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Antioxidative, phytochemical and antimicrobial analysis of juices of eight citrus cultivars grown in Pakistan

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ABSTRACT: Comparative nutritional analysis of citrus varieties cultivated in Pakistan has not been reported. Citrus is consumed all over the world due to its taste and also has pharmacological components. The present investigation evaluated the antioxidant, reducing power, total flavonoids and phenolics, DPPH free radical scavenging, protein kinase inhibition, and the antimicrobial activities of eight Pakistani citrus varieties. Grapefruit showed maximum total antioxidant potential (77 μg AAE/100mg), followed by Kinnow and Shakri. Khatai showed maximum reducing power potential (69.6 μg AAE/100 mg) while Shakri and Grapefruit trailed it. All the varieties showed significant DPPH free radical scavenging activity. Maximum total phenolics in citrus juice were found in Shakri and Kinnow; 26.2 and 25.9 μg GAE/100 mg, respectively. Variation in total flavonoids content was observed as Kinnow>Grapefruit>Shakri>Khatai. All the citrus juices showed mild to moderate antibacterial activity, while Mosambi and Malta contained potent antifungal components. HPLC analysis of citrus juices revealed that catechin was present in all citrus genotypes except Kinnow. The study concludes that citrus juices contain strong antioxidative potential, bear protein kinase inhibitors and can be used as cancer chemoprevention and supportive nutrition.

1. INTRODUCTION

Fruits' biological activities are due to biochemical constituents, and fruits are rich with such components. Among these constituents, phenolics, flavonoids, and other antioxidative molecules are of significant concern. Although antioxidants are the best free radical scavenger, synthetic antioxidants have deleterious effects (Botterweck et al., 2000). Therefore, identification, production, and isolation of natural antioxidants are prerequisites, and fruits are the best source.

The oxidation process occurs in each cell during catabolic and anabolic pathways. During these processes, many free radicals produce that might be dangerous to cells and may cause health problems and life-threatening diseases. The antioxidative molecules and enzymes can scavenge free radicals (Surh & Ferguson, 2003; Tsao & Deng, 2004). It is beneficial to find natural antioxidants that could be used as nutraceuticals.

Citrus belongs to the family Rutaceae has hundreds of genera because of hybrids within the citrus species. Only a few citrus species are named with high confidence, while the confidence level is moderate to low for others. Genus citrus is widely grown worldwide as fruit and ornamental plants. However, tropical and subtropical climate is considered best for their cultivation. Few hybrid citrus varieties can survive in hot and cold environments. Pakistan is among the top ten citrus growing countries in the World. In Pakistan, citrus production has increased from 1898 thousand tonnes in 2001 to 2396 tonnes in 2015. This is about a 26% increase in 14 years at a rate of 1.6% per annum (Sadiq et al., 2018) . About 170 thousand hectares of the land grow citrus trees in Pakistan, and Punjab is the central producing region. The major citrus varieties grown in Punjab are Kinnow and Fruitor (Naz et al., 2007).

Along with taste, texture and delightfulness, citrus juice is a source of phytoconstituents, including flavonoids and phenolics, significant for human nutrition (Kumar et al., 2013). Among them, phenolics and flavonoids are abundant in citrus fruits and have many biological effects, including antioxidative, anti-carcinogenic, cardiovascular and anti-inflammatory (Huang & Ho, 2010). Although some studies have been conducted to evaluate the potential of citrus fruit parts as antioxidative and other activities, little is known as a comparative analysis of citrus juices among varieties. In order to identify the nutritional value of citrus and explore them in the food industry as a source of antioxidants, the antioxidative potential of eight Pakistani citrus cultivars was determined. Antibacterial, antifungal and protein kinase inhibition activities of citrus juices were also evaluated along with HPLC fingerprinting of citrus juices to determine biochemical constituents.

