Dinis *et al.,* (1994).

Evaluation of antidiabetic activity

i. Preparation of animal house for rabbits: Rabbits *Oryctolagus cuniculus* were kept in the animal house earthen pots were kept in animal house for rabbits. Animal house was twenty feet in length, ten feet in width.

Standard conditions were maintained in the animal house regarding its temperature, relative humidity along with dark and light cycle.

ii. Acute oral toxicity: Acute oral toxicity of all essential oils was carried out adopting "up and down procedure" as per given in the OECD guidelines, Anonymous (2002). Rabbits weighing > 750 g ± 20 and aged 8–12 months were divided into four groups, having 5 animals each. The rabbits were kept in separate cages at room temperature for 7 days so as to acclimatize. Weights of overnight fasting rabbits were taken and an oral dose of each essential oil @ 2000 mg/kg was given to one animal of each of the groups. Food was stopped for further 3 hrs and the animals were observed, once during the first 30 min and then for 24 h at regular intervals with special attention in the first 4 h. All animals survived; the same dose was given to the additional 4 animals of each group, and observations were made for 14 days as per OECD guidelines.

iii. Preparation of alloxan diabetic rabbits: The rabbits were made diabetic by injecting intravenously alloxan monohydrate 150-mg/Kg-body weight. This dose permanently destroys the beta cells of pancreas and produces diabetes mellitus. Eight days after injection of the alloxan monohydrate, blood glucose of all the surviving rabbits was determined by checking direct glucose level by glucometer. Water and diet were available to the animals throughout the treatment period. After eight days rabbits confirmed diabetic with blood glucose level from 200-500 mg/100ml were selected for the evaluation for antidiabetic activity.

iv. Experimental plan: After acclimatization for 7 days all rabbits were distributed into 4 groups (A, B, C and D of five rabbits each.

The group **A** acted as control and was not given any treatment, but 10 ml of water. Groups B and C received applications of essential oils. In each of these groups of rabbits, two sub-groups were made as follows. Their glucose level was checked daily after 5, 10, and 24 hours and recorded.

Group B: The group B received an application of a dose of EO from leaves *Citrus reticulata* cv. Honey which was diluted in CMC. This group was further divided in to two sub groups.

B-1: 50µL/kg body weight, of EO from leaves *C. reticulata* cv. Honey which was diluted in 10 ml of 1% CMC in water.

B-2: 200µL/kg body weight, of EO from leaves of *C. reticulata* cv. Honey which was diluted in 10 ml of 1% CMC in water.

Group C: The group C received an application of a dose of EO from rind of *C. reticulata* cv. Honey which was diluted in CMC. This group was further divided in to two sub groups.

C-1: 50µL/kg body weight, of EO from rind of *C. reticulata* cv. Honey which was diluted in 10 ml of 1% CMC in water.

C-2: 200µL/kg body weight, of EO from rind of *C. reticulata* cv. Honey which was diluted in 10 ml of 1% CMC in water.

Group D: Group D acted as positive control group. This group was administered with locally available drug, Glibenclamide (Glib.). This drug was used in 0.5 mg/ml concentration diluted also in 10 ml of 1% CMC in water.

v. Collection of blood: Blood was taken from ear of rabbits with syringe, tested glucose level by glucometer. Glucose level of each subgroup was recorded after 5, 10, and 24 hours daily.

vi. Statistical analysis: One-way ANOVA and Probit-Regression tests were applied, using SPSS 13.0 (statistical soft ware) on the data for statistical analysis to draw conclusions.

Table 1. Percent yield, of essential oils from leaves and rind of *Citrus reticulata* Blanco cv. Murcot (honey).

Plant name	*Citrus reticulate* (leaves)	*Citrus reticulate* (rind)
Weight of specimen (Kg)	3.200	5.220
Weight of oil (g)	3.18	25.27
Percent yield	0.1	0.48
Color of oil	Clear yellowish	Light yellow

Table 2. Glucose level of each subgroup recorded after 5, 10, and 24 hours interval.

