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Evaluation of *in vitro* α- amylase inhibitory kinetics and free radical scavenging activities of *Momordica charantia*

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Abstract : Diabetes is a metabolic disease that has become a serious problem of modern society due to the severe long-term health complications associated with it. Diabetes is also an oxidative stress related disorder and is emerging as a pandemic. One of the therapeutic approaches which involves decreasing hyperglycemia aims at inhibiting the enzyme alpha amylase. Potent inhibitors found in some vegetables, herbs and fruits have been known to be effective for control and prevention of diabetes. The aim of the present study was to investigate the *in vitro* α- amylase inhibitory kinetics and free radical scavenging activities of *Momordica charantia.* Ethyl acetate extracts of *Momordica charantia* seeds and *Momordica charantia* flesh showed effective α- amylase inhibition of 94.2 % and 92.6 % respectively. Mechanism of inhibition and its kinetics studied by the method of Dixon Plot and Cornish-Bowden plot showed that the inhibition was found to be non competitive type by both the extracts. DPPH, Nitric oxide, Hydroxyl radical and Superoxide radical scavenging assays studied showed that ethyl acetate extracts of *Momordica charantia* flesh and seeds have potent free radical scavenging capacity. The combined effect of α-amylase inhibitory and radical scavenging activity exhibited by *Momordica charantia* shows promising scope in control and prevention of complications in diabetes.

Key words : *Momordica charantia*, alpha-amylase inhibition, free radical scavengers.

Introduction

Diabetes is an important public health problem, and it is one of four priority non communicable diseases targeted for action by world leaders. Both the number of cases and the prevalence of diabetes have been steadily increasing over the past few decades [1]. India has today become the diabetic capital of the world with over 20 million diabetes and this number is likely to increase to 57 million by 2025 [2].

Diabetes mellitus is a group of metabolic diseases in which a person has high blood sugar, either because the body does not produce enough insulin, or because cells do not respond to the insulin that is produced. Hyperglycemia generates more reactive oxygen species and attenuates anti-oxidative mechanism through glycation of the scavenging enzymes. Therefore, oxidative stress has been considered to be a common pathogenic factor of the diabetic [3].Diabetes is characterized by the presence of chronic hyperglycemia accompanied by greater or lesser impairment in the metabolism of carbohydrates, lipids and proteins [4].The debilitating effects of diabetes mellitus include various organ failures, progressive metabolic complications such as retinopathy, nephropathy, and neuropathy [5]. More than 90% of diabetic patients suffer from type 2 DM.

Several epidemiological and clinical studies indicate a direct relationship between hyperglycemia and long term microvascular and macrovascular complications, which develop as the disease progresses and gradually decrease quality of life of diabetic patients. Therefore, it is essential to control blood glucose levels during the early stages of the disease [6].

Controlled kinetics of carbohydrate digestion and monosaccharide absorption could be of great value in the avoidance of conditions such as diabetes, obesity, hyper lipoproteinaemia and hyperlipidaemia. In this aspect, amylase and glucosidase inhibitors are of particular importance [7].The World Health Organization (WHO) has recommended the evaluation of traditional plant treatments for diabetes as they are effective, non toxic, with less or no side effects and are considered to be excellent candidates for oral therapy [8].The role of medicinal plants in disease prevention is attributed to their antioxidant properties due to their bioactive constituents [9].

