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BIOPROSPECTING THE UNDERUTILIZED FRUITS: AS A SOURCE OF NATURAL ANTIOXIDANTS

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Abstract

Worldwide, health concerns have led to a substantial increase in the demand for edible plants that endow health benefits over synthetic antioxidants. Therefore, the demand for wild fruits with nutraceutical properties has also increased. India is rich in diversity of fruits, but due to lack of awareness and the challenging trend of artificial supplements, the consumption of underutilized fruits is underrated. Keeping in mind the current necessity and demand for natural antioxidants, this study evaluates the antioxidant parameters (total antioxidant activity (TAA), and ferric ion reducing antioxidant power (FRAP) assays) and radical (i.e, DPPH (1,1-diphenyl-2-picryl hydrazyl) and hydroxyl radical) scavenging activity of the aqueous extracts of two underutilized fruit species, *Annona squamosa* L. and *Dillenia indica* L.. The study observed that the TAA and FRAP and radical scavenging activity of both the fruits were high in comparison to the positive control (*Syzygium cumini*). Compared to *Syzygium cumini* the amount of total phenolic (TPC) and total flavonoid content (TFC) were also high in both the species (i.e., *Annona squamosa* L. and *Dillenia indica*). Furthermore, nutritional analysis through X-ray fluorescence (X-RF) revealed the high content of macro and micro-nutrients for both the study species. The results of the study indicate that both the underutilized fruit species are rich in natural antioxidants and dietary supplements; therefore, these fruits could serve as a promising source of natural antioxidants/ nutraceutical compounds in future.

Keywords: Natural antioxidants, Phytochemicals, Radical scavenging activity, Total antioxidant potential (TAA), Ferric ion reducing antioxidant power (FRAP)

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Introduction

In today's world, it is a need of time to have strong immunity which has led to the rising inclination towards higher consumption of fruits that fulfills the requirement of essential nutrients in human body (Abeysuriya *et al*., 2020). As already acknowledged, the phytochemicals present in fruits exhibit synergetic pharmacological possessions to develop better immunity in humans which directly correlates with the reduction risk of non communicable diseases (NCDs) (Abeysuriya *et al*., 2020; Cha *et al*., 2015). Nowadays, the major cause of NCDs is the exposure towards various biotic & abiotic stress and unwarranted oxidative stresses in the cells resulting from disparity between the quenching and production of free radicals, predominantly reactive oxygen (ROS) and reactive nitrogen (RNS) species in cells (Pizzino *et al*., 2017; Wang *et al*., 2011; Yahia *et al*., 2010; Luximon-Ramma *et al*., 2003). These free radicals represent a class of highly reactive molecules which play significant role in maintaining cellular and immune response functions at low/moderate concentrations, (Halliwell and Gutteridge, 2007), however; at higher concentrations, they can be a damaging cause to various cellular structures and biomolecules, including lipids, proteins, and DNA (Pham-Huy *et al*., 2008). Accumulation of these free radicals has also been linked to several degenerative disorders such as coronary heart disease, cancer and other neurodegenerative diseases in humans. The natural antioxidants like polyphenols are acknowledged as nutraceuticals, and the presence of ascorbic acids in the fruits, possibly perform as non enzymatic pathways to reduce the detrimental radicals which accordingly quench the surplus oxidative stress in cells aiding to human health (Abeysuriya *et al*., 2020; Venkatachalam *et al*., 2014; Pham-Huy *et al*., 2008). The presence of antioxidative compounds in fruits tends to add the antioxidant potential of plasma and diminishes the harm caused by the oxidative stress, therefore higher consumption of natural antioxidants is beneficial for human health (Bopitiya and Madhujith, 2012; Mallawaarachchi *et al*. 2019). In general, higher amount of natural antioxidants consumed can overcome the negative and toxic effects of synthetic antioxidants (Gulcin, 2012). Similarly the secondary metabolites like phenolics, tannins, coumarins, lignans and flavonoids, present in plants possess good antioxidant activity and cause no toxicity in the human body (Soumaya *et al*., 2014).