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2. MATERIALS AND METHODS

2.1. Collection of fruits and preparation of sample

Eight citrus varieties, i.e. Red blood, Malta, Mosambi and Shakri (*Citrus sinensis*), Fruitor and Kinnow (*Citrus reticulate*), Grapefruit (*Citrus paradisii*), and Khatai (*Citrus medica*), were collected directly from the fields (Sargodha division Punjab Pakistan) after leaf and flower identification of the plant with the help of Prof. Dr. Rizwana Aleem Qureshi, Department of Plant Sciences, Quaid-i-Azam University Islamabad. All the fruits were collected at the same level of maturity, fully ripened and ready to eat. Citrus fruits were washed under running tap water to remove any dirt. The fruits were then peeled, and the juices were extracted using the household juicer. The juices were initially filtered through filter paper followed by centrifugation at 12000 rpm for 60 min (Eppendorf 5427R Kisker Steinfurt UK) and finally stored in clean bottles at 4°C till further analysis. In separate glass vials, 10 mL juice of each variety was taken and dried at 50°C on a hot plate (BioBase China). The dried extracts were dissolved in DMSO at a final concentration of 20mg/mL and then sonicated to be used for analysis. All the chemicals used in this study were purchased from Merck Germany, otherwise mentioned.

2.2. Determination of antioxidative potential of citrus juices

Total Antioxidant capacity, total reducing power potential, DPPH free radical scavenging activity, total phenolic and flavonoid contents were determined by the procedure reported by Khan et al. (2017). Ascorbic acid was used as standard in TAC, TRP and DPPH assays. Gallic acid in DMSO was used as a positive control in TPC while Quercetin in TFC.

2.3. Protein kinase inhibition and antimicrobial assays

Protein kinase inhibition, antibacterial and antifungal activities were determined using the standard protocol described by Khan et al. (2017).

2.4. HPLC based fingerprinting

For HPLC analysis, samples dissolved in HPLC grade methanol at 10 mg/mL were filtered through 0.2-micron filters. Chromatographic system consisted of Agilent Chem station Rev.B.02-01-SR1(260) software and Agilent 1200 series binary gradient pump coupled with diode array detector (DAD; Agilent technologies, Germany) having Zorbex RX-C8 analytical column (4.6 x 250mm, 5 μm particle size, Agilent, USA). Two mobile phases were used; phase-I was composed of methanol-acetonitrile-water-acetic acid (10:5:85:1), while phase-II had methanol-acetonitrile-acetic acid (60:40:1). Time gradient of 0-20, 20-25 and 30 min of 0% -50% B, 50% -100% B till 100% B isocratic was used. The injection volume was 20 μL with a 1 mL/min flow rate. Rutin and gallic acid were analyzed at 257 nm, catechin at 279 nm, caffeic acid at 325 nm and quercetin, myricetin, kaempferol at 368 nm.

2.5. Statistical analysis

All tests were performed in triplicate, and the results were analyzed statistically through SPS to determine the average difference between the mean values. The means were further analyzed using ANOVA and LSD at probability level p<0.05. Linear regression was applied for establishing and verifying the correlation between ascorbic acid and Gallic acid for antioxidant activities.

3. RESULTS AND DISCUSSION

3.1. Antioxidative potential of citrus juices

The results show that citrus cultivars have varying antioxidative, biochemical, and biological properties. ROS are reactive chemical molecules produced naturally in biological systems. These are studied due to their arbitrary role in living tissues playing a good and bad side on physiological, signalling and immune response. The higher concentration and production of ROS causes oxidative stress that needs to be coped with using antioxidants, among which many natural candidates are explored for their potential antioxidant activities (Zuo et al., 2015). Antioxidants molecules diminish oxidative stress by reducing or preventing oxidative damage caused by free radicals. Scavenging the free radicals stops disease initiation and helps the cell overcome stress during disease; therefore, intake of antioxidants in daily life is desirable (Lai et al., 2001).

