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EVALUATION OF ANTIDIABETIC, NEUROPROTECTIVE, ANTIHYPERTENSIVE AND HEPTATOPROTECTIVE POTENTIAL OF FLOWERS OF *Cucurbita maxima* DUCHESNE

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ABSTRACT

All parts of the plant, *Cucurbita maxima* Duchesne, especially the flowers and fruits, are consumed as vegetables in different parts of West Bengal. The present work, new of its kind, intends to focus on inhibitory properties of its male flowers against four different enzymes to explore its antidiabetic, neuroprotective, antihypertensive and hepatoprotective potential. The methanolic extract of the dried male flowers inhibited α-glucosidase (IC_{50} value 610.52 ± 60.33 µg/ml), the key enzyme involved in type 2 diabetes. The flower extract also showed acetylcholinesterase inhibitory property (IC_{50} value 460.78 ± 6.01 µg/ml). At 15 µg/ml the extract showed 46.63% ± 1.15 inhibition of angiotensin converting enzyme (ACE), thus suggesting its potential to have anti-hypertensive property. β-Glucuronidase inhibitors are considered to have hepatoprotective property. The activity of the flower extract against this enzyme (64.69% ± 1.29 inhibition at 500 µg/ml) was better than that of silymarin. GC-MS analysis of crude methanolic extract identified 26 compounds as phytochemical constituents of the male flower.

KEYWORDS: *Cucurbita maxima*, Flower, Antidiabetic, Neuroprotective, Antihypertensive, Hepatoprotective Potential

Cucurbita maxima Duchesne is known worldwide mainly for its fruit familiar as pumpkin which is cooked as a vegetable. In India all aerial parts of this plant are consumed with a faith in its food value for health restoration. The antidiabetic (Sharma *et al.,*2013; Al-Shaheen *et al*., 2013), anticancer (Saha *et al.,* 2011a), hepatoprotective (Saha *et al.,* 2011b), anthelmintic, immunomodulatory, antihypertensive, antibacterial, antihypercholesterolomic and antinflammatory activities (Iqbal *et al*., 2001; Caili *et al.,* 2006) of this plant have been put in to record subsequent to investigations concerning aerial parts and fruits. Since the medicinal virtues of the flowers have not yet been revealed the present authors included it in their research programme on evaluation of efficacy of common edible flowers of West Bengal in management of health disorders and refractory diseases. Since the male flowers of *C. maxima* are consumed almost throughout West Bengal as a very favourite dietary item the present work attempts to reveal its inhibitory potential against the enzymes, specifically linked with such dreadful health issues as diabetes, liver disorder, hypertension and neural disorder.

The enzymes α-amylase and α-glucosidase which are linked with type 2 diabetes were selected for exploring inhibitory potential of the flower of *C. maxima* so that concomitantly with inhibition there is decrease in glucose release from starch and delay in carbohydrate absorption in the small intestine, thus creating an opportunity for the patient friendly treatment of diabetes mellitus and obesity (Gallahar and Schneeman, 1986; Murai *et al.,* 2002). Moreover the chemical constituents of the flower extract were analyzed to trace the α-amylase inhibitors that are potentially safer and suitable as alternatives for modulation of carbohydrate digestion and controlling glycaemic index of food products.

Acetylcholinesterase (AChE) which catalyzes the hydrolysis of acetylcholine in cholinergic synapses (Groner *et al*., 2007) was also selected for the present study since activity of this enzyme, according to the cholinergic hypothesis,

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leads to memory impairment in the patients of Alzheimer's Disease (AD) by causing a deficiency in cholinergic function i.e. by reducing the availability of acetylcholine (ACh) in the neuronal synaptic cleft in the brain. In view of this, the present work attempts to identify AChE inhibitors in the flower extract so that on its use there can be enhancement of cholinergic functions and used in the treatment of Alzheimer's Disease (AD) with least side effect (Howes and Houghton, 2003).

Antagonism to another important enzyme, angiotensin converting enzyme (ACE), was also considered for the present study since ACE inhibition is commonly used in therapy to reduce morbidity and mortality of patients suffering from hypertension and related diseases (Miguel *et al*.,2009).

For exploring the hepato-protective potential of a plant, whether edible or not, evaluation of its efficacy as a β-glucuronidase inhibitor is suggested (Shim *et al*.,2000) since β-glucuronidases in mammals hampers glucuronidation which is a major detoxification process by which metabolites are excreted from the body, unless hydrolyzed by the intestinal enzyme β-glucuronidase (Fior *et al*., 2009). As such β-glucuronidase inhibitory potential of the flower of *C.maxima* was also tested.