Momordica charantia L., belonging to the Cucurbitaceae family, also known as bitter gourd, bitter melon, karela and paharkai in Tamil is an economically important medicinal plant [10].Bitter gourd is one of the nature's most bountiful gifts and is one of the discarded vegetables by people, just because of its bitter taste. All parts of the plant, including the fruit, taste very bitter[11]. Different parts of this plant have been used in the Indian system of medicine for a number of ailments besides diabetes [12,13]. Fruits are traditionally used for hypoglycemic activity. Apart from fruits, leaves and seeds are also used [14].The present investigation was undertaken to assess the *in vitro* alpha- amylase inhibitory and anti oxidant potentials of locally available *Momordica charantia.*

Materials and Methods

Plant Collection, Identification and Preparation of Extract

Momordica charantia fruits were collected from local market, identified and authenticated by botanist from Botanical Survey of India, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India.*Momordica charantia*fruits were washed and cut into small pieces. The outer flesh and the seeds were dried, finely powdered and stored in air tight containers at room temperature. Five grams of powdered *Momordica charantia* flesh (MCF) and *Momordica charantia* seeds (MCS) were macerated with 50 ml of different solvents (petroleum ether, chloroform, ethyl acetate, ethanol, acetone and water) for 48 hours, filtered and collected the solvent. The solvent was evaporated in water bath shaker to get dry extract and used for further analysis.

Alpha-amylase Inhibitory Activity

The *in vitro* antidiabetic activity of MCF and MCS in all the six extracts was determined by assaying the inhibitory activity of the enzyme α-amylase by modified method of Bernfield 1955 [15].200 µl of the extract was allowed to react with 200 µl of porcine pancreatic α-amylase enzyme (Sigma-Aldrich 3176) and 100 µl of 200 mM phosphate buffer (pH-6.9). After 20 min of incubation, 500 µl of 0.5% starch was added. The same was performed for the control where 200 µl of enzyme was replaced by the buffer. After incubation for 5 minutes, 500 µl of dinitro salicylic acid was added to both the control and test. The tubes were kept in a boiling water bath for 10 minutes. The absorbance was recorded at 540 nm using a spectrophotometer and the percentage of α- amylase inhibition was calculated using the formula: Inhibition (%) = 100 (Absorbance $_{Control}$ - Absorbance $_{Test\,/}$ Absorbance $_{Control}$)

Among the six solvent extracts ethyl acetate extracts of MCF and MCS showed highest α-amylase inhibition; hence ethyl acetate extract was used for further studies.

Mechanism of alpha amylase inhibition - Dixon Plot

Different concentrations of *Momordica charantia* flesh and seed extracts (200-800 µg) were used for studying the α-amylase inhibition with different substrate concentrations of starch (0.25, 0.5, 0.75, and 1.0%) as given by Dixon 1953[16] and method of Bernfield (1955)[15].

Absorbance values were extrapolated in a standard graph for maltose and the amount of maltose produced in presence of inhibitor was found. This was taken as the product concentration [V].Calculated 1/ [V]

values and plotted graph with concentration of [I] taken along X-axis and (1/ [V]) along Y-axis. Parallel lines indicate uncompetitive inhibition; Lines intersecting in the space above the negative side of X-axis depict competitive inhibition; Lines intersecting on the negative side of X-axis, indicate non-competitive inhibition. From the nature of graph obtained, the mechanism of inhibition was studied. Since mixed and competitive mode of inhibition cannot be differentiated by Dixon Plot, Cornish- Bowden plot was performed to confirm the mode of inhibition.

Cornish- Bowden plot

The experiment was performed as mentioned in Dixon Plot. The difference here lies in plotting a graph with the concentration of inhibitor [I] taken along X-axis and substrate concentration divided by product concentration (S / [V]) along Y-axis.From the nature of graph obtained, the mechanism of inhibition was studied as suggested by Eisenthel and Cornish-Bowden (1974)[17]. Lines running parallel indicate competitive inhibition, intersection in the space above the negative side of X-axis indicates uncompetitive inhibition while intersection beneath the negative side of X-axis indicates mixed inhibition and intersection on the negative side of X-axis indicates non-competitive mode of inhibition.

