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Preliminary evaluation of an underutilized Red Sitaphal (Annona squamosa L.) peel for its phytochemical composition and in-vitro antioxidant activity

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ABSTRACT

Background Red Sitaphal or Custard Apple is an underutilized minor cultivar of the common *Annona squamosa* fruit from Annonaceae family. This red fruit although produced in quite a large number, is neglected in commercial usage and processing sectors in India. It is important to explore the valorization potential of this fruit and its different parts like peels, seeds etc. to increase its consumption and reduce post-harvest losses. This study characterizes the phytochemical profile and antioxidant capacity of Red Sitaphal (*Annona squamosa* L.) peels, an underutilized agricultural by-product.

Methods Through comparative analysis between different peel extracts, we identified significant concentrations of phenols and flavonoids, found in ethanolic extract. DPPH radical scavenging assay revealed a superior antioxidant activity with ethanol extract as compared to other extracts. ***Results*** Phytochemical screening confirmed the presence of alkaloids, polyphenols, and tannins, with ethanol preferentially extracting polar antioxidants while the non-polar solvents recovered terpenoids. Quantitatively, TPC and TFC were found to be 206.36±6.08 mg gallic acid/g & 67.74±2.51mg quercetin/g, respectively which was corroborated by the fairly moderate DPPH radical scavenging activity shown in the peel extracts.

Conclusion These findings report a first comprehensive phytochemical screening for Red Sitaphal and establish their peel by-product as a source of natural antioxidants, suggesting their potential valorization in nutraceutical applications and sustainable waste reduction strategies. The distinct phytochemical profile compared to common green varieties highlights the need for cultivar-specific utilization approaches.

Keywords: *Red Sitaphal, Annona squamosa, fruit peel, phytochemicals, bioactivity, antioxidants, valorization*

1. INTRODUCTION

Annona squamosa Linn. belonging to family Annonaceae is commonly known as “Sitaphalam” in sanskrit; “Sitaphal” in hindi and “Custard apple” or “Sugar apple” in English. Also known as Sweet Sop, and Sharifa, this fruit thrives in many parts of India in its undomesticated form (Patidar et al., 2021). Among the vast number of species included in the Annona genus, only six edible fruit species are found. The evergreen shrub or small tree is found wild and cultivated in various parts of India. The fruits are always variable in shape, oblong, or irregular, about 6 - 9 cm in diameter. Size-wise, it measures between 7 and 12 cm. The fruit is yellowish-green, globose having well-defined areoles easily breaking off in larger portions, with dense pulp that bears many seeds (Jnapika et al., 2019). It is reported that this plant possesses several medicinal properties such as cardiotonic activity, antimicrobial and insecticidal activity, anti-cancerous activity. Several varieties of this fruit can be found across India, the common Green Sitaphal being the mass produced and consumed amongst all. Custard apples have wide variation in form and size of fruit as well as colour of pulp. Due to heterozygosity and cross pollination nature of Annona, there is a large genetic variation in leaf, inflorescence and fruit characters. (Agrawal, 2017; Khanbarad & Bakane, 2020; Kumar et al., 2018; Rajadurai et al., 2022)

Red Sitaphal is an underexplored cultivar of *A. squamosa* mainly grown in organized orchards in regions of Maharashtra and Karnataka. It is characterized by a distinct brownish red exocarp, and lesser percentage of seeds as compared to other varieties (Ghawade et al., 2018). The postharvest system for these fruits is not yet adequately developed, hence these fruits remain