These plants have received significant attention recently, as they are rich in phytochemicals and significantly delay the oxidation process, hence considered a potential source for natural antioxidants (Bakkali *et al*., 2008), furthermore, these are biodegradable and easily available (Karon *et al*., 2011). Fruits are considered as a vital source of essential minerals and antioxidants, apart from the commercial fruits, underutilized fruits play significant role in the nutrition and livelihood of communities. These minor fruits are rich in phytochemicals and nutrients; however, they are poorly consumed and acknowledged. Considering the current food security status, it has become the necessity of time to explore these underutilized plants produces as natural resources of antioxidants for human well-being (Droge, 2002; Peschel and Sahl, 2006).

Although India is a habitat of varied underutilized fruits, these are poorly acknowledged by locals and valued low in market in spite of high nutritional value. The allied reason for this situation could be the poor availability of data on their nutritional

value in comparison to the frequently consumed fruits. It is indeed the time to raise alarm and make these healthier fruits popular at low cost. Therefore, in the present study, the prime objective was to explore important biochemical parameters such as total phenolic content (TPC), total flavonoid content (TFC) and total antioxidant activity (TAA) contributing to human health. The two underutilized plant species, *Annona squamosa* and *Dillenia indica*, especially in the cultivated areas were analyzed, where their potential remained unexplored. The rationale is to encourage the underutilized fruits among the Indian community as healthy and natural alternative sources of antioxidants and nutrition which can also be developed as commercial crops and products considering the food security in future.

Materials and Methods

Collection of Plant Material

The mature ripened fruits of *A. squamosal, D. indica* and *S. cumini* were collected directly from the trees growing in the botanical garden of Panjab University Chandigarh, India. After collection, fruits were washed properly to remove dirt and other foreign particles. The edible portion of each fruit was cut down into pieces, dried under shade and grounded into powder form and further put this material into the sealed plastic bags till further use.

Preparations of Aqueous Extracts

Aqueous extracts from the powders of both the tested fruits were prepared by dissolving 1 gm of powder in 100 ml of distilled water separately for each fruit. The mixture was left for12 hours at room temperature (29°C) followed by filtration, firstly in triple layers of muslin cheesecloth followed by centrifugation at 3000 rpm. The supernatant was stored in the refrigerator at 4°C for determination of various antioxidant and free radical scavenging activities.

Estimation of Free radical scavenging activity

Determination of Free radical scavenging activity (DPPH)

The DPPH radical scavenging activity was determined as per Bozin *et al*. (2006). Briefly, the aqueous fruits extract of (both the sample and positive control) 200 µl were taken to which 3 ml of DPPH (0.1 mM in methanolic solution) was added. The solutions were incubated for 30 min in the dark at 25 ^{O}C. The absorbance of the solutions, including blank (without sample) was read at 517 nm on Shimadzu UV–1800, Japan double beam spectrophotometer. *Syzygium cumini* was used as the positive control. DPPH radical scavenging activity was calculated in percent by using the following formula:

DPPH radical scavenging activity (%) = [(A control – A sample) / A control] × 100 where A means, absorbance / optical density

Hydroxyl radical scavenging assay

The hydroxyl radical scavenging activity of the test plants was determined as per Yu *et al*. (2004). The assay mixture comprised of 0.02 ml of ferrous chloride (0.02 M), 0.5 ml of 1, 10-Phenanthroline (0.04 M), 1 ml of phosphate buffer (0.2 M, pH 7.2) and 1 ml of sample. The reaction was initiated by the addition of 0.05 ml of hydrogen peroxide (7 mM). After 5 min of incubating at room temperature, absorbance was read at 560 nm using UV–1800 double beam spectrophotometer (Shimadzu, Japan). The blank solution consisted of ferrous chloride, 1, 10–phenanthroline and phosphate buffer, whereas control contained everything except the sample. *S. cumini* was used as the positive control. The percent scavenging of hydroxyl radical was calculated using the following formula:
OH radical scavenging activity (%) = [(A sample–A control) / (A blank–A control)] ×100, where A means absorbance / optical density

