Optimization of extraction conditions and evaluation of *Manilkara zapota* (L.) P. Royen fruit peel extract for in vitro α-glucosidase enzyme inhibition and free radical scavenging potential

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**Abstract**

**Background:** Most of the edible portions like peel and skin of some fruits is discarded while consuming it, though they are rich in several health beneficial phytochemicals or nutrients. Many reports from literature are about fruit pulp of (*Sapota*) *Manilkara zapota* (L) P. Royen having high radical scavenging and antioxidant potential, but the studies relating to peel extracts are scanty. Regardless of its commendable phytoconstituents which could have free radical scavenging potential, this fruit peel is as yet still needed to be assessed for in vitro antidiabetic prospects. Hence, the present study aims at evaluating in vitro free radical scavenging and α-glucosidase enzyme hindrance abilities of this fruit peel.

**Results:** With a maximum considerable % extractive yield (18.90%) in 70% ethanol, this study has demonstrated that 70% ethanolic extract of *Manilkara Zapota* (L.) P. Royen Fruit Peel (MZFP) has the highest in vitro free radical scavenging potential as compared to extracts of other solvents viz. n-hexane, chloroform, acetone, absolute ethanol, and water by DPPH and H$_2$O$_2$ assays. In order to optimize the extraction condition parameters, MZFP sample evaluated with three different concentrations of ethanol (40%, 70%, 100%), extraction times (6 h, 9 h, 12 h), and temperatures (40 °C, 50 °C, 60 °C) to get the highest radical scavenging potential. The MZFP when extracted with 70% ethanol, at 50 °C for 12 h, showed higher DPPH (IC$_{50}$ = 0.34 and 88.42% inhibition at 1 mg/ml) and H$_2$O$_2$ (IC$_{50}$ = 32.69 and 65.78% inhibition at 50 μg/ml) radical scavenging potential than absolute and 40% ethanolic extracts, when ascorbic acid was used as a reference standard. While further evaluation for in vitro α-glucosidase enzyme inhibition, 70% ethanolic MZFP extract demonstrated high inhibition activity (IC$_{50}$ = 104.23 ± 1.75 μg/ml) than absolute ethanolic extract (IC$_{50}$ = 111.65 ± 1.57 μg/ml) with a significant difference ($p < 0.05$), when acarbose was taken as reference inhibitor (IC$_{50}$ = 86.93 ± 0.74 μg/ml).

**Conclusions:** Overall results indicated that MZFP 70% ethanolic extract exhibited promising in vitro radical scavenging and α-glucosidase enzyme inhibition potential. Thus, suggesting further studies with isolated phytochemicals from peel to explore its potentials for antidiabetic activity through in vitro α-glucosidase enzyme inhibition.

**Keywords:** *Manilkara zapota* (L.) P. Royen fruit peel, Optimization of extraction conditions, α-glucosidase enzyme inhibition, Antioxidant activity

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In the era of discovering new phytochemicals from natural sources, fruit peels are found to be a new destination from where the beneficial phytochemicals search adds them to utilize them in pharmaceutical, cosmetic industries, or as a food supplement. However, nutritionally valuable components from food industry by-products are nowadays investigated and evaluated for their pharmaceutical potentials [6]. The majority of findings published so far regarding extracting bioactive constituents from fruit peels follow single-step extraction. However, single-step extraction is limited to extract bioactive phytochemicals. So extraction method and fractionation with a specific type of solvent can further add enrichment of biochemical to these extract thus improving their bioactivity considerably [7]. While the yield of such compounds like phenolic and antioxidant content in the crude extract is accompanied by the solvent concentration, extraction time, and temperature [8].