DPPH Radical Scavenging Activity

The reduction capacity of DPPH was determined by decrease in absorbance at 517 nm, which is induced by antioxidants[18]. *Momordica charantia* flesh and seed extracts at various concentrations (200 – 1000 µg) were taken and the volume was adjusted to 100 µl with methanol. 5 ml of 0.1 mM methanolic solution of DPPH was added and allowed to stand for 20 min at 27°C. The absorbance of the sample was measured at 517 nm. Ascorbic acid was used as a standard. Percentage radical scavenging activity of the sample was calculated as follows: % DPPH radical scavenging activity = (control OD-sample OD / control OD) × 100.

Nitric Oxide Radical Scavenging Activity

Aqueous solution of sodium nitroprusside spontaneously generates nitric oxide (NO) at physiological pH, which interacts with O_2 to produce nitrite ions, which is measured at 546 nm[19]. 3ml of 10mM sodium nitroprusside in 0.2 M phosphate buffered saline (pH 7.4) was mixed with different concentrations (200 - 1000µg) of *Momordica charantia* flesh and seed extracts and incubated at room temperature for 150 min. After incubation time, 0.5 ml of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediaminedihydrochloride in 2% H_3PO_4) was added. The absorbance of the chromophore formed was read at 546 nm. Ascorbic acid was used as a standard. Percentage radical scavenging activity of the sample was calculated as follows: % NO radical scavenging activity = (control OD-sample OD / control OD) x 100.

Hydroxyl Radical Scavenging Activity

Hydroxyl radicals were generated from ferrous ammonium sulphate and EDTA. This was detected by their ability to react with ascorbic acid to produce yellow colour complex which was measured at 412 nm [20].Different concentrations of the *Momordica charantia* flesh and seed extracts (200 - 1000µg) were added with 1ml of iron-EDTA solution (0.13% ferrous ammonium sulfate and 0.26% EDTA), 0.5 ml of EDTA solution (0.018%), and 1ml of dimethyl sulfoxide (DMSO) (0.85% v/v in 0.1 M phosphate buffer, pH 7.4). The reaction was initiated by adding 0.5 ml of ascorbic acid (0.22%) and incubated at 80-90°C for 15 min in a water bath. After incubation, the reaction was terminated by the addition of 1ml of ice-cold TCA (17.5% w/v). Three milliliters of Nash reagent (75.0g of ammonium acetate, 3 ml of glacial acetic acid, and 2 ml of acetyl acetone were mixed and raised to 1 L with distilled water) was added and left at room temperature for 15 min. The intensity of the colour formed was measured spectroscopically at 412 nm against reagent blank. Quercetin was used as the standard. The % hydroxyl radical scavenging activity was calculated as follows:% Hydroxyl radical scavenging activity = (control OD-sample OD / control OD) × 100.

Superoxide Radical Scavenging Activity

Superoxide radicals were generated and the assay was based on the capacity of the sample to inhibit farmazan formation by scavenging the superoxide radicals generated in riboflavin-light-NBT system [21]. Each 3 ml reaction mixture contained 50 mM sodium phosphate buffer (pH 7.6), 20 mg riboflavin, 12 mM EDTA, 0.1 mg NBT and various concentrations (200 - 1000µg) of *Momordica charantia* flesh and seed extracts. Reaction

was started by illuminating the reaction mixture with sample extract for 90 seconds. Immediately after illumination, the absorbance was measured at 590 nm. The entire reaction assembly was enclosed in a box lined with aluminium foil. Identical tubes with reaction mixture kept in dark served as blank. Ascorbic acid was used as the standard. The percentage inhibition of superoxide anion generation was calculated as: % Superoxide radical scavenging activity = (control OD-sample OD / control OD) x 100.

Results and Discussion

Inhibition of alpha amylase by various solvent extracts of *Momordica charantia* flesh and seeds were studied and the results are presented in Table 1. Among the six solvent extracts used, ethyl acetate extracts showed maximum inhibition of 94.2% inhibition by *Momordica charantia* seed and 92.6% by *Momordica charantia* flesh extracts.

