

α -Amylase and α -Glucosidase inhibitory activity assessment of *Cucurbita maxima* seeds – A LIBS based study

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Abstract

The aim of the present study was to investigate, α -amylase and α -glucosidase inhibitory activities of the aqueous extract of *Cucurbita maxima* seeds *in vitro* and to correlate their activity with their phytoelemental profile quantitatively assessed by Laser-Induced Breakdown Spectroscopy (LIBS). Diabetes can be managed by controlling postprandial hyperglycemia which can be achieved by inhibiting carbohydrate hydrolyzing enzymes like α -amylase and α -glucosidase. Results reveal that the *C. maxima* seeds have appreciable α -amylase inhibitory activity of $46.03 \pm 1.37\%$ with IC_{50} value at 7.00 ± 0.29 mg ml⁻¹ in addition to substantial α -glucosidase inhibitory effect of $35.11 \pm 1.04\%$ with IC_{50} at 8.11 ± 0.36 mg ml⁻¹. Acarbose was used as a reference. LIBS analysis showed the presence of certain phytoelements viz. Mg, Ca, K and Na which are well known glycemic elements and hence could be responsible for inhibitory activity of carbohydrate hydrolyzing enzymes. Thus, α -amylase and α -glucosidase inhibitory action of *C. maxima* seeds would be responsible for their antidiabetic activity. The glycemic elemental profile further validates their role in controlling diabetes and hence *C. maxima* seeds could be explored as a potential herbal candidate for managing postprandial hyperglycemia causing type 2 diabetes mellitus.

Keywords : *Cucurbita maxima*, α -amylase; α -glucosidase; *in vitro* antidiabetic; LIBS

Introduction

Amylases such as α -amylase and α -glucosidase, are key enzymes in the digestion of carbohydrates. Inhibition of these enzymes helps in managing diabetes by significantly delaying carbohydrate digestion and its absorption. With the continuous growth in the incidence of type 2 diabetes, it is important to explore medicinal plants with α -amylase and α -glucosidase inhibitory activities as potential herbal alternatives. Thus, the objective of this study was to assess the α -amylase and α -glucosidase inhibitory activities of *C. maxima* seeds *in vitro*.

Though, a number of pharmacological approaches are used to control diabetes by different modes of action but decrease in conversion of carbohydrate into glucose and its absorption from the intestine play a vital role in keeping a check on postprandial hyperglycemia [1]. Hence, glycemic control can be achieved using oral herbal agents which interferes either with the conversion of carbohydrates into glucose or with the glucose absorption from the intestine.

Thus, plant based natural inhibitors of α -amylase and α -glucosidase could be developed as phytotherapeutic agents for the treatment of diabetes involving the decrease in postprandial hyperglycemia by inhibiting conversion of carbohydrate into glucose and then its absorption from the intestine. This inhibition reduces glucose absorption through delayed carbohydrate digestion and extended digestion time [2,3].

Some specific elements present even in traces help in controlling the metabolic processes of the biological system [3] therefore, it is in the interest of the scientists to identify the presence of certain set of elements and their role in managing carbohydrate metabolism with special reference of activity of enzymes involved in the process. Recently, Laser-Induced Breakdown Spectroscopy (LIBS) has emerged as a sensitive, reliable and rapid analytical tool for identifying the complete elemental profile of any medicinal plants. LIBS has proved its utility for rapid, *in situ*, real-time analysis of samples present in any phase, viz. solid, liquid and gas. Our research group has reported several LIBS based studies unfurling

the correlation between bioactivity and elemental profile of medicinal plants [4,5].

Inhibition of enzymes by metal ions is of considerable importance and has been studied extensively. Therefore, to identify the complete elemental profile of medicinal plants is the strategy of the present study, in order to find out the elements responsible for inhibitory activity of these enzymes.

Since, *C. maxima* seeds have already been identified by our research group as an antioxidant being rich in polyphenols [5] therefore, these seeds are of our choice for evaluating their *in vitro* inhibitory activities, against α -amylase and α -glucosidase as polyphenols are not only capable of reducing oxidative stress but also of inhibiting carbohydrate hydrolyzing enzymes activity to prevent hyperglycemia [6]. In addition to it, identifying their LIBS based glycemic elemental profile, is also a relevant part of the study.