**Manilkara zapota** (L.) P. Royen fruit from the Sapotaceae family is widely cultivated across the tropical reasons of the world. The various plant parts are being used traditionally for various purposes; leaves are used to treat cough, cold, and diarrhea and gummy latex for making chewing gum [9]. The fruit is used for treatment of pulmonary diseases and the bark to treat dysentery and diarrhea [10]. *Manilkara zapota* fruit’s health benefits are not limited to its edible portion but the non-edible fruit part also plays a beneficial role in providing biologically active principles in it. MZFP contributes to higher amounts of bioactive compounds as compared to its edible pulp [11]. Therefore, the present study was designed to optimize the extraction conditions for extraction of MZFP and to investigate its follow-up during extraction, using various solvents in increasing order of their polarity to extract a high amount of phenolics and antioxidants, along with its in vitro evaluation for the antidiabetic potential. The extract with a high amount of free radical scavenging potential was further fractionated and investigated for its *in vitro* antidiabetic potential through α-glucosidase enzyme inhibition assay.

**Methods**

**Chemicals**

All the chemicals used were of analytical grade, n-hexane, chloroform, acetone, and ethanol (Merck Lif. Sci. Pvt. Ltd., India) and Baker’s yeast α-glucosidase, P-nitrophenyl-α-D-glucopyranoside, and acarbose (Sigma-Aldrich Pvt. Ltd., India).

**Collection and authentication of plant sample**

The mature, wholesome, roughly equal size, and just ripened *Manilkara zapota* (L.) P. Royen fruits were purchased from the local market of Nanded, MS, India (latitude 19.13° N and longitude 77.32° E). The
taxonomic identity, authentication (No.: -BSI/WRC/100-1/TECH./2019/68), and voucher specimen of fruit was deposited at the herbarium of Botanical Survey of India, Pune, India.

Sample preparation and extraction
The fruits were washed thoroughly with deionized water, shade dried, and peeled off with the help of a steel spoon. Peels were coarsely powdered in a pulverizer under room temperature (28°C) at University's Rashtriya Uchchatar Shiksha Abhiyan, Centre for Herbo Medicinal Studies and stored in an airtight container with wrapping in aluminum foil at room temperature in the dark.

After defatting by pet ether, the dried peel powder was individually extracted [12] using different organic solvents with increasing order of their polarity viz. n-hexane, chloroform, acetone, ethanol, and water. Five hundred milliliters of n-hexane was added to 50 g of dried peel powder and shaken in an orbital incubator shaker (Remi RIS 24+) at 150 rpm, 28°C for 2 h. Immediately after cooling down to room temperature using an ice bath, it was centrifuged (Remi Centrifuge, Mumbai, India) at 2500 rpm for 15 min, and Whatman filter paper No. 1 filtered filtrate in petri dishes were evaporated for 3 days [13]. The same procedure was followed for extraction with chloroform, acetone, ethanol, and water. All procedures were carried out at room temperature and obtained extracts were stored in airtight dark bottles at 4°C before further analysis. The extractive yield was calculated as

\[
\% \text{ Extractive Yield} = \left( \frac{\text{dry extract obtained}}{\text{weight of the extraction sample}} \right) \times 100
\]

Evaluation of antioxidant activity
DPPH assay
DPPH assay was performed to assess the radical scavenging activity by n-hexane, chloroform, acetone, ethanol, water, and ethanol aqueous fraction extracts of MZFP. The MZFP extracts samples were dissolved in methanol. Ascorbic acid was used as standard and methanol (5 ml) as blank, while for control equal volume of DPPH in methanol was used. Three milliliters solution of DPPH in methanol (0.2 mM) was mixed with 1 ml of the extracts samples of various concentrations (0.1 to 1 mg/ml). After proper mixing in test tubes incubated in dark for 40 min at room temperature then absorbance was measured at 517 nm [14–16]. The mean values ± SEM of the assay carried out in triplicate were presented. The percentage inhibition was calculated by the formula

\[
\% \text{ inhibition} = \left[ \frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}} \right] \times 100
\]

Where \( A_{\text{control}} \) is absorbance by DPPH solution and \( A_{\text{sample}} \) absorbance by DPPH solution with the extracted sample.

