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Bioprospective of the leaves of *Cucurbita maxima* Duch. ex Lam (family Cucurbitaceae) for ACE inhibitory activity-from farm to phytotherapy

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ABSTRACT

This study aims to evaluate the effect of the *in vitro* angiotensin converting enzymeinhibitory activity of the chloroform extract of *Cucurbita maxima* leaves (CECML). Initially we performed various phytochemical screening, Electron dispersive X-ray Spector photo meter (EDS) for the presence of various trace elements, determination of flavonoid content, total phenolic content and TLC. Preliminary phytochemical screening showed the presence of proteins, amino acids, flavonoids, terpenoids, saponin, carbohydrates, tannins, phyto sterol, glycoside and alkaloids. Volatile oil and fixed oil were found to be absent. Total Flavonoid content of CECML 21.6 mg/g (as quercetin). Total phenolic content 18.2 mg/g (as gallic acid). The presence of cucurbitacin was confirmed by TLC. Trace elements analysis by EDS showed the presence Ca, K, P and Mg. The spectrophotometric method is utilized for determining ACE activity and inhibition *in vitro.* The method involves hydrolysis of the substrate, N-[3-(2- furyl)acryloyl]-frphenylalanylglycylglycine (FAPGG), to NE3-(2-ftlryl)acryloyl]-L-phenylalanine (FAP) and glycyiglycine and measurement of the consequent change in absorbance (A) at 345 nm. The percentage inhibition of 5, 10, 15, 20, 25 µg/ml of CECML concentrations were observed 0.117±2.76, 14.99±0.08, 27.21±0.06, 67.71±1.73 and 91.92±2.02 respectively. The percentage inhibition of 2.5, 5, 10, 20, 30 nM/ml of standard drug captopril concentration were observed 26.09±1.2, 30.13±0.09, 45.52±1.03, 52.19±1.72, 78.20±1.56 respectively. The result was dose dependent and statistically significant ($p<0.001$). It is assumed that this effect may be due to the phenolic content, cucurbitacin and the influence of calcium, potassium, magnesium content and antioxidant activity

Keywords: *Cucurbita maxima*, Cucurbitaceae, Cucurbitacin, ACE inhibitor, hypertension.

1. INTRODUCTION

One in three adults worldwide has raised blood pressure – a condition that causes around half of all deaths from stroke and heart disease (WHO, 2013). It is predicted that this rate would increase by 60% in 2025. Thus, it is not surprising events that hypertension is a silent killer that rarely causes symptoms [1]. Angiotensin I-converting enzyme (ACE, dipeptidyl carboxpeptidase, EC 3.4.15.1) plays an important role in the regulation of blood pressure as well as cardiovascular function. ACE converts the inactive decapeptide angiotensin I into the potent vasoconstricting octapeptide angiotensin II and also inactivates vasodilator, bradykinin [1]. Thus, inhibition of ACE results in a decrease in blood pressure. Many potent synthetic ACE inhibitors such as captopril, enalapril, lisinopril, and ramipril have been widely used in the clinical treatment of

