ANTIOXIDANT AND ANTIDIABETIC POTENTIALS OF *Cucurbita pepo* LEAVES EXTRACT FROM THE GULF REGION

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ABSTRACT

Cucurbita pepo has been known to be used as a folk medicine for treating many disorders such as ulcers, intestinal parasites, urinary tract disorders, and as an anti-diabetic agent in the gulf region. Quantitative analyses of ethyl acetate solvent of *C. pepo* leaves were performed, and the total phenol and flavonoid contents were quantified as 32.6 ± 0.17 mg GAE/g and 80.5 ± 0.02 mg RUE/g, respectively. The results of antioxidant studies by DPPH method had shown that CPLE has an IC_{50} : 49.31 ± 0.21 μ g/mL and in ABTS assay CPLE showed an IC_{50} : 48.67 ± 0.27 μ g/mL. In antidiabetic analysis studies using α -amylase enzyme inhibitory assay, it was found that CPLE exhibited an IC_{50} : 24.99 ± 0.07 μ g/mL contrasted to Acarbose (standard drug) with an IC_{50} : 19.45 ± 0.19 μ g/mL. Also, in α -glucosidase enzyme inhibitory assay, CPLE exhibited IC_{50} : 22.29 ± 0.27 μ g/mL, whereas acarbose exhibited IC_{50} : 16.70 ± 0.99 μ g/mL. CPLE can be used and suggested as a remedy for radical scavenging and diabetes therapy after further clinical investigations.

Keywords: *Cucurbita pepo*, Antioxidant, Anti-diabetic, α -amylase, α -glucosidase.

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INTRODUCTION

Plants throughout history were considered a thorough part of pharmacotherapy; hence the development of natural medicine is focussed.¹ Many medicinal or ancient-used plants have been used as complementary medicine and pharmaceutical manufacturers are still working on plant research to expand information and to conduct novel discoveries. *Cucurbita pepo* is an annual plant that was domesticated throughout the Americas; it belongs to the family Cucurbitaceae. *C. pepo* could also be referred to as squash, pumpkin, zucchini, courgette, and gourd has a total of eight divisions according to their shapes and varies slightly in their compositions, the squash leaves were used in the present study. *C. pepo* leaves are considered edible, especially in Korea, India and in many places of Africa and it has been used as an anti-diabetic agent and also for parasite or worm management in many countries as a folk medicine² and it also proved to show significant improvement in benign prostatic hyperplasia related urinary symptoms.³ The Kingdom of Saudi Arabia globally is considered among the top 10 highest countries according to diabetes mellitus prevalence.⁴

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Recently, research is focused on *C. pepo*, which proved to possess antibacterial⁵, antioxidative⁶, analgesic, and anti-inflammatory activities.⁷ Pumpkin seeds are known to possess active ingredients, magnesium salts, zinc, linoleic acid, stearic acid, oleic acid and stearic acid. Pumpkins also comprise biologically active elements like peptides, proteins and polysaccharides.⁸

Antioxidants, both natural and synthetic play an important role in preventing or delaying cellular damage caused by oxidative stress or free radicals. Most of the biological studies on *C. pepo* were focused on its fruit hull or pulp and there are only limited studies reported on leaves.^{9,10} To our comprehension, this is the preliminary scientific finding incorporating the potential use of *C. pepo* leaves as an antioxidant and an anti-diabetic agent from a Saudi Arabian geographical zone (gulf region). Based on the literature, we planned to investigate the phytochemical contents, antioxidant, alpha (α)-amylase, and alpha (α)-glucosidase inhibitory potentiality of *C. pepo* leaf ethyl acetate extract.

EXPERIMENTAL

Material and Methods

Gallic acid (GA), 2, 2'-Azino-bis (3-Ethylbenzothiazoline-6-sulphonic acid (ABTS), Rutin, intestinal α -glucosidase, and pancreatic α - amylase procured from Sigma-Aldrich Corporation (USA). 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) procured from Cayman Chemical Company (USA). Reagents and chemicals used in the analysis were obtained from Fouz Chemical Company (Saudi Arabia).