**H\textsubscript{2}O\textsubscript{2}-scavenging assay**
The various concentrations (10 to 50 μg/ml) of each of n-hexane, chloroform, acetone, ethanol, water, and ethanol aqueous fraction extracts of MZFP was prepared by dissolving in 4 ml of 0.1 M phosphate buffer (pH 7.4) and added to 0.6 ml of H\textsubscript{2}O\textsubscript{2} solution (43 mM). After 30 min., the absorbance of the reaction mixture was recorded at 230 nm, and then followed at every 10 min. Phosphate buffer (pH 7.4) was used as a blank sample for background subtraction [17, 18]. The % scavenging activity was compared against ascorbic acid as a standard and calculated as

\[
\% \text{ radical scavenging} = \left[ \frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}} \right] \times 100
\]

Where \( A_{\text{control}} \) is absorbance by H\textsubscript{2}O\textsubscript{2} solution and \( A_{\text{sample}} \) absorbance by H\textsubscript{2}O\textsubscript{2} solution with the extracted sample.

Optimization of extraction conditions
In accordance with high extractive yield and antioxidant activity, the optimization of extraction conditions with the ethanol solvent was carried out according to the method of Woo et al. 2013 [12] with some modifications. The first step was initiated by using three different concentrations of ethanol (40%, 70%, and 100%) for extraction. Five hundred milliliters of each above ethanol concentration solvent was added to 50 g of each dried MZFP powders. The mixtures were shaken in an orbital incubator shaker at 150 rpm, 28°C for 2 h. Then the extracts were rapidly cooled to room temperature using an ice bath, after that extracts were centrifuged at 2500 rpm, for 15 min using a centrifuge, and the supernatants were filtered by Whatman filter paper No. 1. The filtrates were then evaporated in a rotary evaporator at 40°C to remove the solvent.

The optimum concentration of ethanol solvent to extract MZFP was identified by comparing the radical scavenging (DPPH, H\textsubscript{2}O\textsubscript{2}) assays. In the second step, with optimum concentration of ethanol, by following the same procedure as above, the extraction was done with three different extraction times viz. 6, 9, and 12 h. While in the third step, with optimum concentration of ethanol and the optimum extraction time, the extraction procedure was done at 3 different temperatures for extraction viz. 40°C, 50°C, and 60°C. The whole extraction process was carried out in a dark environment and the extracts obtained were kept in dark bottles in the freezer (4°C) before further analysis.
Evaluation of in vitro antidiabetic potential

In vitro evaluation for α-glucosidase enzyme inhibition

Assay was performed as per the method described by Wan et al. (2013) and K. Savikin et al. (2018) [19, 20] with slight modifications. An optimum (about 2 ml) volume of 0.1 M phosphate buffer (pH 6.8) was prepared. Each of 200 μl Baker’s yeast α-glucosidase enzyme (0.5 U/ml) extract, MZFP extract samples in the concentration ranging from 25 to 150 μg/ml and phosphate buffer were mixed, kept for pre-incubation at 40 °C for 3 min. To start the reaction, a 200-μl of substrate p-Nitrophenyl-α-D-glucopyranoside (15 mg/10 ml) was mixed into the above mixture and again incubated at 40 °C for 25 min. An 800 μl of 0.2 M sodium hydroxide was added to this mixture to terminate the reaction. For measuring the α-glucosidase enzyme inhibition activity by sample extract, the release of p-nitrophenol from p-nitrophenyl-α-D-glucopyranoside was recorded at 405 nm. Acarbose was used as a reference α-glucosidase inhibitor. The % inhibition was calculated by the formula

\[
\% \text{Inhibition} = \frac{\text{Abs}_{405} (\text{control}) - \text{Abs}_{405} (\text{extract})}{\text{Abs}_{405} (\text{control})} \times 100
\]

Statistical analysis

The antioxidant activity study results were expressed as mean ± SEM of three determinations, while IC_{50} values were calculated by linear regression analysis by program Excel 2010. The α-glucosidase inhibition assay procedures were carried out with three determinations and mean ± SD values were determined. The plots of percent inhibition versus log inhibitor were calculated by linear regression analysis from the mean inhibitory values to estimate IC_{50} values. The presence of significant differences between inhibition by acarbose and extract samples was calculated by one-way ANOVA followed by Tukey’s multiple comparisons test at 95% confidence interval by GraphPad Prism version 9.1 (221) software. While the p values < 0.05 were considered statistically significant.