lesser known for commercial uses (Jadhav et al., 2015). In a comparative study between different cultivars of *A. squamosa* undertaken by Nandi et al. (2018), Red Sitaphal plant was found to have a high mortality rate (81%) indicating its susceptibility to bacterial wilt and eventual death of fruits. This makes this cultivar a highly seasonal crop, thus causing difficulty for the fruits to be explored in different culinary applications or to be processed into shelf-stable products. However, a range of phenolic acids such as p-coumaric acid, o-coumaric acid, 2,4-dihydroxybenzoic acid, caffeic acid, gentisic acid, protocatechuic acid, t-cinnamic acid and ferulic acid has been detected and quantified in Red Sitaphal pulp (Shetty et al., 2020). Polyphenolic compounds are the secondary metabolites which are widely found in plant/plant derived foods and offer several health benefits due to their antioxidant properties (Li et al., 2022). Naturally, there is scanty research conducted on the suitability of the by-products from these fruits for valorization and subsequent scientific investigations on their bioactive properties. Despite its widespread cultivation, the peels of the fruit are often discarded as waste (Saleh et al., 2021). However, fruit peels have been recognized as a rich source of bioactive compounds, including polyphenols, flavonoids, and other phytochemicals, which possess potential health benefits (Kumar et al., 2021). Fruit peels including those from neglected cultivars, offer an economical and sustainable source of bioactive compounds, that can be utilized in various food and pharmaceutical applications (Hernández-Fuentes et al., 2021). Though there has been extensive research on the antioxidant activity and phytochemical content of the most commonly occurring fruits, there is a considerable knowledge gap when it comes to many tropical underutilized fruits that are present in developing countries. India also has an untapped reserve of minor fruits which remain abundant yet underutilized (García-Villegas et al., 2022). To supplement efforts towards the promotion of the role of diet in preventive medicine (Khoo et al., 2016), the peel of Red variety of *Annona squamosa* was chosen to be studied. The red variety of Sitaphal, in particular, is less common and underutilized compared to its green counterpart, offering an opportunity to explore its unique phytochemical profile. Although previous research has focused on the common green variety of Sitaphal, there is a lack of scientific studies on bioactivity and phytochemical characterization of Red Sitaphal which would help uncover its potential value-added applications for enhancing the economic viability of custard apple cultivation and promote food waste utilization. Thorough screening of literature indicated the only known comparative study done by Sekar et al. (2015) which suggested that Red variety of Custard apple had a better phytochemical profile when compared to its green fruit counterpart, although further research on particularly Red Sitaphal fruit extracts is scanty. Given the underutilized nature of Red Sitaphal peels, a comprehensive phytochemical content and antioxidant activity of the fruit peel samples were determined to identify their role as a valuable fruit by-product. Hence, this study was conducted to assess the phytochemical composition, total phenol, total flavonoids and antioxidant potential of Red Sitaphal peel. The results of this study will also provide a baseline data for future research on this fruit and its by-products.

2. MATERIALS AND METHODS

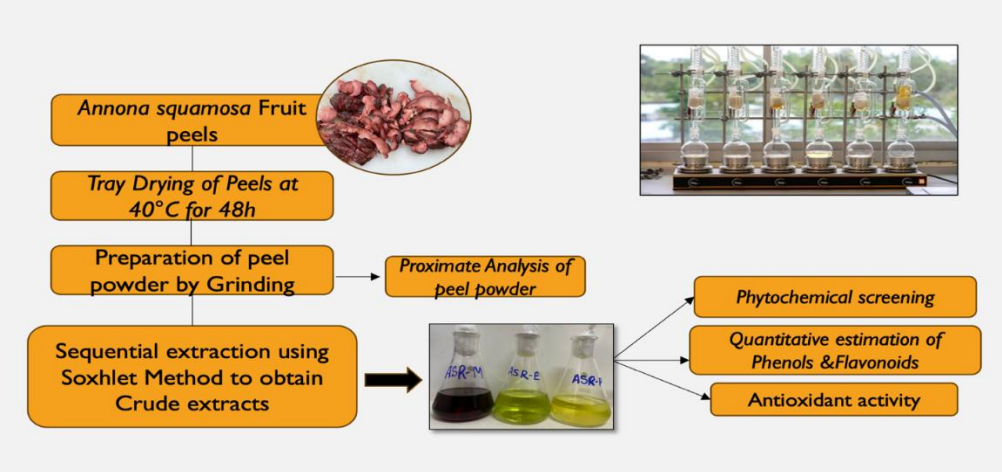

Fig 1. Standardized methodology followed for the experimental design

2.1 Plant material collection

Mature fruits of Red *Annona squamosa* (AS) were procured from the Ex-situ Biodiversity Park, University of Horticultural Sciences – Bagalkot, Bangalore Campus during the months of August and September. After harvesting, the whole fruits were immediately transferred to the Department of Food Technology, MS-RUAS for all further experiments. Twig specimens of AS plant were sent for authentication to FRLHT, TDU University, Bangalore, where it was identified with FRLHT Accession no. 127107.