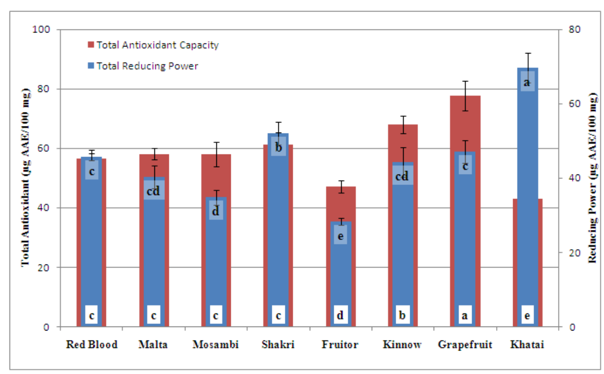

Figure 1. Total antioxidant and reducing power capacity of citrus juices. The alphabetic letters at the base of each bar represent the least significant difference (LSD P<0.05) of total antioxidant potential within citrus species. The alphabetic letters marked at the upper corner of each bar represent LSD value of reducing power potential within citrus varieties.

Among the antioxidant, phytochemicals play a crucial role as they possess preventive disease mechanisms and act as raw materials for orthodox and traditional medicine. They also have several health benefits such as antimicrobial, antihypertensive and anti-inflammatory activity (Oikeh et al., 2013). Citrus fruits are an abundant source of phytochemicals, minerals, vitamins and other compounds (Zhou et al., 2012), possessing a range of biological potential, making it an extraordinary antioxidant agent combating various diseases (Zhang et al., 2015). Determination of antioxidant activity and its correlation with phenolic compounds showed variation among different citrus species. Khatai belonging to *C. medica* exhibited minor activity while the member of *C. reticulate*, Fruitor, also showed low total antioxidant potential (Figure 1). The study correlated to previous studies depicting low radical scavenging capability in citrus, as Rauf et al. (2014) stated. All the three genotypes belonging to *C. sinensis*; Red blood, Malta and Mosambi, exhibited equal total antioxidant potential. The presence of phenolic compounds and vitamin C in fruit juices strongly correlates with higher antioxidant capacity. A study suggested that phenolic compounds dominate the total antioxidant capacity of citrus fruits (Arena et al., 2001). The citrus fruits varieties, maturity level, processing of the material and the methodology of analysis may cause the difference in results.

The reducing ability is a significant indicator of potential antioxidant activity, and the antioxidant activity of plant extracts can be correlated with reducing powers. The wild citrus genotype exhibited maximum reducing power; Khatai (69.6 μg AAE/100 mg) belongs to *C. medica* (Figure 1). Fruitor cultivar exhibited the least reducing power potential, contradictory with Kumar et al. (2013). In citrus varieties, reductones are generally responsible for reducing activity, which donates hydrogen atoms and reduces free radicals by breaking the free radical chain (Rapisarda et al., 1999).

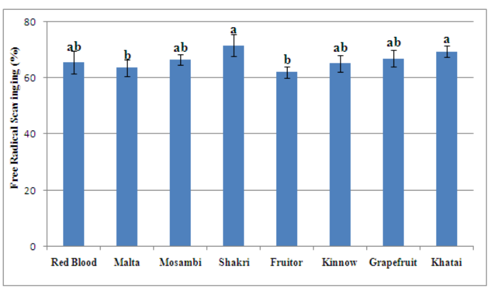

Figure 2. Free radical scavengingactivity (DPPH assay) of citrus juices. The alphabatic letters marked on the bars represent LSD (P<0.05) value withincitrus varieties.