Table1.Alpha -amylase inhibitory activity in different solvent extracts

Solvent Extracts	MCF % Inhibition	MCS % Inhibition
Petroleum ether	44.3±2.54	52.9±1.45
Ethyl acetate	92.6±2.32	94.2±2.55
Chloroform	21.4±1.48	23.5±1.20
Ethanol	58.6±1.60	79.4±2.12
Acetone	10.6±0.35	12.3±0.36
Water	40.9±0.94	32±0.80

Values are mean ± SD of triplicates

Ethyl acetate and methanol extracts were effective than Petroleum ether and chloroform leaf extracts of *Thespesia populnea* in inhibiting the alpha amylase enzyme[22].Ethyl acetate extracts of *Mallotus repandus* stem significantly inhibited α-amylase activity in a dose dependent manner like acarbose. Bioactive compounds present in the ethyl acetate stem extract would have been responsible for multifaceted medicinal property [23].

Mechanism of alpha amylase inhibition of MCF and MCS was studied by Dixon and Cornish- Bowden plot and the results are shown in Fig 1a and 1b for MCF and Fig 2a and 2b for MCS.

Fig1. Mechanism of alpha amylase inhibition by *Momordica charantia* flesh extracts

Type of inhibition-Non Competitive

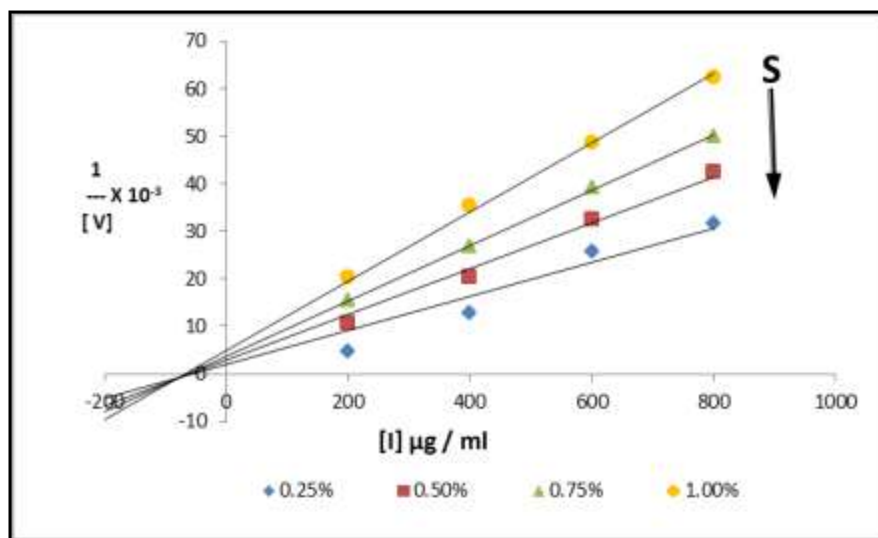

Fig 1a.Dixon Plot

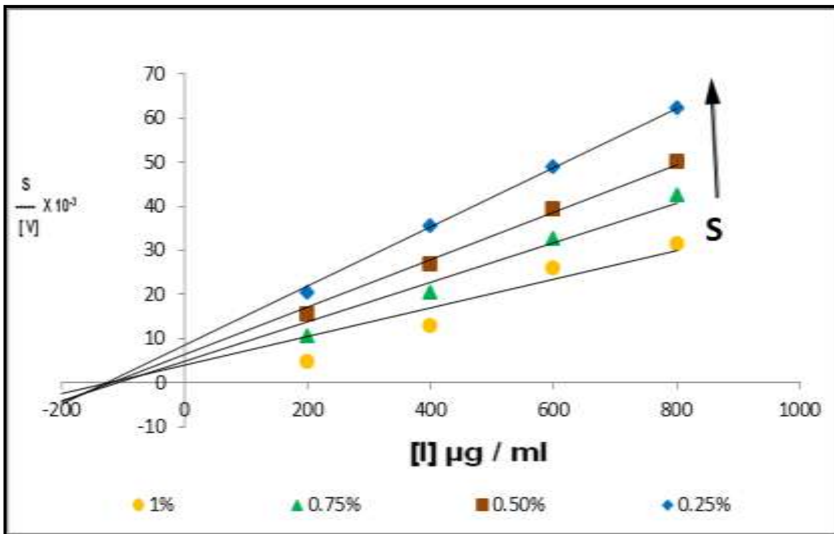

Fig 1b.Cornish-Bowden Plot

Fig 2. Mechanism of alpha amylase inhibition by *Momordica charantia* seed extracts

Type of inhibition-Non Competitive

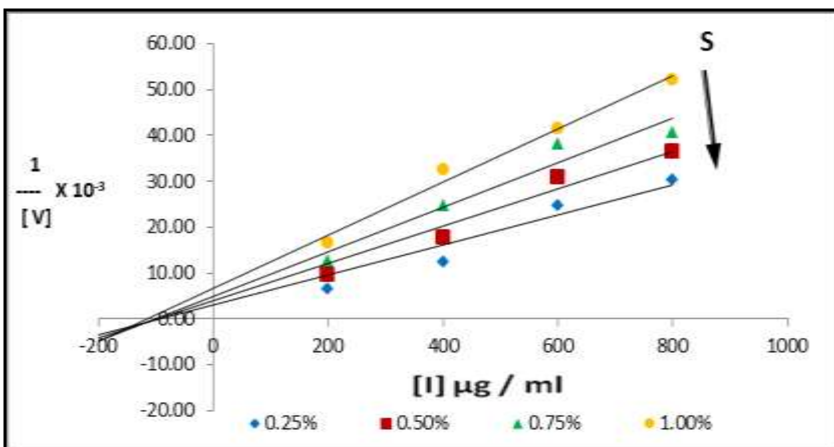

Fig 2a.Dixon Plot

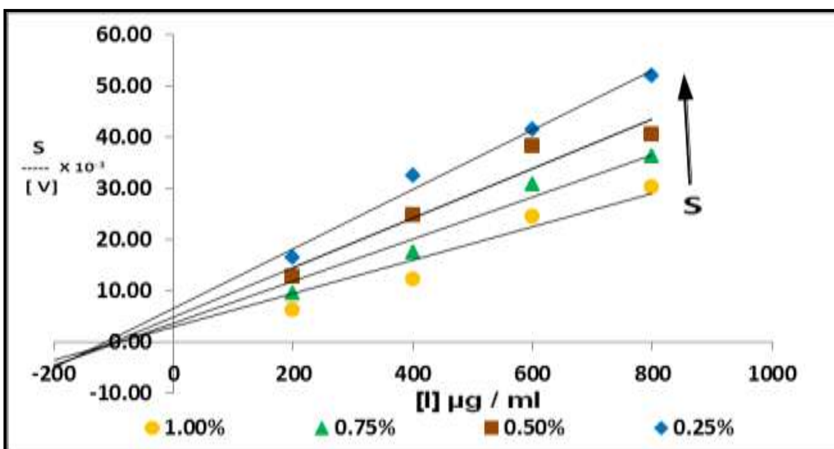

Fig 2b.Cornish-Bowden Plot

Dixon and Cornish- Bowden plots showthat the type of inhibition was non competitive type in both the *Momordica charantia* flesh and seed extracts.In non competitive inhibition, a molecule binds to an enzyme somewhere other than the active site. This type of inhibition reduces the maximum rate of a chemical reaction without changing the apparent binding affinity of the catalyst for the substrate. This suggests that the active component of the extract binds to a site other than the active site of the enzyme and combines with either free enzyme or enzyme substrate complex possibly interfering with the action of both [24].

