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# EVALUATION OF IN VITRO ANTI-DIABETIC EFFECT OF ANNONA SQUAMOSA FRUIT PEEL EXTRACT

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## ABSTRACT

The present work focused on preparing successive solvent extracts of fruit peel of *Annona squamosa* and evaluating its anti-diabetic potential using in-vitro enzyme inhibition assay methods. The extraction of the fruit peel was carried out using n-hexane, ethylacetate, methanol and water. The extraction yield was found to be 4.35%, 10.25%, 23.80% and 19.81 w/w respectively in the above solvent. The findings of the phytochemical analysis suggest the presence of alkaloids, saponin, phenolics, flavonoids and sterols the methanolic and aqeuous extracts. Flavonoids were also found in the ethylacetate extract and the hexane extracts. The calculation of the total phenolic content revealed 7.55, 25.21, 111.81 and 60.53 GAE mg/g of phenolics in the hexane, ethylacetate, methanol and aqueous extracts respectively. The calculation of the total flavonoid content revealed 20.03, 27.79, 92.37 and 49.49 QE mg/g of flavonoids in the hexane, ethylacetate, methanol and aqueous extracts respectively. The extracts were tested preliminary for their anti-diabetic action by assessing their ability to inhibit the activity of α-amylase enzyme. The α-amylase inhibition potential was found to be 1.339 ± 0.523, 12.77 ± 0.494, 38.10 ± 0.135 and 28.26 ± 0.41 % for hexane, ethylacetate, methanol and aqueous extracts respectively. The α-glucosidase inhibition potential was studied using in vitro method. The α-glucosidase inhibition potential was found to be 1.773 ± 0.166, 18.818 ± 0.251, 47.256 ± 0.074 and 33.798 ± 0.438 % for hexane, ethylacetate, methanol and aqueous extracts respectively.

**Keywords: Anti-diabetic, amylase, glucosidase, extraction, *Annona squamosa***

## INTRODUCTION

Diabetes represents a spectrum of metabolic disorders, which has become a major health challenge worldwide **[1]**. For thousands of years plants and their derivatives are being used for the treatment of diabetes. A large number of animal studies to test the claimed activity have demonstrated the anti-hyperglycemic property of many of these plants **[2, 3]**. In addition, clinical trials have shown some plants as useful anti-diabetic agents, but the pure chemical compounds isolated from the crude extracts of these plants neither bear structural resemblance to the anti-diabetic drugs in current clinical use nor have similar mechanisms of action **[4, 5]**. But still the search for a novel anti diabetic drug advocates the utilization of plants as a potential source and can be achieved by application of modern scientific technology and recent knowledge on the physiological changes in case of diabetes **[6]**.

The pharmacological potential of *Annona squamosa* was reviewed from the literature available in various journals over the internet and it was found that flavonoids, phenolics and antioxidants were present in the plant as major constituent in the leaves and fruits of the plant **[7-11]**. No systematic report was made on the plant fruit peel for its anti-diabetic activity. As many of the literature suggest that antioxidant activity and anti-diabetic potential in the fruits and leaves it was decided to perform a systematic study on the fruit peel of *Annona squamosa* extracts and evaluate its anti-diabetic potential (*in vitro*).

## MATERIAL AND METHODS

### Collection and identification of the plant material

The fruits of *Annona squamosa* were puchased from the local fruit sellers of Bhopal, Madhya Pradesh in the month of January and authenticated at botany department of RB Science, Bhopal.

### Preparation of the plant material

The authenticated plant fruits were peeled and the pulp was separated. The peel was rinsed with distilled water to remove impurities, dried under shade and powdered using a blender at low speed. The powdered fruit peel was stored in air tight container until taken for further processing.

### Extraction of phytoconstituents [12]

The powdered flowers were used for the extraction process by hot continuous extraction method using soxhlet apparatus. 85 g of fruit peel powder was evenly placed in the extractor of the apparatus and 350 mL of solvent (n-hexane) was poured over it and was allowed to collect in the attached flask.

Extraction was achieved by heating the solvent at 65°C for 9 h till a colorless solution was collected in the siphon tube of the apparatus. The extract was filtered through Whatman filter and concentrated using rotary vacuum evaporator. The resinous extract was collected and stored in desiccator to remove the excessive moisture**.** The dried extract were stored in desiccator for further processing. The marc was dried and extracted further with ethylacetate, methanol and water using the same procedure.

**Preliminary phytochemical screening [13]**

The extract was evaluated by qualitative phytochemical screening in order to identify the type of plant secondary metabolites present in it. The screening was performed for triterpenes/steroids, alkaloids, glycosides, flavonoids, saponins, tannins, and phenolic acids. The color intensity or the precipitate formation was used as analytical responses to these tests.

