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ESTIMATION OF PHENOLIC CONTENT, FLAVONOID CONTENT, ANTIOXIDANT PROPERTYAND ALPHA AMYLASE INHIBITION ACTIVITY OF POMEGRENATE (*PUNICAGRANATUM*) AND KINNOW (HYBRID OF CITRUSNOBILIS AND CITRUS DELICIOSA) PEELS.

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ABSTRACT

The objective of this study was to assess the antioxidant properties of the peels of selected citrus fruits that are readily available in India. A relation was derived between the antioxidant activity and the alpha-amylase inhibition activity to ascertain their use as a potential nutraceutical product thereby reducing solid kitchen waste. The fruit peel extracts of pomegranate (*Punicagranatum*) and Kinnow (Hybrid of *Citrus nobilis* and *Citrus deliciosa*) were prepared in water. Phenolic content, flavonoid content and free radical scavenging activity were estimated. The total phenolic content was found to be 22.86 mg of GAE /100 g for kinnow and 24.6 mg of GAE/100g for pomegranate. Total flavonoid content was found to be 580 mg of quercetin equivalent/100g for kinnow and 1163.34mg of quercetin equivalent/ 100g for pomegranate. In vitro antioxidant activity was found to be 122.75 mg of Ascorbic acid equivalent/100g for kinnow and 545.75 mg of Ascorbic acid equivalent/100 g for pomegranate. Free radical scavenging activity was calculated using DPPH and was found to be 34.6% for kinnow and 84.2% for pomegranate. Alpha amylase inhibition activity was calculated and IC_{50} value for kinnow was 4.9 mg/mL and for pomegranate was < 2mg/mL. It was inferred that both pomegranate and kinnow peels exhibit excellent antioxidant and alpha amylase inhibition activity and can be used as potential nutraceutical products to cure oxidative stress related diseases like diabetes.

KEYWORDS: DPPH, alpha amylase, oxidative stress related disorders, free radicals, antioxidant.

1. INTRODUCTION

Oxidative stress reflects an imbalance between the damage caused by reactive oxygen species (ROS) and the system's ability to repair that damage by readily detoxifying those reactive species. This results in base damage as well as strand breaks in DNA. Due to these disruptions in normal mechanisms of cellular signaling, oxidative stress can cause various long term diseases like cancer, atherosclerosis, heart failure, diabetes and many more. (Drewnowski and Carneros, 2000).[1]

Type 2 Diabetes is one of the most commonly occurring diseases due to oxidative stress. Type 2 Diabetes is a complex metabolic disorder associated with developing insulin resistance, impaired insulin signaling, β cell dysfunction, abnormal glucose and lipid metabolism, subclinical inflammation and increased oxidative stress. (American Diabetes Association, 2008)[2]; (Arif et al., 2014).[3] These long term metabolic disorders lead to long term pathogenesis conditions including micro and macro vascular complication, neuropathy, retinopathy, nephropathyand consequent decrease in quality of lifethereby increasing the mortality rate. Type 2 diabetes primarily occurs as a result of obesity and not enough exercise. Some people are more genetically at risk than others. Type 2 diabetes makes up about 90% of cases of diabetes. Type 2 diabetes is partly preventable by maintaining a normal weight, exercising regularly and eating properly. Amongst the multiple risk factors underlying the incident of progression of type-2 diabetes, diet is the main modifiable factor (Longhurst, 2017).[4] Diet rich in food with high content of phytochemicals and having high antioxidant activity may be related to lower risk of diabetes type 2. Diabetes is quickly gaining the status of a potential epidemic in India with more than 62 million diabetic individuals currently diagnosed with the disease. India currently faces an uncertain future in relation to the potential burden that diabetes may inflict upon the country. The number of deaths due to diabetes type 2 is likely to be about 4 million per year. The treatment of diabetes with insulin or oral hypoglycemic agent like metformin and meglitinideson long term usage may lead to complications which will further affect the immune system of our body. (Mishra, 2003).[5]

Plant sources have been used to alleviate or cure chronic diseases like diabetes since ages. Kinnow, a hybrid of *Citrusnobilis* ('king') and *Citrus deliciosa* ('willowleaf') commonly

known as keenuand Pomegranate or *Punicagranatum* are fruits belonging to the family "Rutacae" and "Lythraceae" respectively. Pomegranates are cultivated as ornamental trees and shrubs in the Mediterranean region, the Caucasus region, the northern and tropical parts of Africa and the northern parts of India. Kinnows are cultivated as trees in the Punjab regions of Pakistan and India. Both kinnows and pomegranates show a large variety of phytochemicals that are recommended for treatment of chronic diseases like diabetes.