C. maxima Duch. (family: Cucurbitaceae) commonly known as pumpkin is an annual herb. The plant is native to North and Central America [7]. Nowadays they are cultivated in almost all areas of the world. It is used as a vegetable and also in the traditional system of medicine [8]. Their fruits are the most valuable part with high nutritional value [9].

The present study is the first reporting of its type which describes the LIBS based glycemic elemental profile of *C. maxima* seeds and their role involved in α -amylase and α -glucosidase inhibitory activities.

Material and Methods

Plant material

The seeds of *C. maxima* plant were procured from the local market of Allahabad, India and authenticated by Prof. Satya Narayan, Taxonomist, Department of Botany, University of Allahabad, Allahabad, India. A voucher specimen has been submitted to the University herbarium (No. MRL/CM/01). The seeds were washed well with water and dried in shade. The shade dried seeds were powdered and extracted with hot distilled water. Extract obtained was filtered, concentrated and lyophilized till constant weight. The dry powder so obtained was stored at -40 C for further use.

In vitro antidiabetic assay

Carbohydrate hydrolyzing enzyme inhibitory activity assays were performed at different concentrations of extract for α -amylase and α -glucosidase. The results were compared with acarbose, the reference. The IC₅₀ values of extract and acarbose were calculated. A lower IC₅₀ value indicates higher inhibitory activity.

Alpha -amylase inhibitory assay

This assay was carried out using a modified procedure [10]. A total of 250 μ L of extract of varied concentration ranging from 1.0 to 10 mg ml⁻¹ was placed in a tube and 250 μ L of pancreatic α -amylase solution (0.5 mg ml⁻¹) in 0.02 M sodium phosphate buffer (pH 6.9) was added. The mixture was incubated at 25°C for 10 min, after which 250 μ L of starch solution (1 %) in 0.02 M sodium phosphate buffer (pH 6.9) was added. This reaction mixture was again incubated at 25 C for 10 min. The reaction was finally quenched by 500 μ L of a reagent, 96 mM 3,5-dinitrosalicylic acid (DNS), and further incubated in boiling water for 5 min and then cooled to room temperature. The content of each test tube was diluted with 5 ml distilled water and the absorbance was measured at 540 nm in a spectrophotometer. Absorbance of control was also measured. The assay was carried out in triplicate. The results were expressed as percent inhibition of α -amylase activity using the following formula:

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

Alpha-glucosidase inhibitory assay

This assay was carried out according to the method described by Kim et al. [11] using α -glucosidase from *Saccharomyces cerevisiae*. A total of 50 L of extract of varied concentration ranging from 10 to 20 mg ml⁻¹ was placed in a tube and 100 L of α -glucosidase (1.0 mg ml⁻¹) in 100 mM sodium phosphate buffer (pH 6.9) was added. The mixture was incubated at room temperature for 10 min, after which 50 L of substrate solution, p-nitrophenylglucopyranoside (pNPG) (3.0 mM) in 0.02 M sodium phosphate buffer (pH 6.9) was added. This reaction mixture was again incubated at 37 C for 20 min. The reaction was finally quenched by 2 ml of 0.1 M Na₂CO₃. The absorbance of the yellow colored p-nitrophenol, released from pNPG, was measured at 405 nm. Absorbance of control was also measured. The assay was carried out in triplicate. The results were expressed as percent inhibition of α -glucosidase activity using the following formula:

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

Experimental setup for LIBS

LIBS spectra were recorded using laser wavelength of 532 nm with pulse duration of 4 nanoseconds. The sample was focused with a pulsed laser beam from a Q-switched Nd: YAG (Neodymium: Yttrium-Aluminum-Garnet) laser (Continuum Sure-Lite III-10) using a Quartz converging lens of 30 cm focal length. Consequently, the temperature of the locally heated region rose rapidly which led to plasma formation on the sample surface. The light emitted from micro-plasma was collected using an optical fiber tip placed in the vertical plane at 45° with respect to the laser beam and finally fed into an entrance slit of the spectrometer (Ocean Optics LIBS2000+) equipped with CCD (charge-coupled device) and 4 gratings. The initial three gratings had the resolution of 0.1 nm covering the wavelength range from 200–310 nm, 310–400 nm and 400–510

nm, respectively, while the fourth grating, called as broadband grating, covered the wavelength range from 200-1100 nm and had a resolution of 0.75 nm. For recording the LIBS spectra at 1 Hz laser frequency and 100 mJ laser energy, all the four gratings were used simultaneously. The sample solution was prepared by dissolving 1.0 g of lyophilized material in 10 ml of distilled water. Several experiments were carried out by varying the experimental parameters such as laser power, lens to sample distance, the position of collection emission optics with respect to plasma plume to get best S/N (Signal to Noise) and S/B (Signal to Base) ratio. In order to get better S/N ratio average spectra was taken, whereas for enhanced S/B ratio, continuum background was reduced. The optimum results were found at laser energy 100mJ; a lens to sample distance 30 cm, tip of fiber bundle at 45° with respect to the laser beam. Each LIBS spectrum is average of 100 laser shots [12].

Statistical analysis

The entire group of data was statistically evaluated using one-way ANOVA, followed by a post hoc Scheffe's test using the SPSS computer software, version 7.5. The values were considered significant when $P < 0.05$. Experiments were done in triplicate and the mean value was reported as mean \pm S.D.

Results

Alpha -amylase inhibitory activity

Figure 1a, shows the results of percentage inhibition of α -amylase activity by the aqueous extract of *C. maxima* seeds (CMSE). It clearly reveals that the inhibition was concentration-dependent varied from $1.82 \pm 0.01\%$ to $46.03 \pm 1.37\%$ for the lowest evaluated concentration of 1 mg ml^{-1} to the highest evaluated concentration of 10 mg ml^{-1} , respectively. Thus, the maximum α -amylase inhibition produced by CMSE was $46.03 \pm 1.37\%$ at a concentration of 10 mg ml^{-1} .

Figure 1b, depicts the results of percentage inhibition of α -amylase activity by acarbose. It also exhibited the similar concentration-dependent response that varied from $29.25 \pm 1.02\%$ to $98.14 \pm 1.74\%$ for the lowest evaluated concentration of 0.1 mg ml^{-1} to the highest evaluated concentration of 1 mg ml^{-1} , respectively. Thus, the maximum α -amylase inhibition produced by acarbose was $98.14 \pm 1.74\%$ at a concentration of 1 mg ml^{-1} .

Moreover, the IC_{50} values for CMSE as well as acarbose were found to be $7.00 \pm 0.29 \text{ mg ml}^{-1}$ and $0.2 \pm 0.01 \text{ mg ml}^{-1}$, respectively.

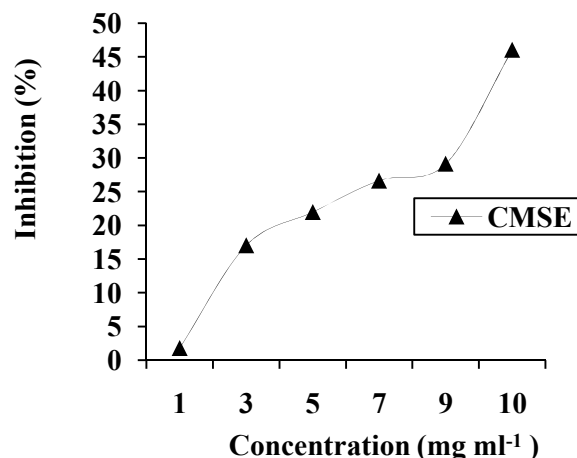


Figure 1a: Inhibition (%) of α -amylase activity by *C. maxima* Seeds. Each value is presented as mean \pm S.D. (n=3)