Hydrogen peroxide (H_2O_2) scavenging assay

It was estimated as per the method of Ruch *et al*. (1989). A solution of 40 mM H2O2 was prepared in 0.1 M phosphate buffer (pH 7.4). Further, 0.6 ml of H2O2 was added to each sample. The absorbance of the solution was read at 230 nm after 10 min against a blank containing phosphate buffer without H2O2. *S. cumini* was used as the positive control. The percent scavenging of H2O2 was calculated by using the formula:
H2O2 radical scavenging (%) = [(A control–A sample)/A control] ×100, where A means absorbance / optical density

Total Antioxidant Activity (TAA)

The antioxidant capacity of extracts was evaluated by the phosphomolybdenum method, according to Prieto *et al*. (1999). To 0.1 ml of sample solution (water extracts), added 1 ml reagent of solution (6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The reaction mixture was incubated at 95 ^{O}C for 90 min. The samples were cooled at room temperature, and the absorbance of the solution was measured at 695 nm using Shimadzu, Japan UV–1800 double beam spectrophotometer. *S. cumini* was used as the positive control. Total antioxidant capacity was estimated in percent by using the following formula:
Total antioxidant activity = [(A sample – A control) / (A sample)] ×100, where A means absorbance / optical density

Determination of ferric reducing antioxidant potential (FRAP) assay

FRAP assay was done by following the method proposed by Oyaizu (1986). For this, the samples (0.2 mL) were mixed with 0.6 ml of phosphate buffer (0.2 M, pH 6.6) and 0.6ml of potassium ferricyanide (1% *w/v*). The mixture was incubated at 50^{O} C for 30 minutes followed by addition of 0.6 ml trichloroacetic acid (10%, *w/v*), and then centrifuged at 3000 rpm for 10 minutes. After centrifugation, 0.6 ml of supernatant was mixed with 0.6 ml of water and 0.125 ml of 0.1% (*w/v*) freshly prepared ferric chloride (FeCl3). The mixture was kept for 10 min, and the absorbance was read at 700 nm using Shimadzu, Japan UV–1800 double beam spectrophotometer against a blank containing ferric chloride and distilled water. *S. cumini* was used as the positive control. The activity was estimated in terms of percent inhibition by using the following formula

% inhibition = [(A sample – A control) / A sample] × 100, where A means absorbance / optical density

Total phenolic content (TPC)

TPC was determined as per Swain and Hills (1959) method using Folin- Ciocalteu reagent (FCR). The extract equivalent to 0.125 ml was mixed into 0.875 ml of distilled water, and 0.5 ml of FCR was added and the contents were shaken thoroughly. After 3 min, 1 ml of 7.5% sodium carbonate (Na2CO3) was added and the mixtures were allowed to stand for 30 min to 1 h. The intensity of the blue color so developed was read at 760 nm using Shimadzu, Japan UV-1800 double beam spectrophotometer against a standard of gallic acid (50 µg ml-1). The concentration of the phenolic compounds was expressed as mg gallic acid equivalents per gram (mg GAE g-1) of the fruit tissue.

Total flavonoid content (TFC)

TFC was determined as per the methodology given by Meda *et al*. (2005). In this, 1 ml of 2% of Aluminum trichloride (AlCl3 dissolved in methanol) was added into 1 ml of water extracts. After 10 min, the absorbance was read at 415 nm on Shimadzu, Japan UV-1800 double beam spectrophotometer. Quercetin was used as a standard. The amount of flavonoid was expressed as mg of quercetin equivalents per gram (mg QE g-1) fruit tissue.

Nutritional analysis through X-ray fluorescence (X-RF)

The dry powder of the test plants was also analyzed for the presence of macro - and micronutrients. These were subsequently identified using WD-XRF (wavelength dispersive x-ray fluorescence, Model: S8 TIGER, Make: Bruker, Germany). The sample was grinded in a vibratory cup mill to make a homogenized fine powder and to achieve a particle size of up to 5 microns. The thickness and diameter of samples were 4 mm and 34 mm, respectively. The time taken for the analysis of each sample was 20 min.