The phytoconstituents present in these fruits have been reported to exert biological effects, including carbohydrate hydrolyzing enzyme inhibition and antioxidant activity. The α-amylase (α-1, 4-glucan-4-glucanohydrolase) or the carbohydrate hydrolyzing enzyme issecreted by the pancreas and salivary glands. It plays a role in digestion of starch and glycogen (Paul et al, 2013).[6] Alpha amylase randomly cleaves the α(1-4) glycosidic linkages of amylose to yield dextrin, maltose, or maltotriose. These are further acted on by α-glucosidases and further degraded to glucose which on absorption enters the blood stream. Rapid breakdown of starch by α-amylase leads to postprandial hyperglycemia due to increased glucose levels in the blood stream. Inhibition of α-amylase limits the elevated blood glucose levels by delaying the process of carbohydrate hydrolysis. (Kamtekar et al, 2014).[7]

Free radicals are atomic or molecular species with unpaired electrons in the outermost bonding orbital and are likely to take part in chemical reactions. Electrons prefer to be in pairs and when an electron is alone in its orbital in will try to take an electron from another atom to become more stable. When the other atom loses its electron it tries on its turn to steal an electron from another atom, often resulting in a dangerous chain reaction. Free radicals can cause damage to our cells (Musa, 2011).[8] Most free radicals are coming from oxygen atoms and are called Reactive Oxygen Species (ROS), such as superoxide ion, hydroxyl radical, hydrogen peroxide and singlet oxygen.(Jain et al, 2016).[9]

Antioxidants are defined as the compounds that can delay, inhibit or prevent the oxidation of oxidizable materials by scavenging free radicals by stopping that chain reactionand diminishing oxidative stress. Oxidative stress is an imbalanced state where excessive quantities of reactive oxygen or nitrogen species (ROS/RNS) overcome endogenous antioxidant capacity, leading to oxidation of a variety of bio macromolecules, such as enzymes, proteins, lipids, DNA etc. oxidative stress is important in the development of chronic degenerative diseases including diabetes type 2 (Patel et al, 2010).[10] Natural

phenolics and flavonoids present in the fruit as a phytochemicals act as an antioxidant and possess ideal structural chemistry for free radical scavenging activities because they have 1) phenolic hydroxyl groups that are prone to donate a hydrogen atom or an electron to a free radical; 2) extended conjugated aromatic system to delocalize unpaired electron (Jin Dai et al, 2010).[11]

The motivation for this project is to prevent and control oxidative stress related diseases using kinnow and pomegranate peels as nutraceuticals and in the process reducing solid kitchen waste. Using these peels as a preventive measure for diabetes would make the use of oral hypoglycemic, redundant and would help people lead a healthy life using a kitchen byproduct that would otherwise go to waste.

2. Materials and equipment

The materials used to conduct the in-vitro assays were -

Fruit peel extract: Peels of 3 kinnows and 3 oranges were dried at 60˚C in an oven for 24 hours and filtered through muslin cloth to make a powder, 0.5g of which was dissolved in 50mL water to make 10mg/mL extract solution for both kinnows and orange peels.

Chemicals used were Gallic acid, Folin-Ciocalteu's reagent, Quercetin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, sodium phosphate buffer, 3,5-dinitro salicylic acid (DNSA), sodium carbonate, sodium nitrate, aluminium chloride, sodium hydroxide, methanol, potassium ferricyanide, trichloroacetic acid, ferric chloride and starch.

Equipment used was oven, vortex, spectrophotometer.

3. METHODOLOGY

3.1 Determination of total phenol content

Total phenolic content was estimated by folinciocalteu's method. 1mL of aliquots and standard Gallic acid (0, 20, 40, 60, 80, 100, 120 µg/mL) was positioned into the test tubes and the volume was made up to 5mL in each test tube with distilled water. 0.5 mL of folinciocalteu's reagent was mixed and shaken (George et al, 2005).[12] After 5 minutes, 1.5 mL of 20% sodium carbonate was added and volume made up to 10mL with distilled water.

It was allowed to incubate for 2 hrs at room temperature, intense green colour was developed. After incubation absorbance was measured at 750 nm using spectrophotometer. The assays were performed in triplicates. The calibration curve was plotted using standard Gallic acid.

The data for Total Phenolic content of fruit peels was expressed as mg of Gallic acid equivalent.