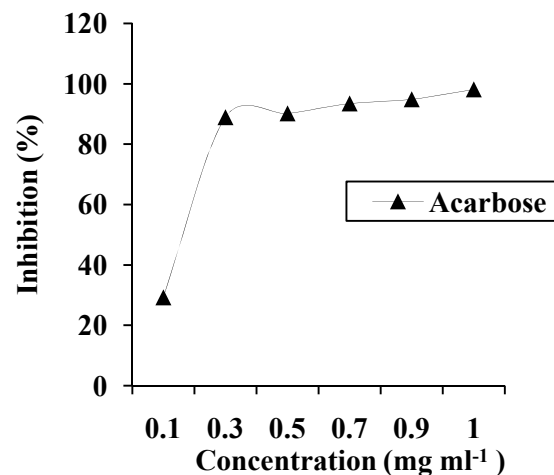


Figure 1b: Inhibition (%) of α -amylase activity by Acarbose. Each value is presented as mean \pm S.D. (n=3)

Alpha-glucosidase inhibitory activity

Figure 2a, reveals the results of percentage inhibition of α -glucosidase activity by the aqueous extract of *C. maxima* seeds (CMSE). It clearly reveals that the inhibition was concentration-dependent varied from $1.82 \pm 0.01\%$ to $35.11 \pm 1.04\%$ for the lowest evaluated concentration of 10 mg ml^{-1} to the highest evaluated concentration of 20 mg ml^{-1} , respectively. Thus, the maximum α -amylase inhibition produced by CMSE was $35.11 \pm 1.04\%$ at a concentration of 20 mg ml^{-1} .

Figure 2b, demonstrates the results of percentage inhibition of α -glucosidase activity by acarbose. It also exhibited the similar concentration-dependent response that varied from $59.31 \pm 1.40\%$ to $95.52 \pm 1.90\%$ for the lowest evaluated concentration of 1 mg ml^{-1} to the highest evaluated concentration of 10 mg ml^{-1} , respectively. Thus the maximum α -amylase inhibition produced by acarbose was $95.52 \pm 1.90\%$ at a concentration of 10 mg ml^{-1} . Moreover, the IC_{50} values for CMSE as well as acarbose were found to be $8.11 \pm 0.36 \text{ mg ml}^{-1}$ and $1.83 \pm 0.01 \text{ mg ml}^{-1}$, respectively.

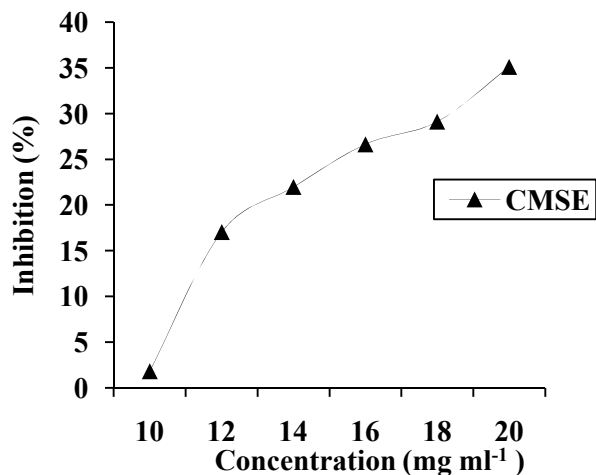


Figure 2a: Inhibition (%) of α -glucosidase activity by *C. maxima* Seeds Each value is presented as mean \pm S.D. (n=3)

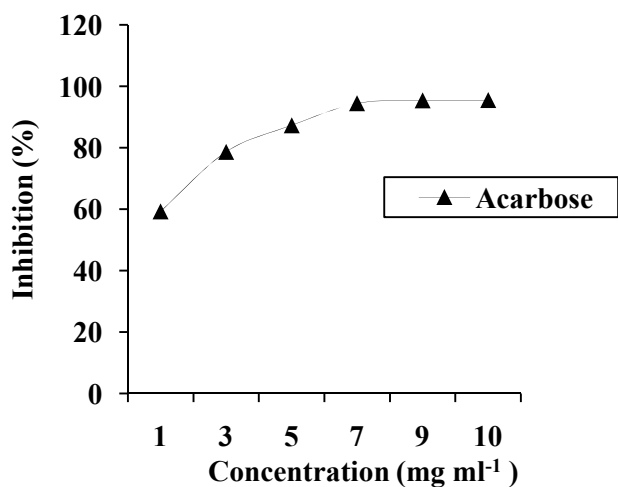


Figure 2b: Inhibition (%) of α -glucosidase activity by Acarbose Each value is presented as mean \pm S.D. (n=3)