	Glucose level in alloxan induced rabbits mg/dl	Glucose level mg/dl		
		5h	10h	24h
Water	222 ± 0.50	225.6 ± 0.50	233.6 ± 0.5	242.4 ± 0.92
Citrus reticulata leaves EO 50µL/kg	200 ± 0.33	137 ± 1	122 ± 0.70	108 ± 0.707
C. reticulata rind EO 50µL/kg	220 ± 0.6	140 ± 0.70	121.8 ± 0.6	120 ± 0.70
Citrus reticulata leaves EO 200µL/kg	200 ± 0.67	107.4 ± 0.67	100.8 ± 0.6	96.2 ± 0.86
Citrus reticulata rind EO 200µL/kg	251 ± 0.85	130.4 ± 0.67	101 ± 0.70	90 ± 0.70
Glib 0.5 mg/ml	342 ± 0.95	137 ± 0.70	131.6 ± 0.5	111.6 ± 0.50

(Mean ± SEM, n=5)

Results and Discussion

ABTS radical scavenging capacity: ABTS radical cation produced as a result of reaction between ABTS and potassium persulfate in aqueous solution at physiological pH has considerable stability and sensitivity towards crude and specific antioxidants. The reduction potential of ABTS radical cation is very similar to that of hydroxyl radical cation. So in test environment it may be taken as equivalent to hydroxyl radical produced *in vivo* during certain disorders and metabolic reactions. ABTS radical scavenging ability of the test samples were evaluated using ABTS radical cation decolourization assay.

Percent inhibition of ABTS radical cation by the two samples was dose dependent (Fig. 1). A significant correlation (R^2 = 0.9974 and 0.985 for CRL and CRR respectively) was found between percent inhibition and amount of the sample.

Due to health-promoting effects of antioxidants a general recommendation to the consumer is to increase the intake of foods rich in antioxidant compounds e.g. polyphenols, flavonoids (Sies, 1985; Dreosti, 1991; Ahmad, 1995; Re *et al.,*1999; Davies, 2000; Finkel, 2000; Kitts, 2000; Lee & Shibamoto, 2000; Liu & Ng, 2000; Lee *et al.,* 2003). Phenolic compounds, especially flavonoids and anthocyanins, are very important antioxidants because of their natural origin and their ability to act as efficient free radical scavengers (Hertog *et al.,* 1993; Hertog *et al.,* 1995; Velioglue *et al.,* 1998; Langley-Evans, 2000; Wang & Jia, 2000). Small berries have been reported to be rich sources of phenolic compounds such as phenolic acids as well as anthocyanins, proanthocyanidins, and other flavonoids, which display potential health promoting effects (Faure *et al.,* 1990; Block *et al.,* 1992; Bomser *et al.,* 1992; Wang *et al.,* 1996; Hakkinen *et al.,* 1999; Fukumoto & Mazza, 2000; Feldman *et al.,* 2001; Bushra *et al.,* 2013).

The present study showed a relatively good relationship between antioxidant activity determined through ABTS radical cation decolourization assay and FRAP Assay.

DPPH, lipid peroxyl and superoxide anion radicals scavenging activities: DPPH and lipid peroxide free radicals have been used to evaluate reducing properties and to assess free radicals chain breaking abilities of phyto-chemicals. Fig. 2. demonstrates the kinetics of DPPH radicals scavenging by CRL and CRR. Both the extracts showed time dependant quenching of DPPH radicals. CRL sample was found to be a better quencher of DPPH radicals than CRR extract. The absorbance continued to decrease with almost a uniform gradient throughout the time span of 30 minutes showing the presence of a good amount of slow reacting antioxidant components in both the mixtures.

In bio-systems, unsaturated fatty acids are always at stake due to free radicals attack on the bio-membranes which results in membrane lipid peroxidation, decrease in membrane fluidity, loss of enzymes and receptor activity and damage to membrane proteins leading to cell inactivation (Dean *et al.,* 1993) Many disorders like hyperglycaemia have been ascribed to development of oxidative stress due to increased lipid peroxide production. Lipid peroxidation values of the extracts were found using linoleic acid emulsion system. Linoleic acid after its aerial oxidation to peroxyl radicals converts ferrous to ferric form. The extent of conversion is assessed through Iron (III) complex with thiocyanate, spectrophotometrically. The antioxidative components in proportionate to their amount halt this conversion by trapping peroxyl radicals. Fig. 3 shows that both the samples had considerable resistance to lipid peroxidation which is quite comparable with that of trolox (10 µM).