3.2 Determination of total flavonoid content

Total flavonoid content was measured by the aluminum chloride colorimetric assay. 1 mL of aliquots and 1 mL standard quercetin solution (100,200,300,400,600,800,1000µg/mL) was positioned into test tubes and 4 mL of distilled water was added to each test tube. 300 µL of 5% $NaNO_3$ was then added to each test tube. After 5 minutes, 300 µL of 10% $AlCl_3$ was added to each test tube. After 1 minute, 2mL of 1M NaOH was added and volume was made upto 10 mL with distilled water in each test tube.

It was allowed to incubate for 1 hour at room temperature. Afterincubation absorbance was measured at 510 nm. The extracts were performed in triplicates. The total flavonoid content was expressed as mg of quercetin equivalent.

3.3 Free radical scavenging assay

The decrease of absorption of the DPPH solution after addition of sample was measured. 0.1 mL aliquots of methanol with different concentrations of peel extract was positioned into a test tube with a control test tube containing 0.1 mL methanol only. 4 mL DPPH solution was added to all the test tubes. The mixture was vortexed and then incubated for 30 minutes. Absorbance of sample and control was measured at 517 nm. The free radical scavenging activity was calculated by the formula:

$$\% \text{ scavenging activity} = [\frac{\text{absorbance of control} - \text{absorbance of sample}}{\textit{absorbance of control}}] \times 100$$

3.4 In-vitro antioxidant assay (FRAP)

10 mL solutions of distilled water with different concentration of ascorbic acid and fruit peel extracts were prepared in test tubes. To these, 1mL of 0.2 M phosphate buffer and 1 mL of 1% potassium ferricyanide was added.

The mixture was incubated at 50C for 30 minutes. Reaction mixture was rapidly cooled and then 2.5 mL of 10% trichloroacetic acid was added to stop the reaction. 2.5 mLaliquots was pipetted out of this and was positioned into test tubes. 2.5 mL water and 0.5 mL of 1% ferric chloride solution was added. The colour changed to green. The mixture was allowed to stand for 10 minutes and then absorbance was measured at 593nm. The results were recorded in terms of mg ascorbic acid equivalent per 100g of dry powder.

3.5 In-vitro inhibitory anti amylase assay

The fruit peel extracts were incubated with 500 µL of α amylase solution at room temperature for about 10 minutes. After incubation, 500µL of 1% starch solution was added and incubated at room temperature for 10 minutes. To this mixture, 1mL of DNSA reagent was added to stop the reaction and was incubated in a hot water bath (85˚C) for 5 minutes. After 5 minutes, reaction mixture colour changed to orange-red and was cooled to room temperature. It was diluted to 5 mL with distilled water. Extracts at different concentrations were performed in triplicates. Individual blank was performed by replacing enzyme with buffer. Control was performed by using the enzyme without the extracts. Absorbance was measured at 540 nm in spectrophotometer. (Kamtekar, 2014).

$$\text{The } \alpha\text{amylase inhibitory activity} = \frac{(Ac+) - (Ac-) - (AS - AB)}{(Ac+) - (Ac-)} \times 100$$

Where, A_c^+ = absorbance of 100% enzyme activity

A_c^- = absorbance of 0% enzyme activity

A_s = absorbance of test sample with enzyme

A_B = absorbance of test sample without enzyme

4. RESULTS AND DISCUSSION

4.1 Determination of Total Phenolic Content (TPC)

The total phenolic content was determined using Folin Ciocalteu's method with Gallic acid as standard. The reagent is reduced to a mixture of blue oxides of tungsten and molybdenum by oxidation of phenols. The blue colouration has maximum absorption in the region 750 nm and proportional to the total quantity of phenolic compounds originally present. The Gallic acid solution of concentration 500µg/mL conformed to Beer's law at 750 nm with coefficient of determination (R^2)= 0.918 and the equation of the standard curve is y = 0.03x – 0.081. The Table 1 below gives the total phenolic content observed in Kinnow and Pomegranate peel extracts.

Table 1: Total Phenolic Content.

Fruit Peel Extract conc. (mg/ml)	Phenolic content (mg of gallic acid equivalent/ g of dry material)
Kinnow – 1.5	22.86
Pomegranate – 0.2	24.60

4.2 Determination of total flavonoid content

The total flavonoid content was measured with aluminum chloride colorimetric assay using quercetin as standard. Aluminum chloride forms acid stable complexes with the C-4 keto groups and C-3 hydroxide group of flavones and flavonoids. The quercetin solution of concentration 5000 µg/mL conformed to Beer's law at 510 nm with coefficient of determination (R^2) = 0.891 and the equation of the standard curve is y = 0.0003 + 0.338. The Table 2 below gives the total flavonoid content observed in Kinnow and Pomegranate peel extracts.