2.2 Sample preparation

The whole fruits were washed thoroughly with distilled water to remove any dirt or contaminants, followed by surface sanitization using 70% ethanol & NaCl solution, respectively. The peels were then separated, cleaned and weighed manually. Fresh peels were dried using a Tray Dryer at 40°C for 2 days, followed by grinding in a mill-grinder and sieving in No. 20 mesh to obtain a fine and homogenous peel powder. The powdered samples were sealed in double layered LDPE zip-loc bags under dark, cool and ambient conditions until further analysis.

2.3 Sequential soxhlet extraction

Extraction of sample was carried out according to the method described by Vázquez et al. (2013). Ethanol, ethyl acetate and petroleum ether were used as solvents in a sequential order of polarity for the extraction in a Soxhlet apparatus, for 8 hours. The extracts were then filtered twice to remove any residue and concentrated under reduced pressure using a rotary evaporator.

2.4 Proximate analysis

The nutritional composition was investigated through proximate analysis of the dried peel sample, which included the determination of moisture content, ash, crude protein, fat, and crude fiber. All the parameters were determined according to the Association of Analytical Chemist (AOAC, 2000) standard protocols. Carbohydrate content was calculated by difference using the formula:

[100 − (moisture% ash + % fat + % protein + % fiber content)]

2.5 Qualitative phytochemical screening

Qualitative phytochemical tests (Sharma et al., 2013; Edem et al., 2016) were conducted to identify the presence of various secondary metabolites in the extracts. All the peel extracts were analyzed for the presence of phytochemicals following standard reagent tests. The tests included:

- Alkaloids: Mayer's reagent was used to detect the presence of alkaloids.
- Polyphenols: Ferric chloride was used to test for phenolic compounds.
- Flavonoids: Shinoda's test was employed to detect flavonoids.
- Tannins: Braymer's test was used to identify tannins.
- Terpenoids: Liebermann-Burchard's test was used to detect steroids and triterpenoids.
- Saponins: Frothing test was performed to identify saponins.

2.6 Total Phenol Estimation

Folin-Ciocalteu assay was used to determine total phenolic compound in sample with slight modification which followed the method described by Vo et al., 2022. Results were expressed as mg gallic acid/g sample.

2.7 Total Flavonoids Estimation

Total flavonoid content was determined by using colorimetric assay following (Zahid et al., 2019). The results were also expressed on a dry weight basis as mg quercetin/g sample.

2.8 DPPH Assay

A modified method (Jiang et al., 2016) was followed. Stock DPPH solution was prepared fresh by mixing DPPH into methanol at a concentration of 100 µmole/L. Approximately, 1 mL of sample extract from solvent extraction was mixed with 6 mL of DPPH solution. The mixture was vortexed for 1 minute and kept in dark for 30 minutes. Absorbance was read at 517 nm of wavelength using UV-Vis Spectrophotometer (Shimadzu, UVmini-1240) against blank of methanol. The result obtained was calculated and expressed in the terms of % DPPH free radical scavenging activity by using the formula stated below.

% Inhibition = [(A control – A sample) / A control] x 100 %

2.9 Statistical analysis

All experimental data obtained in the study were measured in triplicate and calculated as the mean ± SD for n=3. A one-way analysis of variance was carried out with statistical significance at $p<0.05$.