Plant Material

C. pepo leaves were obtained from Rama farms, Al-Muthnab, Al-Qassim, Kingdom of Saudi Arabia. *C. pepo* leaves cultivated collected in a clean bag, washed thoroughly with tap water to remove soil and dirt from their surface. The leaves were distributed and kept to dry in a hot area avoided direct sunlight. After drying, the leaves were grounded and used for extraction through the maceration technique. The *C. pepo* leaves (plant material) were authenticated by Qassim University, Saudi Arabia (Reference No: QA/FOP/02).

Extract Preparation

The maceration method was used for the preparation of *C. pepo* leaves extract (CPLE). Powdered 150 g of leaves were added to 500 mL of ethyl acetate and the mixture was incubated on a rotatory shaker at 100 rpm continually for 5 days under room temperature and a stepwise extraction from the remaining plant material was performed. The procedure was performed in cycles until a colourless supernatant was observed then filtered the extract through a muslin cloth.¹¹ The filtrate obtained was evaporated using a rotary flash evaporator.¹² The resulting extract was freeze-dried and transferred to an airtight container and stored in a desiccator. The percentage yield was calculated, and the extract was used for further studies.

Qualitative Phytochemical Analysis

Preliminary screening of phytochemicals including protein, alkaloids, carbohydrate, steroids, glycosides, non-reducing polysaccharides (starch), flavonoids, phenol, gums, saponin and tannins of *C. pepo* leaves extract using standard biochemical testing methods was performed as described.¹³

Quantitative estimation of Total Phenolic Content (TPC)

The mixture was made by combining each of 0.1 mg/mL ethanolic solution (0.5 mL) of extract, 1% Folin–Ciocalteu's reagent (2.5 mL), along with 0.75% sodium bicarbonate solution (2.5 mL). The standard drug, Gallic acid concentrations, are prepared in concentrations of 0.1 - 1.0 mg/mL in ethanol. The samples and the standard were incubated at 37°C (30 min). The absorbance reading was taken at 765 nm from a UV/Vis spectrophotometer. The samples were done in triplicates and the absorbance means were acquired. The absorbance values were represented as mean $\pm$ standard deviation (SD). The blank solution was prepared concomitantly with ethanol rather than extract solution. The calibration line was plotted using Gallic acid values. The TPC of CPLE was calculated and expressed as Gallic acid equivalent in GAE mg/g.^{12,14} The total phenol content (TPC) can be calculated using eqn.-1:

$$\text{TPC} = (C \times V) / m \quad (1)$$

C= Concentration of GA from the graph calibration curve in ($\mu\text{g/mL}$), V= Sample volume solution (mL), m= extract weight (g).

Quantitative Estimation of Total Flavonoid Content (TFC)

To estimate TFC of CPLE, a modified spectrophotometric method was used. 0.2 mg/mL CPLE prepared using methanol as solvent. Rutin was used as a standard drug and prepared in concentrations ranging from 10-1000 µg/mL in methanol solvent. The reaction mixture is prepared by combining 3 mL of CPLE and 3ml of 2% aluminum chloride solution which was dissolved in methanol. Then it was kept at 37°C for an hour. The absorbance reading was noted at 415nm from UV/Vis spectrophotometer. The samples were done in triplicate and the absorbance mean was acquired and the data were represented as mean ± Standard Error Mean (SEM). The same process was repeated for the Rutin and the calibration line was plotted. The TFC was calculated and termed as mg Rutin equivalents per gram of dry weight (RUE mg/g)^{12,15}. The TFC can be calculated using eqn.-2:

$$\text{TFC} = (\text{C} \times \text{V}) / \text{m} \quad (2)$$

C= Concentration of Rutin from the graph calibration curve in (µg/mL), V= Sample volume solution (mL), m= extract weight (g).