Statistical Analysis

Data was statistically analyzed using SPSS version 15.0 for windows (SPSS Inc., USA). Five replicates for each species were maintained for each concentration, and the data was presented as mean ± standard errors. Further, the significance was checked by one-way ANOVA followed by the separation of the mean values using *post hoc* Tukey's test at $P \leq 0.05$ and $P \leq 0.01$.

Results and Discussion

DPPH radical scavenging activity

In the present study, the ripened fruits of both the underutilized plants were used to determine the radical scavenging activity. The DPPH radical scavenging activity was observed at different concentrations from 0.625 to10 mg ml^{-1} (aqueous extracts), which increased significantly in a dose-dependent manner represented in Fig.1. Both the underutilized species had similar scavenging activity i.e., 5.8 for *D. indica* and 6.7 for *A. squamosa*, (Fig.1) at lowest concentration (0.625 mg ml^{-1}) of aqueous extract. However, at the highest concentration i.e. 10 mg ml^{-1}, the DPPH scavenging

activity was observed to be high in aqueous extract of *A. squamosa* (~ 80%) in comparison to *D. indica* (Fig 1). Further, when the IC_{50} value were compared for both the species with the positive control *S. cumini* (IC_{50} value 4.3 mg ml^{-1}); it was observed that *A. squamosa* (4.2 mg ml^{-1}) had almost similar value to positive control, whereas, the IC_{50} value of *D. indica* (6.8 mg ml^{-1}) was comparatively higher than the positive control.
The relatively low IC_{50} value of *A. squamosa* indicates its superior ability to scavenge DPPH radical similar to the reports that indicate the leaf extract of *A. squamosa* possessed strong potential to scavenge DPPH radical, escalating its restorative value (Chowdhury *et al*., 2021; Baskar *et al*., 2007). Studies also indicate that *D. indica* and *A. squamosa* possess good potential for DPPH radical (Saha *et al*., 2009; Shirwaikar *et al*., 2004).

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity was also studied for both the species, where results clearly depicted that the IC_{50} value in case of *D. indica* extract (5.2 mg ml^{-1}) was higher in comparison to *A. squamosa* (2.8 mg ml^{-1}) and the positive control i.e., *S. cumini* (3.6 mg ml^{-1}). It was observed that the IC_{50} value of the hydroxyl radical scavenging activity was found to be better in the fruits of *A. squamosa* when compared to *D. indica* and positive control (Fig. 2). The results of the present study were corroborated with previous studies and it was concluded that the leaf extract of *A. squamosa* exhibited great scavenging potential for hydroxyl radical (Kalidindi *et al*., 2015; Pandey and Barve, 2011; Bhaskar *et al*., 2007). Hydroxyl radical is known as the most reactive molecule that can damage all the essential biomolecules such as proteins, amino acids and DNA (Yasuda *et al*., 2000). The previous studies have highlighted that the underutilized plant species possess good radical scavenging activity against hydroxyl radicals and can prove to be beneficial to human health (Muthu and Durairaj, 2015; Khan *et al*., 2012). Therefore, as per the results of the study *A. squamosa* can be a great source to defend against hydroxyl radicals and aid to human health.

Hydrogen Peroxide Radical Scavenging Activity

In the present study, it was observed that both the test plants possessed good scavenging activity against H_2O_2 radical in a dose-dependent manner. The IC_{50} values of *D. indica* and *A. squamosa* were calculated as 2.8 mg ml^{-1} and 1.7 mg $ml^{-}1$, respectively. The IC_{50} value of *A. squamosa* was lesser than that of the positive control *S. cumini* (2.6 mg ml^{-1}) indicating its better ability to scavenge H_2O_2 radicals (Fig. 2), reported as similar to the study represented by Kalidindi *et al*., 2015. There are several studies reported on the other underutilized plants bearing good scavenging activity towards H_2O_2 radical, such as *Madhuca longifolia* (J.Koenig ex L.) J.F.Macbr. (Umadevi and Kamalam, 2015), *Solanum nigrum* L. (Srinivasan *et al*., 2012), *Cordia dichotoma* G.Forst. (Singh *et al*., 2010), *Carissa carandas* L. (Verma *et al*., 2015) and *Aegle marmelos* (L.) Correa (Abeysuriya *et al*., 2020). Though, the hydrogen peroxide (H_2O_2) is not a free radical but is a weak oxidizing agent which can easily cross cell membrane. It can also produce singlet oxygen through reaction with superoxide anion or with hypochlorous acid (HOCl) in living systems. However, an antioxidant can interfere in the formation of hydroxyl radicals by directly reacting with H_2O_2 and prevent the decomposition of peroxides, reducing capacity and radical