MATERIALS AND METHODS

The specimens, i.e. the male flowers of *Cucurbita maxima* Duchesne, of the angiospermic family Cucurbitaceae, were collected from local market of Chandannagar in Hooghly district of West Bengal state and its taxonomic identity was authentically ascertained. The specimens were washed under running water and then sun dried. The dried flowers were powdered and extracted with methanol by reflux for 5 hours. The organic solvent after filtration was evaporated to dryness and the crude extract obtained. The methodology for assessing inhibitory potential of the extract is presented enzyme wise in the following.

α-GLUCOSIDASE ASSAY

α-Glucosidase inhibitory property was assayed by modifying the method of Kwon et al. (2008) wherein *p*-Nitrophenyl α-D-glucopyranoside was used as the substrate. A solution (0.006%) of α-Glucosidase (ex. microorganism) was prepared in 0.02 M phosphate buffer (pH 6.3). In each set 0.034 ml of methanolic extract of flower, 0.11 ml of Phosphate Buffer (pH 6.3) and 0.034 ml of enzyme were mixed. After 1 hour of incubation at 25° C, 0.17 ml of p-nitrophenyl α-D-glucopyranoside was added. Again the reaction mixture was incubated for 30 min at 30° C. The enzyme reaction was stopped by adding 0.5 ml of 1(M) Na_2CO_3 solution. Blank set consisted of 0.034 ml of buffer instead of enzyme. The control set was prepared by adding methanol instead of flower extract. The optical density was measures at 405 nm spectrophotometrically. Percentage inhibition was calculated by using the formula: [(Control OD - Test OD)/control OD]×100.

AChE ASSAY

Acetylcholinesterase (AChE) inhibitory property was assayed following the method of Ellman (1961) with necessary modifications. AChE from electric eel was used for assay. Different concentrations of methanolic extracts of flower (0.01ml) were added to 0.02 ml AChE and 1ml of buffer. The reaction was started by adding 0.01 ml 0.5 mM 5,5' dithiobis (2 nitrobenzoic acid) (DTNB) and 0.02 ml of 0.6mM acetylthiocholine iodide solution. The reaction mixture was incubated at 37^0C for 20 minutes. The optical density was recorded

instantly at 412 nm. The percentage inhibition of AChE activity by plant extract was calculated accordingly.

ACE ASSAY

ACE (Angiotensin Converting Enzyme) inhibitory activity was assayed using the method of Cushman and Cheung, 1971 with suitable modifications. As much as 15µl of methanolic flower extract was incubated with 250 µl of 200 mM borate buffer (pH 8.3) containing 2(M) NaCl and 7 mM hippuryl-L-histydyl-L-Leucine (HHL). To this mixture was added ACE from rabbit lung (100 mU) (15µl) and 20 µl distilled water and the reaction mixture was incubated at 37°C for 30 minutes. The reaction was stopped with 250 µl of 1 (N) HCl. The hippuric acid formed was extracted with ethyl acetate (1500 µl). After evaporation of ethyl acetate (1000 µl), hippuric acid was redissolved in 500 µl of distilled water and measured spectrophotometrically at 228 nm. The activity of each sample was tested in triplicate.

β-GLUCURONIDASE ASSAY

β-Glucuronidase inhibition assay was carried out following the method of Kim *et al.*(1999). β-Glucuronidase (50 µl) from bovine liver (6666.6 units/ml in 0.1M phosphate buffer, pH 7.0) and 420 µl of the flower extract of 500 µg/ml concentration in 0.1M phosphate buffer (pH 7.0) were pre-incubated at 37°C for 15 minutes. Following the pre-incubation, 15 µl of β-glucuronidase and p-nitrophenly-β-D-glucuronide (3.15 mg/ml in 0.1 M phosphate buffer, pH 7.0) was added and incubated at 37°C for 50 minutes. The colour developed was read at 405 nm in a spectrophotometer.

DETERMINATION OF IC_{50} VALUES

Regression equations were prepared from the concentrations of the extracts and percentage inhibition in different systems of assay i.e., AChE inhibitory assay, α-amylase and α-glucosidase inhibitory assay. IC_{50} values were calculated from these regression equations. A lower IC_{50} value indicates higher enzyme inhibitory activity.