In-Vitro Antioxidant Studies

DPPH Free Radical Scavenging Method

Antioxidant activities of CPLE were determined using DPPH reagent (2 mM in 95% ethanol) and were kept in the dark for at least 30 min before adding to extract. DPPH was chosen due it is stability as a free radical. Both the extract (sample) and ascorbic acid (standard) were prepared in a concentration range of 10 - 1000 µg/mL, respectively, with ethanol. Sample stock was prepared by adding 500 µL of the extract with 500 µL of the DPPH solution. From the stock solution, variant concentration of sample and standard were prepared using the dilution method. Standard concentrations are also prepared in the same method. Control was prepared using 500 µL of 95% ethanol and 500 µL DPPH solution. All the solutions were kept at 45°C (20 min) and the absorbance reading was taken at 517 nm from UV/Vis spectrophotometer. All assays were carried out in triplicates and the mean of each absorbance was recorded. The values are termed as mean ± SEM (Standard Error Mean)¹⁶. Inhibition % of test compounds were calculated using the eqn.-3:

$$\% \text{ Inhibition} = [(\text{Control Absorbance} - \text{Sample Absorbance}) / \text{Control Absorbance}] \times 100 \quad (3)$$

ABTS Free Radical Scavenging Method

The antioxidant ability of CPLE was further estimated using ABTS scavenging method using the procedure described earlier.^{11,17} The absorbance reading was taken at 734 nm using a UV/Vis spectrophotometer. Accordingly, the procedure was carried out for ascorbic acid (standard) and the % scavenging (free radicals) was quantified from eq.-3.

Antidiabetic Studies

Alpha-Amylase Inhibition Method

Mixture 500 µL of CPLE and acarbose at different concentrations (100 - 1000 µg/mL) were treated each with 500 µL α-amylase solution (0.5 mg/mL) in phosphate buffer (0.2 mM ; pH 6.9) respectively. Both standard and samples were subjected to incubation for 10 min at 25°C. Then, 1% starch solution was added into the solutions continued with incubation for another 10 min at 25°C. Then, dinitro salicylic acid color reagent (1 mL) was added and incubated in water to boil for 5 min. After incubation, they were left for cooling to room temperature. The reaction mixture was further diluted with distilled water (10 mL). The absorbance reading was taken at 540 nm using UV/Vis spectrophotometer. The procedure was done for the control using the solvent without the extract. All assays were carried out in triplicates, and the mean of each absorbance was recorded. The values are expressed as mean ± SEM¹⁸. The % inhibition of α-amylase was quantified from eqn.-3.

Alpha Glucosidase Inhibition Method

A mixture of 1 mL of acarbose and CPLE (100-1000 µg/mL) was mixed with 1mL of α-glucosidase (1 U/mL) at 37°C for 5 min. Accordingly, 2% sucrose (1 mL) in Tris buffer (pH 8) was added and kept for 20 min. The mixture was withheld by water to boil for approximately 2 min. Then, the glucose release was

quantified (glucose peroxidase method) using a standard drug (Acarbose).¹¹ The % inhibition was obtained from eqn.-3.

Statistical Analyses

All *in-vitro* quantitative analysis was done in triplicates and the values were stated as mean $\pm$ SD, while antioxidant and antidiabetic study results were expressed as mean $\pm$ SEM. The inhibition concentration acquired for 50% (IC₅₀) inhibition of enzymes along with free radicals was obtained by a non-linear regression graph plotted between the inhibition percentage *versus* log concentrations on x and y axis respectively, using a Graph Pad Prism Software (Version 5.03).