**Total Phenolic Content [14]**

For total phenolic content determination, 200 µL of sample was mixed with 1.4 mL purified water and 100 µL of Folin-Ciocalteu reagent. After 3 min, 300 µL of 20% aqueous $Na_2CO_3$ solution was added to it and the mixture was allowed to settle for 2 h. The absorbance was measured at 760 nm with a UV-Vis spectrophotometer. Standard solutions of gallic acid (20-100 ppm) were treated similarly to obtain the calibration curve. The control solution contained 200 µL of water and suitable reagents, and it was prepared and incubated under the same conditions as the rest of the samples. Results were expressed as milligrams of gallic acid equivalent (GAE) per 100 g of the dry sample.

**Total flavonoid content [14]**

Determination of total flavonoids content was based on aluminium chloride method. 50 mg quercetin was dissolved in 50 ml methanol, and various aliquots of 25- 150µg/ml were prepared in methanol. 0.1 g of dried extract was extracted with 10 ml methanol, filtered, and make up the volume up to 100 ml. One ml (1mg/ml) of this extract was for the estimation of flavonoid. 1 ml of 2% $AlCl_3$ methanolic solution was added to 1 ml of extract or standard and allowed to stand for 60 min at room temperature; absorbance was measured at 420 nm.

**Evaluation of Anti-diabetic activity**

**α-Amylase inhibition assay**

The extracts from the plant were screened for their α-amylase inhibition potential using the method reported by Ogunyemi *et al*. **[15]**. Briefly, 100 µL of the test substance (prepared by dissolving the extract in DMSO to obtain 25 µg/mL) was reacted with 200 µl of α-amylase and 100 µl of 2 mM of phosphate

buffer (pH 6.9) of 0.02 M sodium phosphate (pH 6.9). Subsequent to incubation for 20 min, 100 µL of 1% starch substrate was added. Blank controls where the enzyme was replaced with the buffer were also prepared alongside. Following incubation of the reaction mixture for 5 min, 500 µl of dinitrosalicylic acid reagent was mixed with the control and the test. These were kept in a water bath at a boiling temperature for 5 min. The absorbance readings were taken at 540 nm, and the percentage inhibition was calculated as follows:

$$\%\ \boldsymbol{amylase\ inhibition} = \frac{Acontrol - Atest}{Acontrol} \times 100$$

**α-Glucosidase inhibition assay**

Each of the extracts were screened for their α-glucosidase inhibition potential using the method reported by Telagari and Hullati **[16]**. Briefly, 50 µL of the test substance (prepared by dissolving the extract in DMSO to obtain 25 µg/mL) was 125 µL of α-glucosidase (0.5 U/mL in phosphate buffer solution, pH 6.9), and 700 µL of phosphate buffer was preincubated at 37 °C for 15 min. 125 µL of 5 mM *p*-nitrophenyl glucopyranoside was added, and the mixture was held for 30 min at 37 °C. The reaction was stopped by adding 1000 µL of 0.2 M $Na_2CO_3$, and the absorbance was read at 405 nm (Yellow color). Blank controls where the enzyme was replaced with the buffer were also prepared alongside. The percentage inhibition was calculated as follows:

$$\%\ \boldsymbol{glucosidase\ inhibition} = \frac{Acontrol - Atest}{Acontrol} \times 100$$

## RESULTS AND DISCUSSION

**Extraction Yields**

The extraction yield was determined by weighing the dried extract and calculating percentage with respect to the weight of the marc used for extraction **(Table 1)**.

**Phytochemical Screening**

The extract was tested for the presence of various categories of phytochemicals and the results are presented in **Table 2**. The findings of the phytochemical analysis suggest the presence of alkaloids, saponin, phenolics, flavonoids and sterols the methanolic and aqeuous extracts. Flavonoids were also found in the ethylacetate extract and the hexane extracts.

**Total Phenolic content**

The extracts were assessed for quantification of the total phenolic content. The result of the total phenolic content of the extract examined using Folin-Ciocalteu method. The calculation of the total phenolic content was done using absorbance of the test sample and the equation of calibration curve of gallic acid **(Table 3)**.

**Total Flavonoid content**

The total flavonoid content was determined taking quercetin as the reference flavonoid

component of the extracts and the results were calculated using calibration curve for quercetin. The absorbance of the samples prepared from the extracts of the plant was recorded to calculate the quercetin equivalent (QE) per gram of extract **(Table 4)**.

**α-Amylase inhibition assay**

The extracts were tested preliminary for their anti-diabetic action by assessing their ability to inhibit the activity of α-amylase enzyme. The α-amylase inhibition potential of the extracts is presented in **Table 5, Figure 1**.