hypertension and heart failure in humans. However, synthetic ACE inhibitors can have side effects including cough, taste disturbances and skin rashes. Therefore, interest in identifying herbs as natural sources of ACE inhibitors has increased. Many ACE inhibitory medicinal plants have recently been reported. Eg: *Piper betle, Jasminum sambac, Cardiospermum halicacabum* and *Tribulus terrestris.* Out of the 73 species investigated, 22 showed more than 50% ACE inhibitory activity. They were selected on the basis of their usage as cardiotonics, diuretics and other uses related to the symptoms of hypertension [2]. Numerous methods for the measurement of ACE activity have been reported, including spectrophotometric fluorometric, radiochemical, high-performance liquid chromatography (HPLC), and capillary electrophoresis methods. These assay methods also were used to obtain information on the inhibitory potency for different ACE inhibitory substances. With respect to radiochemical, HPLC and capillary electrophoresis methods, these methods require specific expensive instruments that are usually not available in common laboratories. The spectrophotometric method is the most commonly utilized in the assays for determining ACE activity and inhibition in vitro [3]. In this study we selected a widely available plant *Cucurbita maxima* (Cucurbitaceae). It is popularly known as Pumpkin, in English and parankikai, sakkaraipoosani in Tamil. It is monoecious, trailing herb, Annual plant, cylindrical stem 10meter length strongly branched, latral branches are longer then vein, tendrils are mostly branched, climbing by latral, 2-5 branched tendrils. Stems rounded soft long running softly pubescence. The leaves are reniform, simple, not lobed, softly hairy, occasionally with white plotches, 5-8-13 cm in size, green colour, finely toothed margin, deeply cordated, 3-veined from the base and petiole 5-20 cm long. It was reported that fresh leaves contains β-carotene 743.9±35.0 μg/gm dry weight, lutein-1534.448μg/gm dry weight, Neoxanthine-327.813.7μg/gm dry weight Vialoxanthine-818.528μg/gm dry weight [4], protein-18.6%, crude fibre-9.46%, lipid-4.46%, carbohydrate-51.3%, moisture-7.21% and ash-8.95%. tannin-0.220, phylate-10.3, phenolic-0.15, phytin phosphorus-2.9, oxalate-1.42mg/100gm dry weight and saponin-0.85, flovanoid-0.51% Minerals percentage Na-19.5, K-32.4, Ca-15.4, Mg-11.5, Zn-17.7, Iron-5.22 lead- <0.001, Cd<0.001, Mn-5.6, Cu-0.37, P-65.8, Na/K-0.602, Ca/Mg-1.34, K/Na-1.66,Ca/P-0.234mg/100gm of the leaf dry weight and [K(Ca/Mg)-2.41meq ratio] [5]. *C.maxima* leafs contain 569mg/100gm of methionine and it is good source of Vitamin A, C,E and B and iron [6]. Leaves are used in nervous disorder, cancer, helminthiasis, cooling effect in the body, gastrointestinal problem, joint pain, cold, piles, dysentery, oedema, skin disorder, leprosy, rheumatoid arthritis, chicken pox [7], anaemia, malaria [8], anti-HIV [9], diabetic, inflammation, immunomodulatory, antibacterial [11], hypertension [20], burns, scalds, inflammation, abscesses, boils, migraine, neuralgia, haemoptysis [13], diuretic, refrigerants, prostate, problem, ascarisis [11], aerial parts are used for hypoglycaemic [14], antioxidant [15], hepatoprotectivity [16], antidiabetic [17], anti cancer [10, 18], caridoprotectivity [19-23]. In short, there is good level of traditional and experimental evidences to support various claims and advantages of this widely available plant. As mentioned earlier several reports have been published on the effects of the plant extract and chemical constituents on different biological activities *in vitro* and *in vivo*. There have been a plethora of reports on the role of cucurbitacin for their cytotoxic, hepatoprotective, cardiovascular and anti diabetic effects [24]. Cucurbitacin a terpenoid possess immense pharmacological potential and may prove to be lead molecule for future research. The objective of this study was to determine the antihypertensive activity through *in vitro* inhibition of ACE activity.

2. Materials and Methods

Sonicator (Analytical lab supplier), Vortex mixture (Serwell instruments), pH meter (Thermo), Incubator (Serwell instruments), homogenizer (Serwell instruments), UV-Vis spectrometer (Shimatzu 1800) boric acid (Sigma), dimethyl sulphoxide (Thomas baker) FAPGG(Sigma), pig lung, borate buffer pH 8.2. For the determination of trace element by Hitachi Scanning electron Microscope 3000 H model.

2.1. Collection and authentication of the leaves of *C.maxima.*

The leaves of the healthy plant *C.maxima* selected for our study was collected from Keelvani, Erode Dist, Tamilnadu, India during the month of July 2017 and was authenticated by Dr.Stephen, Department of Botany, American college, Madurai.

2.2. Preparation of extract

The shadow dried leaves was powdered, sieved in a No.60 mesh and macerated with chloroform. The filtrate evaporated under vacuum. The residue obtained (CECML) was stored in the refrigerator until further use.

2.3. Preliminary phytochemical screening

Preliminary phytochemical screening was carried out to find out the presence of various

phyto constituents using standard procedures [25-27].

2.5. Estimation of flavonoid content

The flavonoid content of plant extract was estimated by aluminium chloride method. In this method, aluminium chloride complexes with flavonoids of C3-C5 hydroxyl group and to produce intense colour in acidic medium. The intensity of the colour is proportional to the amount of flavonoids and can be estimated as quercetin equivalent at wavelength of 415nm [28-30].

2.6. Estimation of total phenolic content

The total phenolic content of the CECM was determined by Folin Cio-calteau reagent. This reagent consists of phosphotungstate and phosphomolybdate mixture which is reduced to mixture of blue molybdenum and tungsten oxides while phenolic content of the extract was oxidized. The intensity of colour is proportional to the amount of phenolic content of the extract and which was measured at 765nm. The total phenolic content in CECML was expressed as milligrams of gallic acid equivalent (GAE) per gm of extract [31-32].