RESULTS AND DISCUSSION

Numerous biological studies on *C. pepo* were centered on its fruit hull, seeds, pulp¹⁹ and there are only partial studies reported on leaves in south.²⁰ This is the first report incorporating the potential use of *C. pepo* leaves from a Saudi Arabian geographical zone. *C. pepo* leaves extract was prepared from three macerations in a step-wise manner using ethyl acetate as a solvent, the percentage extraction yield of CPLE in our study was found to be 30% w/w. Ethyl acetate was used as a solvent since it showed the highest flavonoid content among all other solvents of extraction of *C. pepo* leaves in a previous study.²¹ Preliminary screening of phytochemicals of CPLE was conducted and proved the presence of phenols, proteins, flavonoids, alkaloids, saponins, glycosides, gum, carbohydrates, and starch using standard biochemical testing methods¹³. These results are similar to a previously conducted study on phytochemical constituents of *C. pepo* leaves extract using ethyl acetate solvent conducted in India. Phenol and flavonoid both are important phytochemical constituents that have been found in many plants, including vegetables and fruits. The polyphenol content correlates to the antioxidant effect of the plant.²² Polyphenols are used as antioxidants in food industrials and pharmaceutical manufacturers because of their beneficial health effects. Phenol and flavonoid as polyphenols had anti-diabetic, anti-hypertensive, anti-inflammatory, cardiac, and cerebrovascular protection effects along with its antioxidant effect^{23,24} For the present study, the Folin-Ciocalteu procedure was processed to estimate the TPC, and Gallic acid (reference standard) was used. The calibration curve was presented in a range of 10-1000 $\mu\text{g/mL}$ (Fig.-1a). The regression value was quantified as $R^2=0.9786$ and the TPC were estimated as 32.6 ± 0.17 mg GAE/g. The Rutin reagent was used as a standard in the TFC estimation. Subsequently, the calibration curve of TFC was set in a range of 10-1000 $\mu\text{g/mL}$ (Fig.-1b). The regression value estimated was quantified as $R^2=0.9822$. The TFC in CPLE was estimated as 80.5 ± 0.02 mg RUE/g.

Fig.-1: Standard Calibration Curve (n=3) (a) Gallic Acid for TPC (b) Rutin for TFC

An antioxidant assay using DPPH and ABTS analyses was chosen as sensitive, convenient, and rapid methods to determine radical scavenging abilities²⁵, and the results were presented in Fig.-2a and Fig.-2b. DPPH assay estimated to have a comparably good antioxidant activity with an IC₅₀ : 49.31 ± 0.21 $\mu\text{g/mL}$ contrasted to standard ascorbic acid with an IC₅₀ equals 33.66 ± 0.17 $\mu\text{g/mL}$. As well as ABTS analysis showed an IC₅₀ : 48.67 ± 0.27 $\mu\text{g/mL}$ contrasted to the standard, which showed an IC₅₀: 33.39 ± 0.11 $\mu\text{g/mL}$.

Fig.-2: Radical Scavenging Effect of Ascorbic Acid and CPLE (n=3) (a) DPPH Method (b) ABTS Method

Amylases and glucosidases enzymes act as carbohydrates digestive enzymes that lead to an increase in the digested glucose level in diabetic patients. The inhibition of alpha glucosidase and alpha amylase is especially beneficial in delaying the digestion and absorption of carbohydrates without affecting insulin secretion hence, reducing the risk of development of diabetes mellitus.²⁶ In this assay, the enzymatic inhibitory activities were observed using acarbose as a standard drug. The percentage inhibition versus concentration of both CPLE and standard is depicted in Fig.-3a and Fig.-3b. CPLE showed a good alpha amylase inhibitory activity with an IC_{50} of 24.99 ± 0.07 $\mu\text{g/mL}$ compared to the standard drug, acarbose which showed an IC_{50} : 19.45 ± 0.19 $\mu\text{g/mL}$. Also, in α -glucosidase enzyme inhibitory assay, CPLE showed an IC_{50} : 22.29 ± 0.27 $\mu\text{g/mL}$, whereas acarbose showed an IC_{50} equal to 16.70 ± 0.09 $\mu\text{g/mL}$.

Fig.-3: % Enzyme Inhibition of CPLE and Acarbose (n=3) (a) α -amylase (b) α -glucosidase.

CONCLUSION

The *in-vitro* studies on ethyl acetate extract of *Cucurbita pepo* leaves in the present research had concluded the extract potentiality of both antioxidant and anti-diabetic properties. The extract resulted in a high polyphenols content of both the flavonoids and phenols that correlate to the reduction of free radicals or antioxidant activities. Moreover, the extract had shown a significantly high inhibition of alpha glucosidase and alpha amylase enzymes that are beneficial in dietary carbohydrates-induced hyperglycemia. Antioxidant and antidiabetic activities exhibited by natural plants may have the capability to produce the same effect and even better efficacy that could be safer than synthetic agents hence, further investigations on clinical studies on CPLE must be conducted in future studies.

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