Several disorders such as cancer, diabetes, degenerative diseases etc., are manifested by free radicals produced during biochemical processes. DPPH free radical scavenging activity of citrus juices was observed between 62.1 to 71.7% (Figure 2). The three varieties belonging to *C. sinensis* (Red blood, Malta and Mosambi) showed approximately the same results, while genotypes belonging to *C. reticulate* showed variation among scavenging activity. Kumar et al. (2013) also reported that *C. reticulate* exhibited higher radical scavenging activity than C. sinensis. While in this study, some varieties of *C. sinensis* like Shakri, Mosambi and Red blood showed higher values, and those of *C. reticulata* variety Kinnow had lower values comparatively but exhibited higher than Malta which belongs to *C. sinensis* and Fruitor, which is a member of *C. reticulata*. This might be due to geographical variation in the cultivation of citrus varieties. Caro et al. (2004) also reported variation in DPPH scavenging activity of citrus juices. The increase in the Kinnow value than Fruitor might be due to the improved quality of Kinnow because it is a hybrid of two species (*Citrus deliciosa* x *Citrus nobilis*). The antioxidant activity of an agent is associated with the ability of the component to retain cell structure and its function by eradicating free radicals, preventing oxidative damage and inhibiting lipid peroxidation. (Cai et al., 2004; Ke et al., 2015). With citrus fruits, their antioxidant activity is linked to ascorbic acid content and total phenolics rather than flavanone glycosides (Rekha et al., 2012).

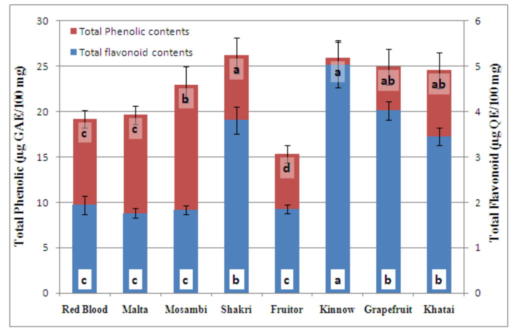

Figure 3. Total Phenolic contents and total flavonoid contents of citrus juices. The alphabetic letters at the base of each bar represent the least significant difference (LSD P<0.05) of total phenolic contents within citrus species. The alphabetic letters marked at the upper corner of each bare present LSD value of total flavonoid contents within citrus varieties.

The phenolic compounds as antioxidants have radical scavenging potential by suppressing free radical formation or protecting through a defence system (Kamran et al., 2009). In the present investigation of citrus varieties, variation in total phenolic contents was observed among the citrus varieties ranging from 15.3 to 26.2 μg GAE/100 mg (Figure 3). These observations were analogous to Kumar et al. (2013) and Rapisarda et al. (1999). Citrus is a rich source of phenolic acids having varying levels of scavenging. The antioxidant property of citrus is correlated to the ortho-substitution of a benzene ring and the dehydrogenation of hydroxyl groups (Dai & Mumper, 2010). It has been reported that antioxidant activities significantly increase with phenolic contents, and this observation has been made in many fruits and vegetables (Kamran et al., 2009).

Flavonoids inhibit ROS due to decreased peroxide formation, activating antioxidant enzymes of a living system, and counteracting lipid oxidation. Among the most commonly studied flavonoids of citrus are naringenin, hesperidin and naringin (Valls et al., 2021). The variety Kinnow showed maximum total flavonoid contents (5.04 μg QE/100 mg) compared with its companion genotypes Shakri and Fruitor (*C. reticulate*). Grapefruit contained 4.02 μg QE/100 mg total flavonoid contents (Figure 3), which is the member of *C. paradisii*. The citrus varieties, sweet in taste, contained common flavonoids, i.e., Malta, Fruitor, and Mosambi. However, one sweet variety Shakri contained a moderate amount of flavonoids, and this value is approximately the same as in wild citrus Khatai. The presence of phenolics and flavonoids in natural products is beneficial because such components fight against pathogens, increase immunity, reduce the free radicals, keep down the aging process, and others (Ali et al., 2021). The results show that citrus cultivars have varying concentrations of these metabolites and can quench radicals.