Alpha-glucosidase inhibitors from ethyl acetate extracts of *Luculia pinciana* branches showed strongest inhibitory activity. Out offive active compounds isolated and identified, four of them showed non competitive type of α-glucosidase inhibition[25]. *Picralima nitida* leaf extracts exhibited non competitive mode of alpha amylase inhibition[26].

The results of DPPH and nitric oxide free radical scavenging capacity of MCF and MCS are shown in Fig 3 and Fig 4. Scavenging activity of the plant extracts was found to be dose dependant. The scavenging activity of DPPH by plants was found to be higher in MCS with 59% and MCF 55% at 1000 µg /ml. Standard ascorbic acid exhibited scavenging activity of 62% at 1000 µg/ml.Nitric oxide scavenging activity was found to be 53% in MCF and 56% in MCS extracts when compared to ascorbic acid standard with 77% scavenging effect.

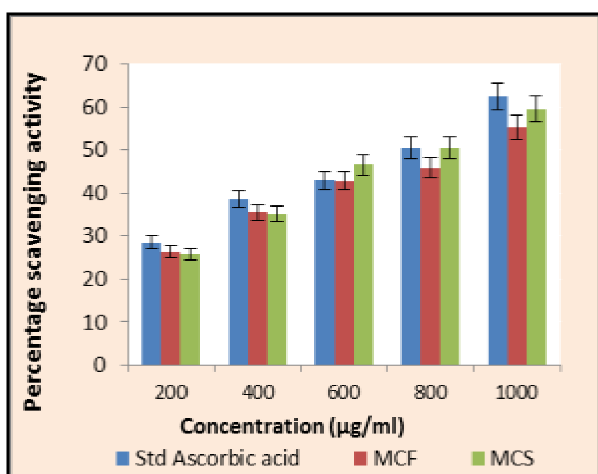

Fig 3.DPPH Radical Scavenging Activity

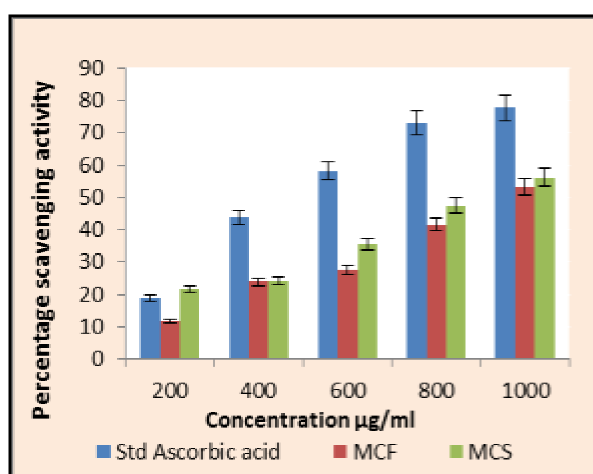

Fig 4.Nitric oxide Radical Scavenging Activity

Values are mean ± SD of triplicates

DPPH radical scavenging activities of the extracts depend not only on plant type but also upon the extraction solvent [27].DPPH radical scavenging effect had been reported in ethanol and ethyl acetate extracts of whole fruits of *Momordica charantia*[28].Ethyl acetate extracts of whole plant *Calycopteris floribunda* showed moderate Nitric oxide radical scavenging activity when compared with standard ascorbate[29]. Ethanol and ethyl acetate extracts of aerial parts of *Cordia retusa* showed nitric oxide scavenging activity[30].

The results of hydroxyl radical and super oxide radical scavenging capacity of MCF and MCS are shown in Fig 5 and Fig 6.