3. RESULTS & DISCUSSION

3.1 Proximate analysis

Proximate analysis was performed by using dried peel powders. The determined parameters were moisture, ash, crude protein, crude fat, crude fiber, and total carbohydrate content (Table 1). One of the most important parameters considered

during storage of any dried fruit powder is the moisture content (Kolla et al., 2021). The moisture content of the Red Sitaphal dried peel was 5.44±0.05%, which indicates a low moisture obtained via tray drying method. This helps to increase the storage stability of the sample while reducing microbial contamination.

Table 1: Proximate composition of tray-dried peel powder

Parameter	**Values*** (%)
Moisture	5.44±0.05
Ash	13.05±0.15
Fat	4.53±0.06
Protein	6.09±0.10
Carbohydrate	62.12±0.50
Fiber	8.76±0.23

***Values (mean ± SD) are average of three samples, analyzed in triplicate**

High moisture content in fresh sample decreases the quality of stored samples because the water content may possibly cause microbial growth (Kumar & Kalita, 2017). In addition, the sample showed a high ash content after drying, thereby increasing the nutrient concentrations. Moreover, the increased ash content after drying can also be explained by the low volatility of minerals, which are not destroyed by heating. The ash content represents the total amount of minerals present in a food (Yao et al., 2017).

The fat content in food matrix can be related to the oxidative rancidity, which may affect the storage life of food (Ojo et al., 2014). The peel powder showed a considerably low fat %, indicating its shelf stability. Furthermore, a protein content of 6.09±0.10% was found in the sample. Oxidative rancidity is thought to affect food storage life, likely attributed to the fat content of the respective food matrix (Ojo et al., 2014). The low fat percentage (4.53±0.06%) in the peel powder is indicative of its higher shelf life and lesser chances of developing rancidity. The sample was found to contain protein at 6.09±0.10%. This finding shows that tray drying does not effectively preserve the protein content of red A. squamosa peels (Kolla et al., 2021). Fruits, vegetables, and seeds are among the plant-based foods that provide dietary fiber to humans. The quantity of cellulose, hemicellulose, and lignin in a food makes up the crude fiber in a food (Lopez-Nunez et al., 2017). A fiber % of 8.76±0.23 demonstrates that the dried peels have a considerable amount of fiber. Fiber consumption has been positively correlated with decreasing the risk of metabolic disorders, such as cardiovascular disease, colon cancer, and diabetes (Dias et al., 2020). The carbohydrate content of the peel powders was 62.12±0.50%. These results indicated that drying increased the carbohydrate content in dried red A. squamosa peels. Similar results were obtained by research groups who studied the effect of drying on the nutritional composition of fruits (Silva et al., 2020).

3.2 Phytochemical screening

Many bioactive compounds that could be involved in health activities were obtained from the A. squamosa peel extracts (Table 2) as established by the qualitative phytochemical screening. The presence of such a diverse array of phytochemicals in Red Sitaphal peel aligns with previous studies on other Annona species and fruit peels in general (Chimbevo & Essuman, 2019; Shetty et al., 2020).

Table 2: Qualitative phytochemical analysis of Red Sitaphal peel extracts

Chemical Category	**PE extract**	**EA extract**	**Eth extract**
Phenols	+	+	++
Saponins	-	-	-
Tannins	-	+	+
Flavonoids	+	+	++
Alkaloids	+	+	+
Terpenoids	++	+	-

Key: ++ (strongly present), + (present), - (absent)

The qualitative phytochemical screening revealed the presence of alkaloids, polyphenols, flavonoids, and terpenoids, which are known for their various biological activities (Dias et al., 2020), including antioxidant, antimicrobial, and anticancer properties. While ethanol as a solvent is able to extract phenols and flavonoids as polar compounds, the non-polar solvents can extract secondary metabolites such as terpenoids (Isidore et al., 2021).