Results

Extractive yield

The water (19.25%) and 70% ethanolic (18.20%) MZFP extracts showed the highest extractive yield, while the lowest in n-hexane extract (7.50 %) (Table 1).

Evaluation of antioxidant activity

DPPH radical scavenging activity

The DPPH scavenging activities of solvents extract with increasing solvent polarity are stated concerning to the IC_{50} values. At 0.1 to 1 mg/ml, the scavenging capacity of various MZFP extracts in solvents with increasing polarity is depicted in Fig. 1. At 1 mg/ml the ascorbic acid showed 98.42% inhibition. However, the absolute ethanolic extract exhibited a high 85.64%, while a least of 75.64% inhibition was by n-hexane extract amongst the other extracts. The absolute ethanolic extract has a least IC_{50} value of 0.38 mg/ml amongst the other extracts, thus it had exhibited a high potential for DPPH radical scavenging (Table 2).

While with the aqueous fractions of ethanol, the highest of 88.42% inhibition was found at 1 mg/ml by 70% ethanolic extract, with a least IC_{50} value of 0.34 mg/ml showing it’s the highest potential to scavenge DPPH radicals than absolute ethanolic extract (Fig. 2. and Table 2).

H_{2}O_{2}-scavenging assay

The present IC_{50} value states the H_{2}O_{2} scavenging activities of various solvent extracts. At 10 to 50 μg/ml, MZFP extracts exerted solvent polarity-dependent scavenging. The absolute ethanolic extract demonstrated high (63.45%) while n-hexane extract showed a least (44.74%) inhibition at 50 μg/ml as matched to other extracts. The least IC_{50} value (34.52) by absolute ethanolic extract revealed its high scavenging potential amongst the other extracts (Fig. 3 and Table 2). While in the case of the aqueous fractions of ethanol a least IC_{50} value (32.69) by 70% ethanolic extract, with the highest of 65.78% inhibition at 50 μg/ml demonstrated it’s the highest scavenging potential (Fig. 4, Table 2).

Extraction conditions optimization

Extraction condition parameters, like solvent concentration, extraction time and temperature strongly influences on yield of antioxidants and plant phenolics. For individual extraction parameter, each of ethanolic extracts (40%, 70%, 100%) was subjected to DPPH and H_{2}O_{2} scavenging assays as identifying markers to optimize extraction condition parameters and consequently observing these assays, optimal conditions were selected. The extraction of MZFP with 70% ethanol at 50 °C for 12 h shows a high % inhibitions activity with a least IC_{50} value for these two assays Fig. (5).

| Solvent     | Initial weight (g) | % Extractive yield (w/w) |
|-------------|--------------------|--------------------------|
| n-hexane    | 50.00              | 7.50                     |
| Chloroform  | 50.00              | 10.98                    |
| Acetone     | 50.00              | 11.40                    |
| Ethanol     | 50.00              | 18.20                    |
| 70% ethanol | 50.00              | 18.90                    |
| 40% ethanol | 50.00              | 17.60                    |
| Water       | 50.00              | 19.25                    |

Table 1 The initial weight of MZFP sample and percentage extractive yield in different organic solvents
Evaluation of in vitro antidiabetic potential
α-glucosidase enzyme inhibition assay
The results were presented as percent inhibition of enzyme activity by MZFP extracts at varying concentrations (Fig. 6), while a low IC$_{50}$ values elucidate the higher inhibitory potential of tested extracts (Table 3). At 150 μg/mL the % inhibition for known α-glucosidase inhibitor—acarbose was 96.78%, while by absolute ethanol and 70% ethanol was 74.00 % and 77.08 % respectively. Acarbose shows 86.93 ± 0.74 μg/ml of IC$_{50}$ value, while 70% ethanolic extract has exhibited notable IC$_{50}$ value of 104.23 ± 1.75 μg/ml as compared to absolute ethanolic extract (IC$_{50} = 111.65 ± 1.57$ μg/ml) with a statistical significance between them when acarbose as a positive control for this assay (Graph 1).