GC-MS ANALYSIS

GC-MS analysis was carried out following the method of Kind *et al*. (2009) with little modification. HP-5MS capillary column (Agilent J & W; GC Columns (USA) (length 30 m plus Duraguard 10 m, diameter 0.25 mm narrow bore, film 0.25 µm) was used. The analysis was performed under the following oven-temperature - programme: injection in sandwich mode with fast plunger speed without viscosity delay or dwell time, oven ramp 60ºC (1 minute hold) to 325ºC at 10ºC/minute, 10 minute hold before cool – down, 37.5 minute run time. The injection temperature was set at 250º C; the MS transfer line at 290º C and the ion source at 230º C. Helium was used as the carrier gas at a constant flow rate of 0.723ml / min (carrier linear velocity 31.141 cm/sec). The dried extracts were derivatized after using methoxyamine hydrochloride (20 mg/ml in Pyridine) and subsequently with N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) to increase volatility of metabolites. 2 µl FAME (Fatty Acid Methyl Esters) markers [a mixture of internal Retention Index (RI) markers was prepared using fatty acid methyl esters of C8, C10, C12, C14, C16, C18, C20, C22, C24 and C26 linear chain length, dissolved in chloroform (HPLC) at a concentration of 0.8 mg/ml (C8-C16) and 0.4 mg/ml (C18-C30)] was added (Kind *et al*. 2009). Derivatized samples (1 µl) were injected via the split mode (Split ratio 1:5) onto the GC column. Prior to analysis the method was

calibrated with the FAME standards available with the Fiehn GC / MS Metabolomics library (2008) (Agilent ChemStation, Agilent Technologies Inc., Wilmington, USA). Automated mass spectral deconvolution and identification system (AMDIS) was used to deconvolute GC-MS results and to identify chromatographic peaks. Identification of the metabolites was carried out by comparing the fragmentation patterns of the mass spectra and retention times with entries of mass spectra and retention time in Agilent Fiehn Library. The relative response ratios of all the metabolites were calculated after normalizing the peak areas of the compounds by extract dry weight.

RESULTS AND DISCUSSION

Methanolic extract of C. *maxima* flower showed inhibition against the enzyme α-glucosidase in a dose dependent manner (Fig. 1). The IC_{50} value calculated was 610.52 ± 60.33 µg/ml (Fig. 3). The flower also inhibited the enzyme AChE. Percentage inhibition was proportionate to the concentration of the extract (Fig. 2). IC50 value for AChE inhibitory activity was 460.78 ± 6.01 µg/ml (Fig. 3). The flower extract inhibited the enzyme ACE by 46.63% ± 1.15 at the concentration of 15 µg/ml (Fig. 4). The flower may as well be considered to have hepatoprotective potential as it inhibited β-glucuronidase (64.69% ± 1.29 at 500 µg/ml) (Fig. 4). Silymarin, the commercial inhibitor of β-glucuronidase, showed IC_{50} value of 794.62 µg/ml. The result suggested that the flower extract of *C. maxima* had stronger β-glucuronidase inhibition activity than that of silymarin.