Alkaloids have been defined to have potential analgesic as well as antispasmodic actions, which may supplement the traditional therapeutic use of the plant (MAHAWAR et al., 2019). All extracts were found to contain alkaloids known for their diverse pharmacological effects, thereby suggesting wide-spectrum extraction, regardless of solvent polarity (Nugraha et al., 2019). The presence of polyphenols also indicates antioxidant activity which will protect cells from damage due to free radicals. The flavonoids available in the extracts also improve the potential benefit of A. squamosa peels in medicine as anti-inflammatory and cardioprotective. The detection of tannins suggests astringent qualities that may aid in reducing inflammation and wound healing (Vargas et al., 2020). Presence of terpenoids also suggest that the extracts have a variety of pharmacological actions, including possible antibacterial and anticancer properties. Saponin, on the other hand, was not detected in any of the extracts. This can potentially alleviate toxicity issues since saponins at high doses can occasionally cause adverse effects (Sharma et al., 2021).

These compounds impart various physiological activities such as antioxidant, anti-inflammatory, anticancer, and antimicrobial activities leading to beneficial effects in overall human health. Furthermore, polyphenols are powerful antioxidants that constantly appear, which indicates that they are more available within the Red Sitaphal peels and can be efficiently extracted using different solvents. The identification of flavonoids, well known for their anti-inflammatory and cardiovascular benefits, further points to the health-promoting capacities of the peels (Ibrahim et al., 2017). All of these findings suggest that the peels of A. squamosa may possess valuable bioactive substances to be used in traditional medicine as well as pharmaceutical and nutraceutical applications.

3.3 Estimation of Total Phenol & Total Flavonoid content

Total phenol content (TPC) and total flavonoid content (TFC) act as key indicators for the assessment of antioxidant activity in most plant extracts. These data give preliminary information about the quantifiable phytochemical composition of red Sitaphal peels and their possible health benefits. For estimation of phenol content, the solvent extracts PE, EA, and Eth of red *A. squamosa* peel were tested and the results are shown in Table 3. The TPC of the extracts are expressed in milligrams of gallic acid equivalent per gram of the extract, thus providing a standard for comparison.

Table 3. Total phenols (mg gallic acid/g) of the samples by using different extraction solvent

Type of extract	TPC*
Ethanol	206.36±6.08
Pet. Ether	91.30±5.48
Ethyl Acetate	111.39±1.22

***Values (mean±SD) are average of three samples, analyzed individually in triplicate**

Out of all the extracts, the highest phenol content was found in the ethanol extract (206.36±6.08). This difference could be attributed to the polarity difference in the solvents; ethanol is more polar and hence can extract more phenolic compounds than less polar solvents as demonstrated in similar studies with fruit peel extracts (Yang et al., 2021). Literature suggests that Folin-Ciocalteu method, despite its simple protocol and low cost, does not show absolute specificity toward phenolic compounds as it has the potential to react with other reducing agents present in the extract, such as certain sugars, ascorbic acid (Moteriya, 2015), and even some aromatic amines. Hence, the values should be considered as an overall estimation of the reducing capacity of the extract instead of an absolute measure of phenols present (Khoddami et al., 2013). However, the high levels of total phenols provide an evidential support for antioxidant potential of the peels that can be explored further to derive phenol-rich extracts with specific use cases.

The total flavonoids were also estimated quantitively (Table 4) from the different extracts and the values were expressed in terms of milligrams of quercetin per gram of each extract as a standard metric of comparison. Flavonoids being a sub-class of polyphenols are secondary plant metabolites with antioxidant, anti-inflammatory, and anticancer activities (Tazi et al., 2024). Among all the samples, ethanol extract was found to have the highest flavonoid content (67.74±2.51) followed by a moderate amount in the ethyl acetate extract (32.22±1.87). Ethanol as a solvent is efficient in extracting flavonoid compounds, as demonstrated in several other studies (Alvionita & Oktavia, 2019).

Table 4. Total flavonoids (mg quercetin/g) of the samples by using different extraction solvent

Type of extract	TFC*
Ethanol	67.74±2.51
Pet. Ether	27.68±0.71
Ethyl Acetate	32.22±1.87

*** Values (mean±SD) are average of three samples, analyzed individually in triplicate**

The TPC of Red Sitaphal peels is notably higher than many other fruit peels. For example, mango peels have a TPC of about 27.51 mg GAE/g, and grapefruit peels have a TPC of approximately 27.22 mg GAE/g (Suleria et al., 2020). Sugar apple peel extract from another Annona species have been reported to have a TPC ranging from 33.8 to 140.4 mg GA/g (Manochai et al., 2018).