scavenging (Yildirim *et al*., 2000).The underutilized species like *A. squamosa* can be significantly beneficial in the process.

Total antioxidant activity (TAA)

The total antioxidant activity (TAA) for both the species was determined and *D. indica* was reported to be higher at all the concentrations except at the concentration 5 mg ml^{-1} when compared to *A. squamosa*, (Table.1). TAA of *D. indica* ranged from 25−90% whereas, that of *A. squamosa* varied from 13−88% over concentration range of 0.625−10 mg ml^{-1}. The IC_{50} values, of both the test plants were found to be lesser in comparison to the *S. cumini* used as positive control (Table.2). According to Chowdhury *et al*., 2021, the lesser IC_{50} values of the test plants exhibit better antioxidant activity than that of positive control (Table.2). Similar to our results Kamboj *et al*., 2019, describes *D. indica* as a good antioxidant which could possibly be considered against diabetic complications due to its therapeutic value. The present study is supported by the several findings on underutilized plant, that assessed TAA and recognized these fruits as a rich source of natural antioxidants (Abeysuriya *et al*., 2020; Mallawaarachchi and Pushpakumara *et al*., 2019; Chauhan and Kapfo 2013; Özen, 2010**)**.

Ferric reducing antioxidant power (FRAP) assay

In the FRAP assay, the antioxidants present within the samples are treated as reductants in a colorimetric reaction (redox-linked), the reducing power of the antioxidants is imitated in the values, where antioxidant reacts with Fe3+-TPTZ and a colored complex Fe2+ TPTZ is produced, which is deliberated at 593nm (Huang *et al*., 2005). Our study established a significant increase in the percent inhibition of FRAP with increasing concentration in both the aqueous extracts the test plants. At highest concentration (10 mg ml^{-1}), maximum percent inhibition was reported in *A. squamosa* (76%). The FRAP inhibition for *A. squamosa* and *D. indica* was approximately 9% and 18%, respectively at the lowest concentration (0.625 mg ml^{-1}). Further, the IC_{50} value for both the underutilized species was calculated and compared with the positive control (Table. 2). On comparison the minimum IC_{50} value (1.8 mg ml^{-1}) was observed in case of *S. cumini* used as positive control followed by *A. squamosa* (5.2 mg ml^{-1}) and *D. indica* (5.4 mg ml^{-1}). These observations are in close agreement with the previous study which indicate that the wild underutilized fruits possess eminent reducing potential (Kavitha *et al*., 2015; Aklima *et al*., 2014; Rawri *et al*., 2013).

Phytochemical properties

***Total phenolic content* (TPC) *and Total Flavonoid content* (TFC)**

Phenolic compounds are significantly important for our health as they can stimulate the reduction of hazardous factors associated with the physiological and degenerative diseases (Nayak *et al*., 2019; Aadil *et al*., 2013). Similarly flavonoids are exceptionally assorted compounds with enormous diversity in pharmacological activities like antioxidant effect and inhibition of cell proliferation (Bravo, 1998).

The amount of total phenolic content in the fruit extracts of *D. indica* and *A. squamosa* were observed and mentioned in Table.3. The TPC was estimated maximum (~14 mg

GAE g^{-1}) in *D. indica* whereas *A. squamosa* had comparatively lower value (~12 mg GAE g^{-1}). The flavonoid content in *D. indica* was estimated to be 70 mg QE g^{-1} and 54 mg QE g^{-1} in *A. squamosa*. It is suggested that the phenolic compounds are the major antioxidant components, and their antioxidant activity is directly proportional to the total content present (Kalidindi *et al*., 2015; Do *et al*., 2014). Therefore, in support to our study, it can be predicted that the presence of the total phenolic content and the total flavonoids in the extracts of *A. squamosa* and *D. indica* might have contributed to their antioxidant activity (Kalidindi *et al*., 2015).