**α-Glucosidase inhibition assay**

One of the enzymes that the body uses is alpha-glucosidase, which is responsible for converting carbs into glucose. To reduce blood glucose levels, medications called alpha-glucosidase inhibitors (AGIs) delay the intestinal digestion of carbs. Type 2 diabetes is treated with AGIs. The alpha glucosidase enzyme controls the rate of carbohydrate digestion in the intestines, which in turn regulates blood sugar levels. Therefore, inhibiting this enzyme can be a crucial strategy for managing postprandial hyperglycemia (high blood sugar after meals) in individuals. An alpha glucosidase inhibition test evaluates the ability of a compound or drug to block the activity of this enzyme. The α-glucosidase inhibition potential was studied using in vitro method and the results are presented in **Table 6, Figure 2**.

**Table 1: Extraction yield**

| Solvent | Weight of extract | Weight of Marc | Yield (%) | Appearance | Texture |
|---|---|---|---|---|---|
| hexane | 3.7 | 85 | 4.35 | Brown | Powder |
| ethylacetate | 8.2 | 80 | 10.25 | Green | powder |
| methanol | 16.9 | 71 | 23.80 | Dark brown | sticky |
| aqueous | 10.3 | 52 | 19.81 | Dark brown | sticky |

**Table 2: Observations of phytochemical screening**

| Chemical Tests | Observation | Hexane extract | Ethylacetate extract | Methanolic extract | Aqueous extract |
|---|---|---|---|---|---|
| **Alkaloids** | | | | | |
| *Mayer's reagent* | cream colour precipitate | - | - | + | + |
| *Hager's reagent* | yellow colour precipitate | - | - | + | + |
| *Wagner's reagent* | reddish brown precipitate | - | - | + | + |
| *Dragendorff's reagent* | reddish brown precipitate | - | - | + | + |
| **Glycosides** | | | | | |
| *Froth test* | Frothing is seen | - | - | + | + |
| *Kedde's Test* | No color | - | - | - | - |
| *Bontrager's Test* | Rose pink or red color in the ammonical layer not found | - | - | + | + |

| | | | | | |
|---|---|---|---|---|---|
| *Keller-Kiliani* | No color in acetic acid layer | - | - | - | - |
| **Phenols/Tannins** | | | | | |
| *Ferric chloride* | Blue green color | - | - | + | + |
| *Gelatin Solution* | White precipitate | - | - | + | + |
| *Alkaline reagent test* | Yellow to red precipitate | - | - | + | + |
| *Vanillin HCl test* | Purplish red color | - | - | + | + |
| **Flavonoids** | | | | | |
| *Shinoda test* | red color | + | + | + | + |
| *Alkaline reagent test* | Yellow color that turns red on acidification | + | + | + | + |
| *Zinc HCl reductino test* | red color | + | + | ++ | + |
| **Proteins** | | | | | |
| *Millon's Test* | white precipitate, turns red on heating | - | - | + | + |
| *Ninhydrin Test* | Violet color | - | - | + | + |
| **Sterols/triterpenoids** | | | | | |
| *Liberman-Burchard Test* | Brown ring at junction Upper layer turns green | - | - | + | + |
| *Salkowski Test* | Yellow color in lower layer | - | - | + | + |

**+ indicates a positive test, - indicates a negative test**

**Table 3: TPC of the extracts**

| Extract | Absorbance | TPC (GAE mg/g) |
|---|---|---|
| Hexane | 0.039 | 7.55 |
| Ethylacetate | 0.122 | 25.21 |
| Methanol | 0.529 | 111.81 |
| Water | 0.288 | 60.53 |

**Table 4: TFC of the extracts**

| Extract | Hexane | Ethylacetate | Methanol | Water |
|---|---|---|---|---|
| Absorbance | 0.015 | 0.167 | 0.548 | 0.295 |
| TFC (QE mg/g) | 2.03 | 27.79 | 92.37 | 49.49 |

**Table 5: α-amylase inhibition capacity of extracts**

| Extract (25 µg/mL) | % Inhibition of α-amylase |
|---|---|
| Hexane | 1.339 ± 0.523 |
| Ethylacetate | 12.77 ± 0.494 |
| Methanol | 38.10 ± 0.135 |
| Aqueous | 28.26 ± 0.41 |