Table 2: Total Flavonoid Content.

Fruit Peel Extract conc. (mg/ml)	Flavonoid content (mg of quercetin equivalent/ g of dry material)
Kinnow – 7.2	580.0
Pomegranate – 4	1163.34

4.3 Determination of antioxidant properties (FRAP)

In vitro antioxidant assay was done by ferric reducing antioxidant power method using ascorbic acid as standard. Reducing power is a measure of the ability of the extract to reduce Fe^{3+} to Fe^{2+}. Substances which have reduction potential react with potassium ferricyanide to form potassium ferrocyanide and then react with ferric chloride to form ferric-ferrous complex. The ascorbic acid solution of concentration (60-480 µg/mL) conformed to Beer's law at 593nm with coefficient of determination (R^2) = 0.976 and the equation of the standard curve is y = 0.004x + 0.104. The Table 3 below gives the mg of Ascorbic acid equivalent of Kinnow and Pomegranate peel extracts.

Table 3: Antioxidant Activity.

Fruit Peel Extract conc. (mg/ml)	Mg of Ascorbic acid equivalent/ g of dry material
Kinnow – 10	122.75
Pomegranate – 5	545.75

4.4 Determination of Free radical scavenging activity

Free radical scavenging activity was determined by DPPH. The activity was measured as % scavenging activity calculated for sample observations against control observations. $DPPH^+$ is one of the few stable commercially available organic nitrogen radicals and is known to acquire a hydrogen atom from an antioxidant. The presence of antioxidant is proportional to the disappearance of $DPPH^+$ in test samples. The colour turns from purple to yellow after

absorbing hydrogen from an antioxidant and converting to DPPH. $DPPH^+$ shows a strong absorption maximum at 517 nm. The Table 4 below gives the percentage free radical scavenging activity of Kinnow and Pomegranate peel extracts.

Table 4: Free Radical Scavenging Activity.

Fruit Peel Extract conc. (mg/ml)	% free radical scavenging activity
Kinnow – 10	83.2
Pomegranate – 1.5	84.2

4.5 Determination of alpha amylase inhibition activity

Alpha amylase is an enzyme that catalyzes the hydrolysis of α – 1,4 – glycosidic linkages from the non-reducing ends of polysaccharides to yield maltose units. Alpha amylase inhibitor is a protein which binds with alpha amylase enzyme and forms an inhibitor complex that stops the formation of maltose thus reducing alpha amylase activity. This helps in reduced postprandial glucose rise. The Table 5 below gives the IC_{50} value of Kinnow and Pomegranate peel extracts.

Table 5: Alpha amylase inhibition activity.

Fruit Extract	IC_{50} value
Kinnow	4.9 mg/mL
Pomegranate	<2 mg/mL

4.6 Correlation

Quantitative estimation of the fruit peel extracts of kinnow and pomegranate showed a presence of plant phenolics and flavonoids. Various oxidative diseases such as diabetes are known to occur due to enhanced oxidative stress or reduced antioxidant activity. To draw a relation between the amount of phytochemicals present and the antioxidant and alpha amylase inhibition activity shown by the peel extracts, correlation coefficients were calculated. The Table 6 gives the correlation coefficients and the determination coefficients in the brackets. The correlation coefficient shows the linear dependence of two sets of data whereas the determination coefficient gives us the explained variation of one set of data on the other set. The results showed high values of correlation between the two.

Table 6: Correlation between phytochemical content and anti- amylase & antioxidant activity.

		TPC	Flavonoid
Anti-amylase	Kinnow	0.863(0.744)	0.999(0.998)
	Pomegranate	0.904(0.817)	0.996(0.992)
DPPH	Kinnow	0.893(0.797)	0.929(0.863)
	Pomegranate	0.969(0.938)	0.999(0.998)

5. CONCLUSION

This study indicated that the extracts obtained from kinnow and pomegranate peels have excellent antioxidant properties and alpha amylase enzyme inhibition activity. Natural phenolics have been found to intervene at all stages of stress related diseases like diabetes. The presence of high quantity of phenols and flavonoids makes these fruit peel extracts extremely viable as a preventive measure for oxidative stress related diseases. The statistical analysis also showed the dependence of antioxidant and alpha amylase inhibition on the amount of phytochemicals (phenols and flavonoids) present. The extracts have been prepared in water to ensure their use as nutraceuticals. They can be used to prevent and control oxidative stress related diseases commonly occurring in humans and also toreduce the amount of solid kitchen waste by putting it to good use.

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