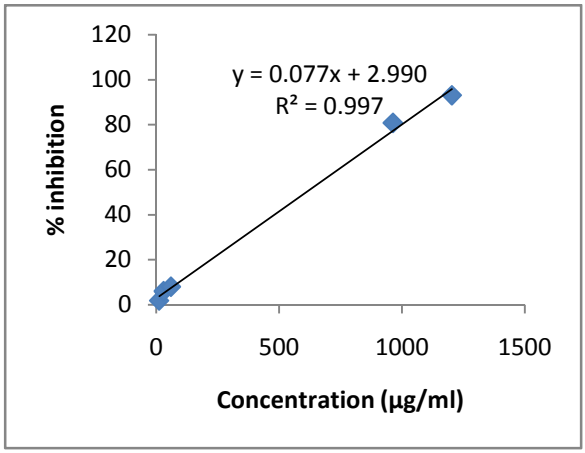

Figure 1: Inhibition of α-glucosidase by *C. maxima* flower extract

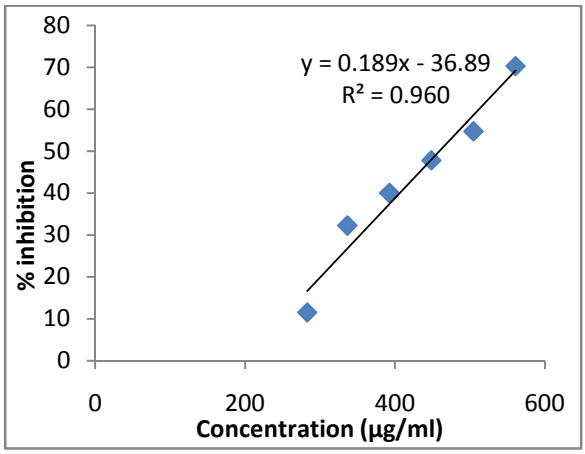

Figure 2: Inhibition of acetylcholinesterase by *C. maxima* flower extract

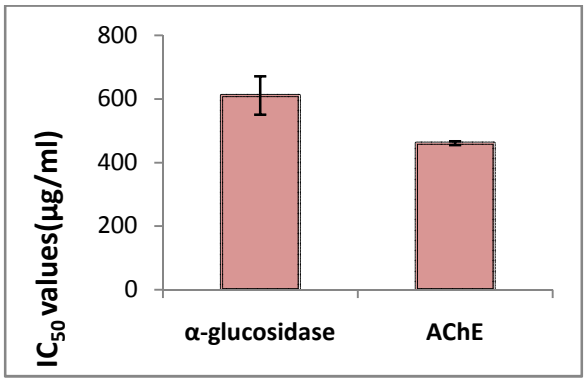

Figure 3: α-Glucosidase and AChE inhibition by *C. maxima* flower extract

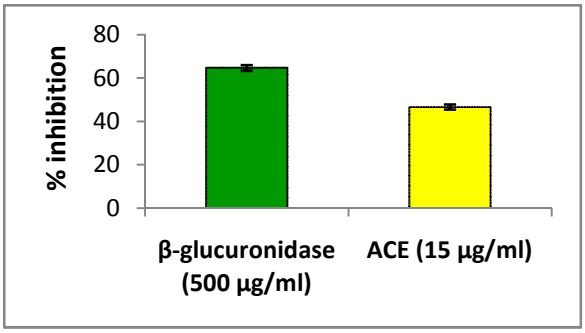

Figure 4: β-Glucuronidase and ACE inhibition by *C. maxima* flower extract

GC-MS data analysis of the crude flower extract was also performed for exploring the number and nature of compounds present therein. The extract was found to have 26 different types of compounds such as 9 organic acids, 7 sugar and sugar alcohols, 4 amino acids, 5 fatty acids and also porphin (Table 1).

Table 1: Phytochemical constituents of the methanolic extract of the flower

Metabolites		Expected Rt	Obtained Rt
Organic acids	**Glyceric acid**	10.74	10.86
	Glycolic acid	7.05	6.87
	Gluconic acid	18.30	18.39
	2-Isopropylmalic acid	13.84	14.01
	L(+)Lactic acid	6.85	7.00
	D-Malic acid	12.79	12.91
	Succinic acid	10.51	10.59
	Tartaric acid	14.59	14.34
	Tartronic acid	11.52	11.73
Amino acids	**Beta-alanine**	12.04	12.11
	L-Glutamic acid	13.23	13.35
	Glycine	10.46	10.52
	L-Valine	9.15	9.25
Sugars and sugar alcohols	**Glycerol**	9.94	10.06
	Melezitose	29.88	29.72
	D-Mannitol	17.81	17.98
	D-Sorbitol	17.90	18.06
	Sucrose	24.00	24.18
	D-(+) Trehalose	24.75	24.95
	D-Threitol	12.95	13.21
Fatty acids	**4-Guanidinobutyric acid**	13.35	13.40
	6-Hydroxy caproic acid	14.79	14.87
	Lauric acid	12.06	11.82
	Palimitic acid	18.86	18.94
	Stearic acid	20.68	20.71
Other	**Porphine**	10.77	10.93

Association of various organic acids, sugar, fatty acid and amino acid together in the flower is likely to have a synergistic effect on activities inhibiting enzymes.

CONCLUSION

The results of this *in-vitro* study clearly indicated that methanolic extract of *C. maxima* male flowers had considerable inhibitory activities against α-glucosidase (the key enzyme involved in type 2 diabetes), acetylcholinesterase (the key enzyme involved in memory impairment in Alzheimer's Disease), angiotensin converting enzyme (ACE, the enzyme responsible for inducing hypertension) and β-glucuronidase (the enzyme responsible for liver disorder) thus suggesting its anti-diabetic, neuroprotective, anti-hypertensive and hepatoprotective potential respectively. These inhibitory potentials when found combined in one edible plant organ add much prophylactic and therapeutic virtues to its dietary value so as to be worthwhile in managing the diseases and disorders as mentioned and in providing the biochemical rationale for further clinical studies.

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