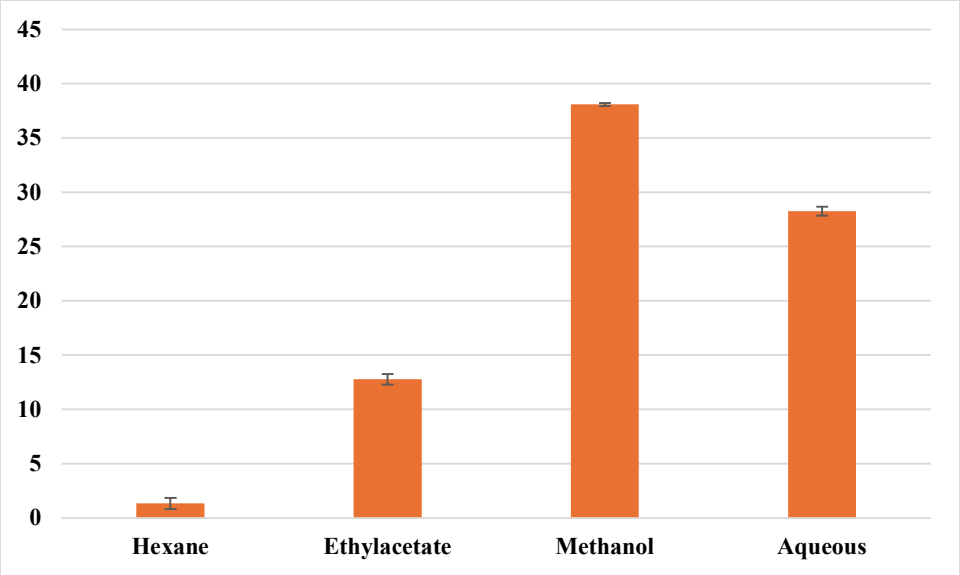


**Figure 1: α-amylase inhibition by extracts**

**Table 6: α-glucosidase inhibition by extracts**

| Group | %Inhibition of glucosidase |
|---|---|
| Hexane | 1.773 ± 0.166 |
| Ethylacetate | 18.818 ± 0.251 |
| Methanol | 47.256 ± 0.074 |
| Aqueous | 33.798 ± 0.438 |

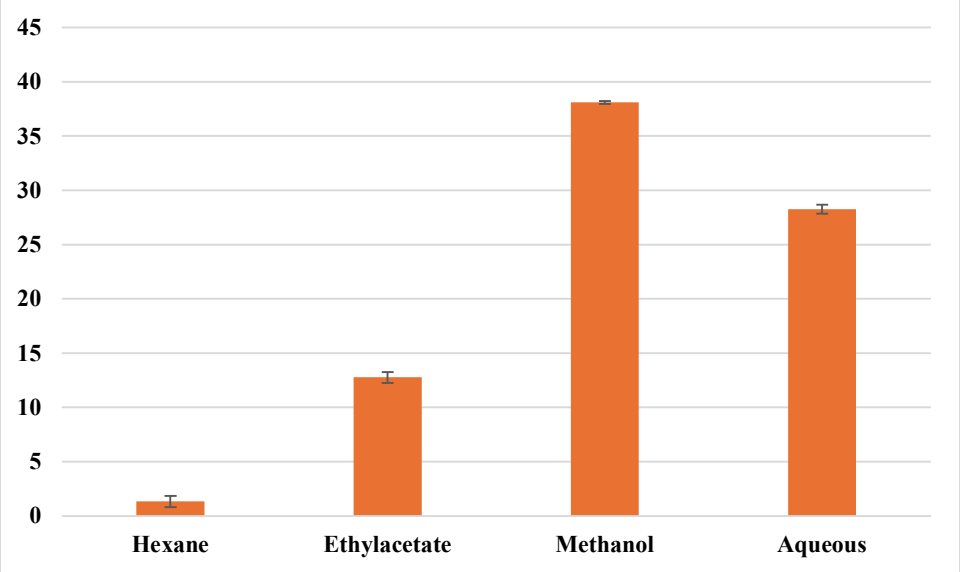


**Figure 2: α-Glucosidase inhibition by extracts**

## CONCLUSION

The objective of this work was to obtain extracts of *Annona squamosa* fruit peel using hexane, ethylacetate, methanol and water as the solventa; identify the class of secondary metabolites present in the extract; determine

the total phenolics and flavonoids in the extract; and evaluate the anti-diabetic property of the extract using α-amylase and α-glucosidase inhibition assays. The results obtained from the study led to the conclusion that the fruit peel of the plant possess phenolics and flavonoids that are beneficial in maintaining the lipid profile as well as the glucose levels in STZ induced diabetes in reducing the activity of enzymes α-amylase and α-glucosidase. Hence it could be concluded from the study that *Annona squamosa* fruit peel possesses anti-diabetic activity and could be explored for the particular constituents responsible for their action.

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