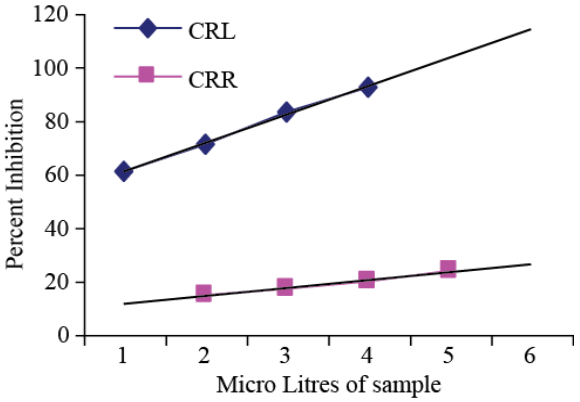

Fig. 1. Comparison of dose dependent percent inhibition of ABTS radical cation by essential oils from leaves and rind of *Citrus reticulata* Blanco cv. Murcot (honey).

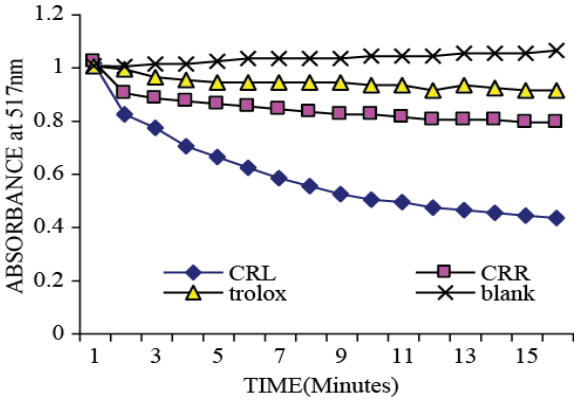

Fig. 2. Kinetics of DPPH radical scavenging by CRL and CRR extracts.

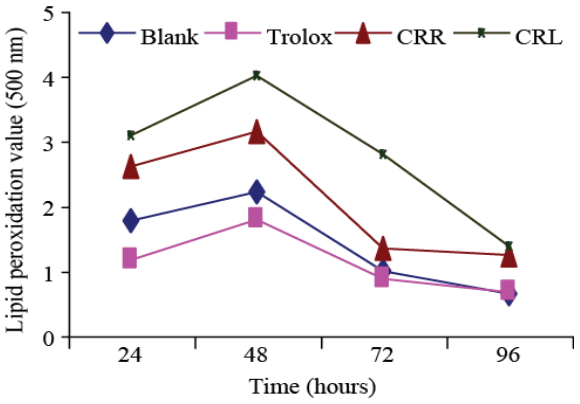

Fig. 3. Resistance of CRR and CRL samples to lipid peroxidation.

Superoxide (O_2^-) anion radical is one of the important ROS which is produced first after oxygen is taken inside the body. The subsequent dismutation of O_2^- leads to the formation of other injurious ROS. So the capacity of samples to scavenge ROS can play a very crucial role in determining the overall strength of antioxidant activity. The percent superoxide anion radical scavenging activity was found to be 15.25 and 48.75 percent for CRL and CRR sample, respectively.

Ferric Reducing and metal chelating activities: The FRAP values were found to be 1.2 and 0.173 mM $FeSO_4$ for CRL and CRR, respectively by measuring the change in absorption at 593nm. The metal chelating activity was found by determining the chelating activity of the sample with ferrous ion in the presence of ferrozine (a ferrous chelating agent) at 562 nm spectrophotometrically. The percent inhibition of the complex formation was found to be 56.10 and 61.82 for CRL and CRR, respectively.

Antidiabetic effect: Both low and high concentrations of essential oils have exhibited hypoglaecemic effect. Immediate drop in the blood glucose levels can be seen. Effect of high dose of essential oil is about almost equal to the low dose. However results of both doses i.e. 50 and 200 µL/kg of essential oils (Table 2.) are far better than the effect of Glib (positive control). All these findings suggest that essential oils may be acting through some mechanism to improve the receptor-responsiveness to insulin causing increased sugar uptake by the tissue. It is very difficult to comment on the exact mechanism of hypoglycemic activity of essential oils, since the study was not designed accordingly.