Discussion
The percentage extractive yield from MZFP sample was found to be high in 70% ethanolic, and water extracts, while the lowest in n-hexane extract. This states its collinearity with polarity of solvents; various literature survey reveals the variance in extractive yield with different solvents [21–23]. Antioxidants scavenge 1, 1-diphenyl-2-picrylhydrazyl radical by donating protons and forms reduced DPPH. The reaction solution loses its color from purple to yellow due to electron pairing off and this reduction is measured by a decrease in absorbance at 517 nm. The decrease in intensity of the purple color is the indication of the amount of DPPH radical scavenged, which is determined in the form of IC$_{50}$ values [24]. The 70% ethanolic extract showed a least IC$_{50}$ value of 0.34 mg/ml, screening it is a high potential to scavenge DPPH radicals, amongst other extracts. These findings represent a linear correlation with phenolic contents and DPPH scavenging potential. Phenolic compounds accompany antioxidant activity, perform a good free radical scavenging and metal-ion chelation [25]. Sample extract with high phenolic content expresses the higher scavenging activity [26]. The DPPH scavenging potential of MZFP probably is due to its high variant of phytochemicals including a high amount of total phenolics, total flavonoids, or due to attribution of gallic acid, ellagic acid, quercetin, and catechin [27–29].

Hydrogen peroxide can inactivate some enzymes by oxidation of active thiol group and it also may get converted into toxic hydroxyl radicals once it enters into the cell, consequently initiating much more of its toxic effects [30]. Thus, cells need to scavenge hydrogen peroxide residing in a cell. The 70% ethanolic extract exhibited the highest of 65.78% inhibition at 50 μg/ml with a least IC$_{50}$ value of 32.69 when compared amongst other extracts. These H$_2$O$_2$ scavenging effects by MZFP extracts may attributable to its high contents of total phenolics and other biologically active constituents like gallic acid, ellagic acid, quercetin, and catechin [31].
While extracting phytoconstituents from plant samples, it is critical to recover them with the same time and temperature conditions, so the solvents used and type of phytoconstituents in samples are another two most important factors [24]. The solvent concentration, extraction time, and temperature strongly influence the extractive yield of antioxidants [32]. However, taking DPPH and \( \ce{H_2O_2} \) scavenging assays as identifying markers for optimizing the conditions for these parameters, the 70% ethanolic extract showed a high % inhibition activity with a least IC\(_{50}\) value for these two assays at 50 °C when extracted for 12 h.

In the case of type II diabetes, one of the therapeutic approaches is to delay the absorption of glucose by inhibition of \( \alpha \)-glucosidase, a carbohydrate-hydrolyzing enzyme in the small intestine. Inhibition of this enzyme retards the release of d-glucose from complex carbohydrates which grounds for a reduced postprandial plasma glucose level and consequently postprandial hyperglycemia [33]. Many recent studies prove that plant phytochemicals express anti-diabetic activity through \( \alpha \)-glucosidase inhibition. The 70% ethanolic extract has exhibited significant \( \alpha \)-glucosidase inhibition by IC\(_{50}\) value of 104.23 ± 1.75 \( \mu \)g/ml as compared to absolute...
**Fig. 4** 
H$_2$O$_2$-scavenging activity by ethanolic aqueous fractions of MZFP extract. (Values are expressed as mean ± SEM of three determinations)

**Fig. 5**  
Flow chart of Extraction condition optimization

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**Crude Drug Sample (MZFP)**

Defatting by Pet ether

Individual Extraction with

| n-Hexane | Chloroform | Acetone | Ethanol | Water |
|----------|------------|---------|---------|-------|