The underutilized fruits comprises excellent amount of TPC and TFC. The polyphenolic compounds available in fruits are accountable for several pharmacological activities such as anti-inflammatory and antiglycemic properties and act as prevailing antioxidants (Abeysuriya *et al*., 2020). There are several studies indicate that phenolic compounds can be helpful in inhibiting the development of cancer and various heart disorders in humans (Arts and Hollman, 2005; Li *et al*., 2012).

Nutrient (minerals) profiles

Different macro- and micronutrients in the fruits of both the tested plants are presented in Table.4. The absence of sodium (Na) was observed in the fruits of *A. squamosa* and *D. indica*. The amount of phosphorus (P) was high in the fruits of *D. indica* (0.30%) and relatively low in *A. squamosa* (0.27%). Similarly, the content of potassium (K), calcium (Ca), magnesium and sulphur (S) was also observed to be more in the fruits of *D. indica* in comparison to that of *A. squamosa* (Table.4). The iron (Fe) content on the other hand, was found to be higher (0.86%) in the fruits of *A. squamosa*. The manganese content (Mn) in underutilized fruits, *D. indica* and *A. squamosa*, was 11 ppm and 12 ppm, respectively. The results showed that the different parts of the underutilized fruits have rich contents of phenolics, flavonoids and minerals and can be used as cheap or easy source of nutrients at the local level. These can help in providing food security and eliminating malnutrition from the country. As per a report, more than 6000 children below the age of five die every day in India due to lack of vitamin A, iron, iodine, zinc, and folic acid (Anonymous, 2006). In order to fulfill these deficiencies, underutilized fruits can serve as best and cheaper source of essential nutrients. Many of the indigenous fruits and vegetables are still not in use due to the lack of awareness, lower market demand and erratic bearing. Historically, wild fruits have been playing a significant role in human diet, especially in the form of carbohydrates, proteins, vitamins, minerals, dietary fiber and possess enormous medicinal potential. These plants could be further utilized as a source of macro- or micronutrients. The present study is also supported by (Saha *et al*., 2015), who reported that many underutilized vegetables have good content of calcium, magnesium, potassium, sodium, manganese, iron, copper and zinc. Similarly, (Gajanana *et al*., 2010) reported that various underutilized fruits like, *Phyllanthus emblica* L., *Grewia asiatica* L., *Tamarindus indica* L., *Aegle marmelos* (L.) Corrêa, *Syzygium cumini* (L.) Skeels, *Carissa carandas* L. play an important role in overcoming the problem of malnutrition due to their high nutritional value.

Conclusion

This study confirms that the underutilized fruits of both the species are rich in phytochemicals, possessing a strong radical scavenging activity and comparably high antioxidant capacity. Along with high antioxidant potential these minor fruits are prosperous in various macro-and micronutrients. Hence, the study suggests that underutilized fruits can be used as an efficient alternative and promising source of antioxidants in comparison to synthetic or artificial antioxidants. These fruits are great source of nutrients at lower values. Therefore, through awareness and commercializing these fruits can be beneficiary in fighting deficiency diseases and eradicating malnutrition in marginal communities, especially in the developing nations, and provide sources of livelihood to the local people. Additionally, being a rich source of natural antioxidants, these underutilized fruits could also serve as a source of natural antioxidants/nutraceuticals and help in promoting and securing better health in human populations and reducing the risk of many NCDs and nutrient deficiencies.

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Authors Contribution

Daizy Rani Batish and Harminder Pal Singh contributed to the study design and conception. Material preparation, data collection and analysis were performed by Anu Sharma and Anita Sharma. First draft of the manuscript was written and edited by Anu Sharma and Ikramjeet Sidhu. All the authors contributed to the manuscript.

Conflict of interest: The authors declare that there is no conflict of interest

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