Antibacterial and antifungal activity: Agar well diffusion method was performed to calculate zones of inhibition for both the samples. Antimicrobial activities (Table 3) showed that essential oils from rind of *C. reticulata* cv. Honey was effective than essential oils from leaves. ZI was 2.2cm against *Streptococcus equi Bacillus cereus*. Similarly 2.1cm ZI was against *Staphylococcus aureus*, *Enterobacter aerogenes* followed by 1.9 cm against *E. coli* in comparison with standard. As far as antimicrobial activities against Fungi is concerned both EOs were either less effective or nearly equally effective in comparison with standard drug, Chloramphenicol. Notable was EO from *Citrus reticulata* rind with ZI measuring 2.2 cm against *Rhizopus oligosporus*.

The essential oils from different parts of members of Rutaceae included in this study are mixture of compounds, rich in Terpenoids like Monoterpenes, Diterpenes, Sesquiterpenes and Terpene alcohols and their derivatives (Nakatani 1994). Essential oils exhibit bactericidal activity by damaging the membrane and causing cell death (Sliva *et al.*, 2011). Essential oil affected variously rupturing cell walls and membranes resulted, causing severe cell damage, ultimately leading to bacterial death (Yu *et al.*, 2009). Essential oils and their monoterpenoid components impose their toxic effects by virtue of lipophilic nature partition from an aquous phase into membrane architecture (Sikkema *et al.*, 1994 and Sikkema *et al.*, 1995). Farnesol and Geraniol interaction increased the growth-inhibitory activity but suppressed its ability to damage cell membranes, which is one of the predominant features of the growth-inhibitory activity of farnesol (Ali *et al.*, 2013; Togashi *et al.*, 2008).

Finally it can be concluded that essential oils can replace synthetic antimicrobials being cost effective, easily available and without possible side effects as imposed by synthetic ones.

Table 3. Antimicrobial and antifungal activity of essential oils from leaves and rind of *Citrus reticulata* Blanco cv. Murcot (honey).

Plant part/standard	Zone of inhibition (cm) of bacterial strains[a]								Zone of inhibition (cm) of fungal strains[b]				
	MR	SA	SE	EA	PA	EC	ST	BC	AN	PC	SC	RO	RM
Citrus reticulata cv. honey leaves*	1.2	NZ	1.6	2.2	0.6	1.2	1	0.8	2	2	1.8	1.2	2.2
C. reticulata cv. honey rind*	1.8	2.1	2.2	2.1	1.8	1.9	1.6	2.2	1.3	1.8	2	2.2	1.8
Chloramphenicol** (positive control)	2	1.2	1.6	1.6	2	0.8	2.4	1	2.5	2	2.1	2	2.6
Negative control (water)	0	0	0	0	0	0	0	0	0	0	0	0	0

*100 microlitre of each oil was used in the experiment **Standard chloromphenicol 100microgram per 100micro liter

[a]Abbreviation of bacterial strains; MR: *Micrococcus roseus*, SE: *Streptococcus equi*, EA: *Enterobacter aerogenes*, PA: *Pseudomonas aeruginosa*, EC: *E. coli*, ST: *Salmonella typhimurium*, BC: *Bacillus cereus*, SA: *Staphylococcus aureus*,

[b]Abbreviation of fungal strains AN: *Aspergillus niger*, PC: *Penicillium chrysogenum*, SC: *Saccharomyces cerevisae*, RO: *Rhizopus oligosporus*, RM: *Rhodotrula minuta*

Conclusion

The data presented here shows that *Citrus reticulata* Blanco cv. Murcot (Honey) extracts have great antioxidant and radical scavenging activity and thus may be used as a good source of natural antioxidants. The *in vivo* efficacy of *Citrus reticulata* Blanco cv. Murcot (Honey) oils against diabetes mellitus or other degenerative diseases may be partially attributed to radical scavenging and antioxidant activity of the plant.

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