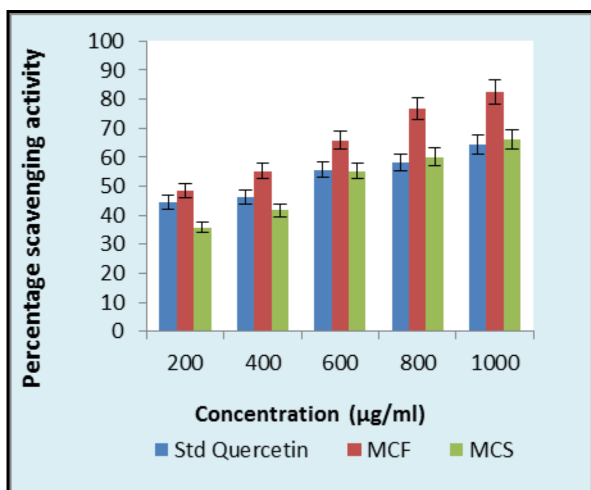

Fig 5: Hydroxyl Radical Scavenging Activity

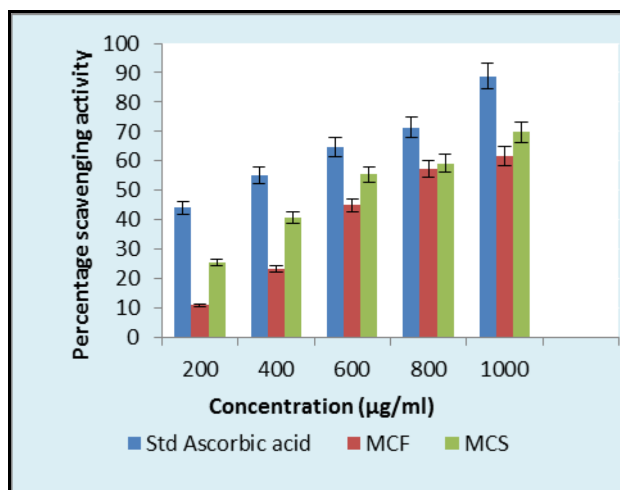

Fig 6: Superoxide Radical Scavenging Activity

Values are mean ± SD of triplicates

The hydroxyl radical scavenging activity of MCF and MCS, were found to be 82% and 66% when compared to standard quercetin which showed about 64% inhibition. The superoxide radical scavenging activity of MCF and MCS were found to be 70% and 62% when compared to standard ascorbic acid which showed about 89% inhibition.

The highly reactive hydroxyl radicals can cause oxidative damage to DNA, lipids and proteins. Hydrogen peroxide is a weak oxidizing agent that inactivates a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. It can cross cell membranes rapidly; once inside the cell, it can react with Fe^{2+} and Cu^{2+} ions to form hydroxyl radicals and this may be the origin of many of its toxic effects. Thus, removing hydrogen peroxide as well as O_2^- is very important for protection of food systems[31]. Ethyl acetate extracts of whole plant *Calycopteris floribunda* exhibited maximum hydroxyl radical scavenging activity than petroleum ether and methanolic extracts[29].

Superoxide anion radical is a precursor to active free radicals that have the potential of reacting with biological macromolecules and there by inducing tissue damage [32]. Ethanol and ethyl acetate extracts of whole fruit of *Momordica charantia* displayed a dose dependent activity in inhibiting the superoxide radicals against reference agent curcumin [28].Phytochemical screening of ethyl acetate extracts of *Momordica charantia* fruitsrevealed the presence of phenols, flavonoids, terpenoids and tannins[33].These phytochemicals present in the extracts might be responsible for alpha amylase inhibitory and free radical scavenging activities.

Conclusion

From the present work, it could be concluded that the solvents play a vital role in the extraction of the plant constituents. The ethyl acetate extracts of flesh and seeds of *Momordica charantia* showed potent alpha amylase inhibition and the mechanism of inhibition was found to be non competitive. The various antioxidant mechanisms exhibited by *Momordica charantia* may be attributed to their effectiveness as good scavengers of free radicals. This may be due to the active compounds present in ethyl acetate extracts. Hence, *Momordica charantia* might be useful in the control of hyperglycaemia and due to its potent antioxidant properties may help in prevention of complications in diabetes.

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