2.7. Determination of trace elements

Percentage of various trace elements like Ca, Mg, K, Na etc was determined by Energy dispersive X-ray spectrometer (EDS) built in scanning electron microscope (SEM) Back scattered electron images in the SEM display compositional contrast yet results from different atomic number of the elements and there distribution [33-35]. Sample for SEM analysis were mounted on the specimen stub using carbon adhesive sheet. Small sample were mounted with I sq. cm glass slide and kept in carbon adhesive sheet. Samples were coated with gold to a thickness of 100 AO using hitachi vacuum evaporator. Coated sample were analysed ina Scanning electron Microscope

2.8. TLC profile of the CECML

CECML was applied as a band on aluminium sheet pre coated with slica gel 60 GF 254. The plates were developed in the mobile phase Toluene: Ethyl acetate (25: 75) to a distance of 10 cm. Vanillin sulphuric acid was used as a spraing agent. Spots absorbed under UV 360nm [36].

2.9. Determination of ACE inhibitory activity

Enzyme

Enzyme from dried pig lung acetone powder was collected and dissolved in Tris-HCl buffer. It was centrifuged and then aliquoted in to vials under sterile conditions and stored at -80º C Substrate solution: FAPGG was prepared with buffer (1mM) Captopril: Stock solution (4.6 mM) was prepared with 80mM borate buffer. Sample preparation: 200mg of sample was weighed and dissolved in 1000µl of DMSO.

Optimum conditions for enzyme reaction

In the kinetic ACE method, a practical and optimal buffer is borate buffer at pH 8.2 (37 0 C) A lag phase is detected in this reaction, and a 5min incubation of substrate and enzyme is suggested before the kinetic measurement. The substrate FAPGG concentration is maximized at 1mM/L and the measurement wavelength is 345nm to ensure the linearity of measurement. The proposed procedure uses a 1:9 enzyme: substrate volume ratio.

Procedure

1. 300µl of enzyme was added with 2700µl of buffered substrate in a 13 X 100mm test tube which is pre warmed at 37 ^{0}C and it was incubated in water bath for 10min.
2. Test sample (100µl) was also incubated for 10min.
3. The samples were swirled together and the mixture was transferred into a pre warmed quartz cuvette.
4. Allow to stabilize for 2 min before measuring the absorbance (Table 1).
5. The change in absorbance during 10min was observed kinetically at 345nm.

Table - 1: Plate lay out

	EB (µl)	SB (µl)	E300 (µl)	CECML (µl)	Control (µl)
Buffer	2800	400	100	-	-
Enzyme	300	-	300	300	300
Substrate	-	2700	2700	2700	2700
CECML	-	-	-	100	-
Control	-	-	-	-	100

EB-Enzyme blank, SB-Substrate blank, E300-Activity at 300µl enzyme, Control-DMSO as vehicle, CECML: CHCl3 ext of *C.maxima* leaves

Percentage inhibitions were calculated. ACE inhibitory activity is calculated as follows:

$$\Delta \text{ Absorbance} = \text{Final absorbance} - \text{Initial absorbance}$$

$$\text{Percentage of inhibition} = \Delta \text{ absorbance-Inhibition} / \Delta \text{ absorbance}$$

Graph was plotted at different concentrations. Data expressed as ± SEM statistical significance was determined by one way Anova.

3. Results

Pale green viscus CECML (19.5%w/w) was obtained. Preliminary phytochemical screening of appropriate solvent extract of the leaves showed the presence of alkaloids, steroids, tannins, proteins and aminoacids, flavonoids, terpenoids, saponin, glycosides, carbohydrates and absence of volatile oil, mucilage, fixed oil. Flavonoid content of CECML in terms of quercetin was found to be 21.6 mg/g. Total phenolic content of CECML in terms of gallicacid was found to be 18.2 mg/g. Estimation of the elements by EDS showed the following mg weight percentage O- 46.72, Mg- 0.76, P- 0.12, S- 0.07, Cl- 0.22, K- 1.50, Ca- 2.82, C- 40.40, N- 4.11, Al- 0.22, Si- 3.06 and atomic percentage O- 42.65, Mg- 0.46, P- 0.06, S- 0.03, Cl- 0.09, K- 0.56, Ca- 1.03, C- 49.12, N- 4.29, Al- 0.12, Si- 1.59. The Co TLC of CECML showed 3 spots of R_f value 0.03, 0.36, 0.82 respectively (the R_f value of cucurbitacin was 0.36 (correlated with reported value) (36). The presence of cucurbitacin in the CECML was confirmed by Co TLC. The percentage inhibition of 5, 10, 15, 20, 25 µg/ml of CECML concentrations were observed 0.117±2.76, 14.99±0.08, 27.21±0.06, 67.71±1.73 and 91.92±2.02 respectively (figure 1).