LIBS based Elemental Profile of *C. maxima* Seeds

Figure 3(a) and 3(b), show the LIBS spectra of the CMSE in the spectral ranges of 200–500 nm and 600–850 nm respectively. Relative concentrations of elements present in CMSE have been evaluated by measuring the intensity of the selected lines in triplicate from the LIBS spectra of the sample. Table 1 and 2 indicate the relative intensity ratios of different phytoelements of the sample (CMSE). Table 1 shows the relative intensities of elements in spectral range (200-500 nm) with respect to C (247.8 nm) while, Table 2 shows the relative intensities of elements in spectral range (600-850 nm) with respect to O (777.4 nm).

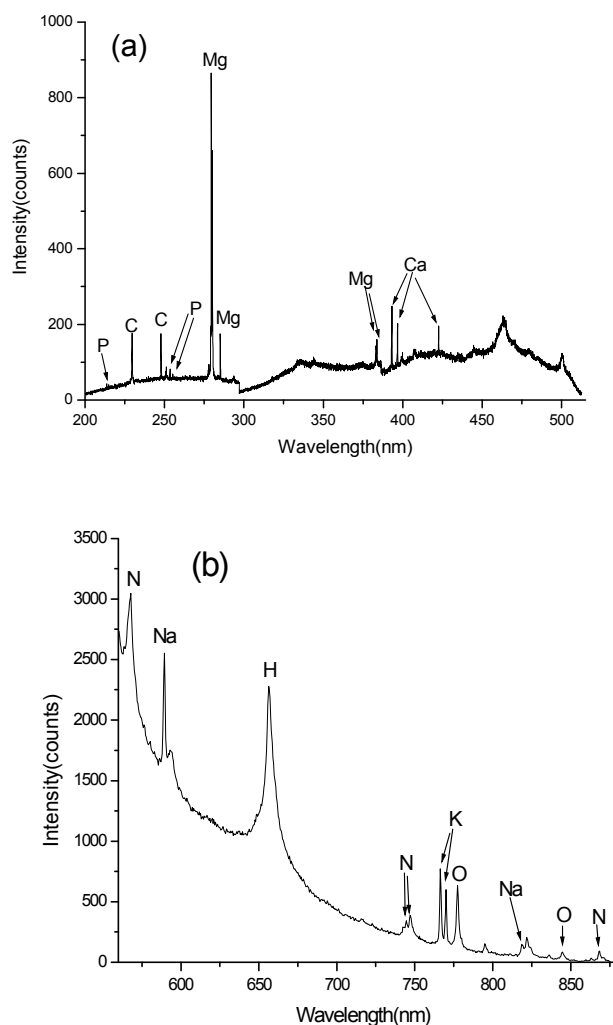


Figure 3: LIBS of *C. maxima* seeds in the range of (a): 200–500 nm and (b): 600–850 nm

Table 1. Intensity ratio of different elements of *C. maxima* with respect to C (247.8 nm) Spectral range (200-500 nm)

Element	Wavelength (nm)	Element/Ref	Intensity Ratio by C (247.8 nm)
Ca	393.366	Ca/C (247.856)	2.12952
Mg	279.553	Mg/C (247.856)	6.11145
P	253.561	P/C (247.856)	0.21837
C	247.856	C/C (247.856)	1

Table 2. Intensity ratio of different elements of *C. maxima* with respect to O (777.4 nm) Spectral range (600-850 nm)

Element	Wavelength (nm)	Element/Ref	Intensity Ratio by O (777.4 nm)
Na	589.592	Na/O (777.417)	1.33342
K	766.49	K/O (777.417)	0.75596
N	567.956	N/O (777.417)	1.85389
H	656.271	H/O (777.417)	9.2443
O	777.417	O/O (777.417)	1

Discussion

Several medicinal plants have been reported to have antidiabetic activity and hence, the use of herbal drugs as complementary and alternative therapy to existing medications for the treatment of diabetes is growing worldwide. In recent years the popularity of alternative medicine has increased for various reasons [13].