DPPH and H$_2$O$_2$ scavenging assay

Active extract (Ethanolic) with a least IC$_{50}$ value

Optimization of Extraction Conditions  
(Solvent concentration, Time and Temperature) for Ethanol solvent

Ethanolic concentrations

| 40% | 70% | 100% |

70% ethanolic extraction at 50$^\circ$ C for 12 h shows a least IC$_{50}$ value for DPPH and H$_2$O$_2$ assays
ethanolic extract. These findings of the moderate α-glucosidase inhibitory effects by 70% ethanolic MZFP extract inclined us to believe that it may be attributed to the abundance of the varied extent of phytochemicals in this fruit peel, which includes phenolics like gallic acid, ellagic acid, catechin, quercetin, kaempferol; flavonoids, and other compounds such as alkaloids, tannins, and saponins [31].

**Conclusions**

After optimizing the extraction conditions for MZFP, extract with a high amount of antioxidants and capable of radical scavenging was obtained with 70% ethanol at 50 °C for 12 h. The absolute ethanolic and 70% ethanolic MZFP extracts showed a marked radical scavenging potential through DPPH and H₂O₂ in vitro assays amongst the other extracts viz. n-hexane, chloroform, acetone, and water. The 70% ethanolic extract exhibited a least IC₅₀ value indicating its high potential towards radical scavenging. While with α-glucosidase enzyme inhibition assay, the 70% ethanolic extract of MZFP exhibited moderate inhibition of α-Glucosidase when compared with a standard inhibitor acarbose.

However, this preliminary investigation data provides a basis for future research where isolated compounds, as well as in vivo studies should be carried out to generate the possibilities towards exploitation for use of *Manilkara zapota* fruit peel as an alternative or complementary herbal source for the management of postprandial

**Table 3** α-glucosidase enzyme inhibition (%) by MZFP extracts

| Concentration (µg/ml) | Acarbose | 70% Ethanol | Ethanol |
|----------------------|----------|-------------|---------|
|                      | % inhibition | % inhibition | % inhibition |
| 25                   | 10.99    | 86.93 ± 0.74*µg/ml | 07.9  | 104.23 ± 1.75*µg/ml | 07.89  | 111.65 ± 1.57*µg/ml |
| 50                   | 24.99    | 19.33  | 32.18  | 48.44  | 61.42  | 77.08  |
| 75                   | 41.15    | 32.18  | 28.63  | 44.42  | 56.68  | 74.00  |
| 100                  | 59.76    | 48.44  | 44.42  | 56.68  | 74.00  |          |
| 125                  | 79.16    | 61.42  | 56.68  | 74.00  |        |          |
| 150                  | 96.78    | 77.08  | 74.00  |        |        |          |

*Mean ± SD of three replicate values, significant at p < 0.05

**Comparision of IC₅₀ values of In-vitro α-Glucosidase enzyme inhibition**
hyperglycemia in type II diabetes through α-glucosidase enzyme inhibition. The results of the present study indicate the possibility of an economical source for the production of potential supplement(s) of the antioxidant and α-glucosidase inhibitor from the natural source.

Abbreviations
MZFP: Manilkara zapota (L.) P. Royen fruit peel; DM: Diabetes mellitus; DPPH: 2-Diphenyl-1- picrylhydrazyl; H2O2: Hydrogen peroxide; IC: Inhibitory concentration

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Study involving plants
1. The plant material was authenticated by Dr. Ingle Priyanka A., Scientist ‘C’ Botanical Survey of India, Pune, India. The herbarium specimen was deposited in Plant Herbarium Division, Botanical Survey of India, Pune, India. 2. As per the local and national guidelines and legislation, and the required or appropriate permission and/or licenses for the study.

Authors’ contributions
PK carried out all experimental work, analysis, and interpretation of study results, and inscribed the major part of manuscript. SD was associated in supervising and advising experimental work. VN and SS performed noteworthy contribution in structuring the manuscript. All authors go through the manuscript in detail and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations
Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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