Table 1
Antibacterial activity of citrus fruit juices against selected bacterial strains

	E. aerogens	*S. typhimurium*	*M. lutues*	*B. bronchiseptica*	*S.aureus*
			Zone of inhibition (mm)		
Red Blood	6.5 ± 0.1^c	7.5 ± 0.2^b	7 ± 0.1^a	6.5 ± 0.1^b	7 ± 0.1^a
Shakri	7 ± 0.1^b	6.5 ± 0.2^c	6.5 ± 0.1^b	6 ± 0.1^c	6.5 ± 0.1^b
Fruitor	6.5 ± 0.1^c	6.5 ± 0.8^c	0.00	7.5 ± 0.7^a	6.5 ± 0.1^b
Malta	12 ± 0.2^a	9 ± 0.1^a	6.5 ± 0.1^b	6.5 ± 0.1^b	7 ± 0.1^a
Kinnow	6 ± 0.1^d	6.5 ± 0.7^c	7 ± 0.1^a	7.5 ± 0.1^a	6.5 ± 0.1^b
Grapefruit	6.5 ± 0.1^c	7.5 ± 0.1^b	6.5 ± 0.1^b	0.00	6.5 ± 0.1^b
Khatai	6.5 ± 0.1^c	0.00	7 ± 0.1^a	6.5 ± 0.1^b	0.00
Mosambi	7 ± 0.2^b	6.5 ± 0.1^c	6.5 ± 0.1^b	6.6 ± 0.1^b	6.5 ± 0.1^b

The alphabetic letters on each value within the column represent the least significant difference (LSD P<0.05) of antibacterial activity within citrus species.

3.2. Antimicrobial Assay

Malta presented intense activity against *E. aerogens* and *S.typhimurium*. The minor activity was shown against *Micrococcus luteus* by Red blood, Khatai and Kinnow, while no activity was found by Fruitor (Table 1). Aromatic and saturated compounds found in juices might be involved in antimicrobial activities and isolated from methanol and ethanol extracts (Bibi et al., 2011). Abeysinghe et al. (2007) reported that *C. paradisii* juice was active against *Staphylococcus aureus*, but *Proteus vulgaris* and *Pseudomonas aeruginosa* were resistant. Similarly, Ž Cvetnić et al. (2007) found no inhibition effect of *C. paradisiion* on the growth of Gram-negative micro-organisms. The lower ZOI against gram-negative bacteria is mainly linked to the periplasmic space comprising the peptidoglycan-and lipopolysaccharide layer of gram-positive bacteria. The gram-negative bacteria prevent antibiotic and environmental substances from penetrating and degrading the content that enters through their enzymatic potential (Oikeh et al., 2016). In citrus juices, naringin, citric and lactic acids, d-limonene, etc., influences antibacterial activity. The presence of phenolics and flavonoids may also contribute to microbial growth inhibition (Rizvi et al., 2013). Only Malta and Mosambi showed mild activity against fungal strains (Table 2). Aibinum et al. (2007) have reported that most fungal strains are susceptible to citrus juice extract with a minimum inhibitory concentration of 256 mg/mL. Somewhat a similar trend was observed against microbial strains using essential oils of citrus (Saeb et al., 2016).

The results obtained from the protein kinase assay depicts that the citrus has some potential to fight cancer. All the citrus genotypes showed a bald zone of inhibition of *Streptomyces* from 6.5 to 7.5 mm except Grapefruit (Figure 4). *Streptomyces mycelia*, when grown on a solid medium with nutrient-deficient produce spores (Stowe et al., 1989). Phosphorylation involving protein kinases occurs during this process; however, the dysfunction of kinases may disrupt this process (Meijer et al., 2004). Due to these reasons, kinases inhibitor search is a new target.