The antidiabetic activity of *C. maxima* seeds has been reported in ancient Indian literature [14] but their scientific evaluation has not been reported till date. Moreover, information regarding *in vitro* α -amylase and α -glucosidase inhibitory activities of *C. maxima* seeds is also not available. Additionally, the value of the present study gets augmented as it also deals with the identification of phytoelements responsible for its glycemic profile. There are several reports which deal with its phytochemical analysis but there are no reports till date on its phytoelemental screening and specifically on its correlation with its antidiabetic efficacy.

Recently, the LIBS based results of antioxidant elemental profile of CMSE has been reported by our research group for the first time [5] and it has motivated us to evaluate the glycemic elemental profile of CMSE by assessing its α -amylase and α -glucosidase inhibitory activities *in vitro* and then by correlating it with the presence of certain LIBS screened phytoelements.

The inhibition of enzyme activity by metal ions is of considerable importance and has been studied extensively [15]. The LIBS based identified elements such as Ca, Mg, K and Na are physiologically important and play a crucial role in many metabolic processes. It was reported that they are helpful for the therapy of diabetes [16].

Thus, α -amylase as well as α -glucosidase inhibitory activities revealed by CMSE could be correlated with the presence of Ca, Mg, Na and K, as these elements have been reported to display significant inhibitory activity of carbohydrate hydrolyzing enzymes. The intensity of the observed spectral lines corresponding to major and minor elements present in the extract is not only indicative of

their concentration, but it also assists in measuring the extent of their role in the management of diabetes.

One way of explaining the 'essential' role of Ca ion in α -amylase is that it appears to stabilize its active site by primary binding [17]. By contrast, reports of the enzymatic hydrolysis by several kinds of α -amylases being inhibited by relatively high concentrations of Ca ions due to secondary binding at the catalytic site in the enzyme have been published [18].

To understand the 'essential' role of Ca ion in a α -glucosidase activity inhibition is equally important because of its involvement in type 2 diabetes mellitus. It is reported that direct binding of Ca ion to the enzyme, induces structural changes which inhibits enzyme activity and hence Ca ion could act as a potent inhibitor of α -glucosidase for the treatment of type 2 diabetes mellitus. It has also been reported that α -glucosidase activity gets inhibited in a dose-dependent manner on incubation with varying concentrations of Ca ions and it gets completely inactivated at a concentration of 500 mM Ca ion [19].

The presence of Mg ions reduce the α -amylase activity up to 50%. This reduction in the enzyme activity was mainly due to the chelation of Mg ions at specific site of the enzyme molecule [20]. However, K inhibits almost completely the activity of neutral α -glucosidase, whereas, Na is diuretic in nature and plays an important role in the transport of metabolites. Hence, Na/K ratio for food is an important factor in the prevention of hypertension and arteriosclerosis, where Na enhances and K depresses blood pressure [21].

Thus, the results reported here provided an insight into how phytoelements influence the enzyme activity as they modulates the inhibition to prevent the substrate binding to the enzyme. They inhibit either individually or synergistically.

Conclusion

The present study has its own significance being relevant at global level, since, it deals with the LIBS based identification of phytoelements which contribute significantly towards the α -amylase and α -glucosidase inhibitory activities of CMSE and hence these seed could be explored further for the treatment of the most common, yet serious, metabolic disorder viz. diabetes mellitus, especially in type 2 which is the most prevalent type of diabetes. The significant presence of some specific trace elements viz., Ca, Mg, Na, and K in the extract, as evident from LIBS spectra, thereby suggest that, these elements may be responsible for the α -amylase and α -glucosidase inhibitory activities exhibited by CMSE. It could therefore be presumed that diabetic patients should increase their intake of Ca, Mg, Na, and K as it will help them in controlling their metabolic disorder of carbohydrates.

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Conflict of Interests

The authors declare no conflict of interest.

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