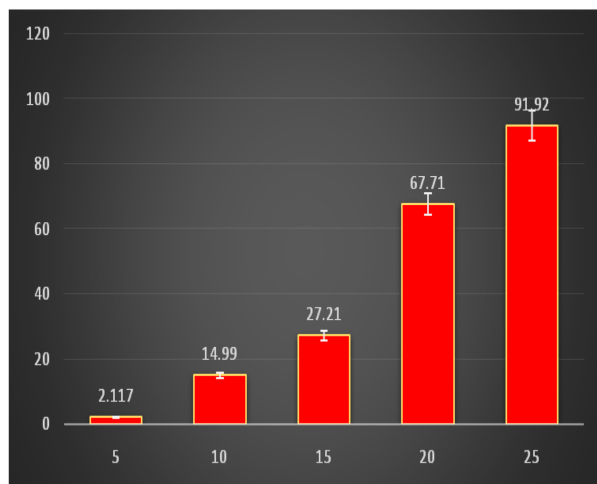

Figure – 1: ACE inhibitory activity of CECML (p<0.001).

The percentage inhibition of 2.5, 5, 10, 20, 30 nM/ml of standard drug captopril concentration were observed 26.09±1.2, 30.13±0.09, 45.52±1.03, 52.19±1.72, 78.20±1.56 respectively (Figure 2).

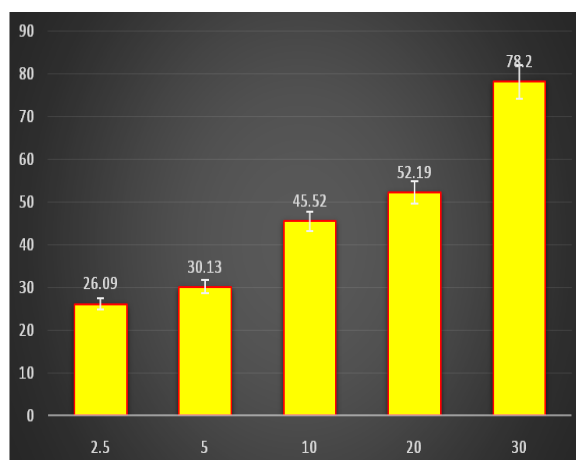

Figure – 2: ACE inhibitory activity of Captopril (p<0.001).

4. Discussion

As reported earlier several reports have been published on the effects of the plant extract and chemical constituents on different biological activities *in vitro* and *in vivo*. Preliminary phytochemical screening of the leaves showed the presence of biologically active constituents like flavonoids, terpenoids, steroids, tannins etc. Remarkable quantity of flavonoids and total phenolic content, various trace elements were observed. Cucurbitacins which are structurally diverse triterpenes, are found in many cucurbitaceous plants possesses immense pharmacological potential including cardio vascular effect [24]. Cucurbitacin contain a basic 19-(10-9β)-abeo-10α-lanost-5-ene ring skeleton. The difference of terpenes cucurbitacins from steroidal nucleus lies in the fact that in basis structure of cucurbitacin a methyl group is located at C-9 rather than C-10. The presence of cucurbitacin was identified by TLC.

The spectrophotometric method is the most commonly utilized in the assays for determining ACE activity and inhibition *in vitro*. The method involves hydrolysis of the substrate, N-[3-(2- furyl)acryloyl]-frphenylalanylglycylglycine (FAPGG), to NE3-(2-ftlryl)acryloyl]-L-phenylalanine (FAP) and glycyiglycine and measurement of the consequent change in absorbance (A) at 340 nm or 345 nm. On enzymic hydrolysis, FAPGG forms FAP and glycyiglycine. FAPGG and FAP absorb at 345 nm; glycylglycine does not. A decrease of 1.0 mmol of FAPGG per liter causes an increase of 1.0 mmol/L in FAP, and the change in absorbance measures the net absorbance change due to this decrease in FAPGG and increase in FAP. Because FAPGG absorbs more strongly than FAP, the net absorbance is a negative change. The results (Figure 1, 2) clearly showed the dose dependent *in vitro* ACE inhibitory activity of CECML and it is

comparable to the standard drug captopril ($p<0.001$). It is assumed that this effect may be due to the phenolic content, cucurbitacin and the influence of calcium, potassium, magnesium content and antioxidant activity.

5. Conclusion

It can be assumed that research focused on this unattended medicinal herb from the nature can prove to be of immense significant in generating scientifically validated data with regard to their efficacy and possible role in various diseases.

Conflict of interest statement

We do not have any conflict of interest.

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