Table 2
Antifungal activity of citrus fruit juices against selected fungal strains

Zone of inhibition				
	fumagatus	***Mucor***	***A.niger***	***A.flavis***
Red Blood	0	0	0	0
Shakri	0	0	0	0
Fruitor	0	0	0	0
Malta	6.5 ± 0.7	7 ± 0.7	7.5 ± 0.7	7.5 ± 0.7
Kinnow	0	0	0	0
Grapefruit	0	0	0	0
Khatai	0	0	0	0
Mosambi	7.5 ± 0.7	7.5 ± 0.7	7.5 ± 0.7	7.5 ± 0.7

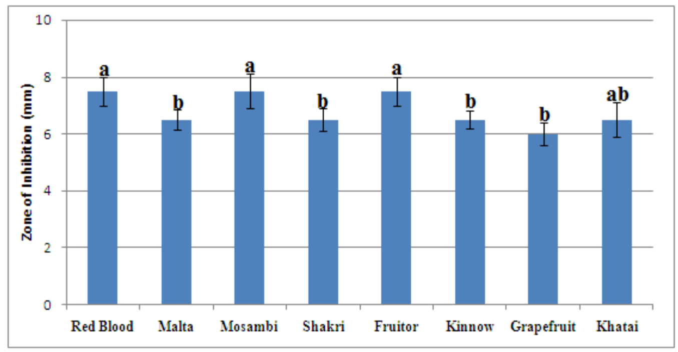

Figure 4. Protein kinase inhibition assay of citrus juices.

Table 3
HPLC base quantification of gallic acid (mg/L) and catechin (mg/L) in citrus fruit juices

Sample	Gallic acid	Catechin
Malta	0	454.3
Grapefruit	0	1227.5
Fruitor	0	358.1
Kinnow	14.7	0
Mosambi	0	144.4
Red Blood	0	60.5
Shakri	0	378.5
Khatai		1892.2

3.3. HPLC based fingerprinting

HPLC analysis was conducted to quantify catechin, myricetin, rutin, quercetin, apigenin, kaempferol, gallic acid and caffeic acid in citrus juices of citrus varieties. The analysis reveals that gallic acid was only present in Kinnow while catechin was present in all cultivars except Kinnow (Table 3). Catechins occur in many plants. Catechins induce apoptosis in human lymphoblastic leukaemia Molt 4B cells, promyelocytic HL-60 cells, stomach cancer KATO III cells, and many others (Isemura et al., 2000). Citrus juices are helpful in diseases, i.e. coronary and asthma. The chemical constants present in citrus juices may vary in species, geographical location, cultivation environment, and other physiological factors influencing the number of chemical constituents (Surh & Ferguson, 2003).

Apart from the aforementioned antioxidant agents within citrus, it has known to possess' high amount of coumarin, limonoids, terpenoids and phenolic acids. The increasing numbers of compounds identified in citrus suggest a structure-activity relationship and mechanism of antioxidant activities among citrus varieties. Literature suggests five possible pathways of the antioxidant mechanism of citrus involving inhibition of oxidant enzymes, inhibiting or reducing ROS production, directly and indirectly, interaction with signalling pathways, chelating transitions metals causing minor oxidative damage and ingredient synergism mechanism (Zou et al., 2016)

4. CONCLUSIONS

This comparative study was performed to access total phenolics, total flavonoids, DPPH scavenging activity, total antioxidant, reducing power potential and antimicrobial potential of citrus juices of different cultivars to gain insight into the nutraceutical content that could easily be explored in the food and beverage industry. The high phenolic content contributes much to the antioxidant potential of these varieties, among which Grapefruit, Kinnow and Shakri are consumed to a large extent. Due to their antioxidant capacity, these local cultivars can be used to prevent many diseases. The occurrence of protein kinase inhibitors adds much value as they can play an essential role in cancer chemoprevention and depict the use of citrus juices as supportive nutraceuticals during cancer treatment. Almost all the varieties showed moderate antibacterial and significant antifungal activity that could be further exploited to identify particular components that could be used as preservatives and to eliminate food borne pathogens to improve shelf life and enhance food safety.

CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

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AUTHOR CONTRIBUTIONS

SN - Research concept and design; AS, JSA - Collection and/or assembly of data; MZ - Data analysis and interpretation; MZ - Writing the article; MZ - Critical revision of the article; MZ - Final approval of the article.

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