It is evident from the phenol and flavonoid estimation that the highest yield for both was observed in samples with ethanol extract (206.36±6.08 mg gallic acid/g & 67.74±2.51mg quercetin/g, respectively). This consistently suggests that ethanol is more effective in extracting phenolic compounds and flavonoids from red Sitaphal peels. Thus, it can be said that the Red Sitaphal peel is an abundant source of phenolic compounds and promises to be a good alternative as a natural antioxidant, requiring further investigation of its biochemical constituents and validation of potential health benefits via in vitro and in vivo studies.

3.4 Antioxidant activity

The percent inhibition of DPPH radical reflects the antioxidant activity of the extracts (Gülçın & Alwasel, 2023). The DPPH assay results exhibited considerable variability in the percent inhibition across the different solvent extracts (Table 5).

Table 5: DPPH inhibition % of the samples by using different extraction solvent

Types of Solvent	Values*
Ethanol	68.67±2.09
Pet. Ether	20.49±1.91
Ethyl Acetate	23.59±3.09

*** Values (mean±SD) are average of three samples, analyzed individually in triplicate**

In the dried peel extracts, ethanol extract demonstrated superior antioxidant activity with the highest inhibition % of 68.67±2.09, and the lowest being in petroleum ether extract (20.49±1.91%). This finding supports the notion that the phenolic and flavonoid compounds present in the ethanol extract are primarily responsible for its antioxidant properties. The high percentage of inhibition indicates that the ethanol extract of red Sitaphal peels can be explored as a valuable source of natural antioxidant in different applications.

This study reveals that Red Sitaphal peels contain a diverse array of phytochemicals, with ethanol proving to be a more effective solvent for extraction. The high phenolic and flavonoid content in the methanol extract correlates with its strong antioxidant activity, suggesting that these compounds contribute significantly to the observed free radical scavenging properties. These findings align with previous studies indicating that fruit peels are rich in bioactive compounds (Rojas-García et al., 2022). The presence of alkaloids, polyphenols, and flavonoids in the peel extracts is consistent with the phytochemical profile of other Annona species (Kazman et al., 2022). The superior antioxidant activity of the ethanol extract indicates that peels could be a valuable source of natural antioxidants. This finding has implications for the development of functional foods and nutraceuticals, as well as potential applications in food preservation.

The presence of alkaloids, polyphenols, flavonoids, and tannins in the peel extracts aligns with previous studies on Annona species, suggesting that these compounds contribute to the observed bioactivities. The red variety of Sitaphal, being less common and underutilized, offers a unique opportunity to explore its phytochemical profile and potential applications. Further research targeted towards identification, purification as well as quantification of bioactive fractions and specific compounds from the Red Sitaphal is required. This can help establish the active molecules responsible for the observed

antioxidant activity and validate their application in different mediums.

4. CONCLUSION

The findings suggest through experimental analysis that the extracts obtained from Red Sitaphal peels possess considerable antioxidant activity, which can be attributed to the high diversity of phytochemical constituents found in this fruit and its peel. Therefore, peels from this fruit can be considered an important source of bioactive compounds with potential in-vitro antioxidant effects.

Red Sitaphal, an underutilized variety of *A. squamosa* can be explored for its peel by-products, which possess significant bioactivity. The ethanol extract, in particular, demonstrates promising properties that could be exploited for various applications. Further research is needed to isolate and characterize specific bioactive compounds and explore their potential health benefits under in-vitro and in-vivo settings. Overall, this preliminary study characterizes the minor fruit variety of Red Sitaphal and underscores its potential as a valuable source of natural antioxidants and highlights the need for advanced methods in future research to understand its full potential as a functional ingredient and establish the underlying mechanism of actions of the phytoconstituents involved.

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