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Anti Diabetic Screening of Methanolic Extract of *Citrullus lanatus* Leaves

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ABSTRACT

Anti diabetic screening of Methanolic extract of *Cirullus lanatus* (MECL) was done by various *in vitro* methods. Preliminary phytochemical screening, total phenolic and flavonoid content, tannin content and estimation of Vitamins (B_1, B_2 and C) were done by standard procedure. TLC & HPTLC of MECL was done by using Quercetin, Gallic acid and Catechin as a standard. Screening of *in vitro* anti diabetic activity was done by Inhibition of α-amylase & α-glucosidase enzyme assay, Glucose uptake in yeast cells and Non - enzymatic glycosylation of Hemoglobin assay. Preliminary phytochemical screening showed the presence of carbohydrates, triterpenoids, proteins, alkaloids, tannins, saponins, flavonoids, sterols and absence of glycosides, volatile and fixed oil. The amount of Phenolic content (in terms mg GAE/g of extract), Flavonoids (mg Quercetin equivalent/g of extract) and Tannin content (mg Tannic acid/g of extract) present in the MECL were found to be 47.05±0.338, 89.99±0.30 and 290.9 ± 0.12mg/g respectively. The amount of Vitamin B_1, B_2 and C of MECL were found to be 56.28 ± 0.004, 245.37 ± 0.06 and 34.00 ± 0.009mg/g respectively. TLC & HPTLC qualitative estimation confirmed the presence of Quercetin, Gallic acid and Catechin in MECL. In Non-enzymatic glycosylation of hemoglobin assay, IC_{50} value was found to be 65.648µg/ml and 59.762µg/ml for MECL and α-Tocopherol respectively. MECL exhibited significant inhibition of glycosylation as compared with the standard drug α-Tocopherol. Glucose uptake assay in yeast cells, IC_{50} value was found to be 77.031µg/ml and 67.408µg/ml for MECL and Acarbose respectively. MECL showed greater efficiency in increasing the glucose uptake by yeast cells as compared to standard drug Acarbose. Inhibition of α-amylase enzyme assay, IC_{50} value was found to be 58.558µg/ml and 47.880µg/ml for MECL and Acarbose respectively. MECL showed significant α-amylase activity as compared to Acarbose. In α-glucosidase inhibition assay, IC_{50} was found to be 627.270µg/ml & 482.188µg/ml for MECL and Acarbose respectively. Overall MECL showed significant *in vitro* anti diabetic activity may be due to Phenolic, Flavonoid, Vitamin and Tannin content. MECL may be used as good candidate for diabetic treatment. Furthermore, *in vivo* studies are required for providing scientific information on the plant.

Keywords: *Citrullus lanatus*, Cucurbitaceae, Water melon, *in vitro* anti diabetic activity.

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INTRODUCTION

Diabetes Atlas published by International Diabetes Federation (IDF) estimated that 40 million persons with diabetes in India in 2007 and this number are predicted to raise 70 million people by 2025. WHO has predicted that, India will be the world's diabetes capital by 2025. It is gradually becoming more dangerous than AIDS. There is an ample evidence to suggest that preventive measures to reduce the burden of diabetes are needed[1]. The WHO Expert Committee on diabetes has recommended that traditional medicinal herbs be further investigated. Although several therapies are in use for treatment, there are certain limitations in allopathic drugs. The limitations of allopathic drugs include taking the medicines throughout life time, high cost therapy, side effects such as hypoglycemia, weight gain, gastrointestinal disturbances and liver toxicity etc. To date, over 400 traditional plant for diabetes have been reported, although only a small number of these have received scientific and medical evaluation to assess their efficacy[2].

Citrullus lanatus is well known as Watermelon plant (Family - Cucurbitaceae). Water melon is popular in indigenous system of folk medicine. It is a trailing annual plant with several herbaceous, firm and stout stems[3-5]. The leaves of *Citrullus lanatus* is used as anti-inflammatory, analgesic, gonorrhoea, mosquitocidal and anti microbial property[6-8]. Cucurbitaceae plants are known to contain therapeutic compounds such as triterpenes, sterols, cucurbitacin and alkaloids [9].

The plant *Citrullus lanatus* has been selected (specially the leaves) for the present investigation on the basis of the ethnomedical information and the review of literature as the plant is widely cultivated throughout India.

Studies on the leaves of *Citrullus lanatus* are still lacking. Hence to exploit its potential use prompted the present study to investigate the leaves of this plant with clear scientific protocol. The leaf extract of *Citrullus lanatus* contain bioactive compounds such as flavonoid, phenolic compound, tannin, triterpenes, sterols, alkaloids and vitamins. The extract may serve as a lead medicinal plant to synthesize various semi-synthetic drugs to treat various life threatening disease such as diabetes, cancer etc. Anti diabetic activity of *C.lanatus* seeds[10,11] & leaves of *C.colocynthis* [12] were previously reported. It prompted us to screen anti diabetic activity of *C.lanatus* leaves.

MATERIALS AND METHOD

Chemicals:

Methanol, Sodium carbonate solution (10%w/v), Folin Ciocalteu Reagent (1N), 10% Aluminum chloride, 1M Potassium acetate, 95%v/v Ethanol, 2, 4-Dinitro phenyl hydrazine (0.2%), 85% Sulphuric acid, Toluene, Ethyl acetate, Hemoglobin, 0.01M Phosphate buffer (pH 7.4), 0.5%

Potassium permanganate, 30% Hydrogen peroxide, 40% Sodium sulphate, α-Tocopherol, α-glucosidase enzyme, 0.2M Tris buffer, Glucose oxidase solution, α- Amylase enzyme, Gentamycin, 1% w/v of soluble starch solution, Iodine in potassium iodide solution, Acetate buffer, 0.01% Potassium dichromate solution, Glucose (5 & 10mM), Folin-Denis reagent and Baker yeast were used in this experiment.

Instrument:

Shimadzu UV Visible spectrophotometer (Model 1800). HPTLC: CAMAG TLC Scanner 3 "Scanner3-070408" S/N 070408 (1.41.21) was used for detection and CAMAG Linomat 5 sample applicator was used for the application of the track. Twin trough plate development chamber was used for development of chromatogram. Software used was win CATS 1.4.3

Collection and preparation of extract:

The leaves of *C.lanatus* were collected and washed thoroughly and dried in shade. The shade dried leaves were powdered and sieved in a No.60 sieve and used for the further studies. About 500g of the dried powdered leaf of *C.lanatus* was defatted with 1.5L Petroleum ether (60-80°C) by maceration. The solvent was removed by filtration and the marc was dried. To the dried marc 1.5L of Methanol was added and the extraction was performed by triple maceration (72hr process). It was then filtered and the combined filtrate was evaporated to a cohesive mass using rota vapour.

Qualitative chemical tests for the leaf powder and crude extracts [13-15]

Preliminary phytochemical screening of MECL was carried out to find out the presence of various phytoconstituents using standard procedure.

QUANTITATIVE ESTIMATION OF PHYTOCONSTITUENTS

Estimation of total phenolic content[16-18]

Phenols are widespread in nature and are important constituents of medicinal plants. They range from simple structures with one aromatic ring to highly complex polymeric substances such as tannins, flavonoids, Anthraquinone and coumarins. Phenolic substances are water soluble and they have been reported to have multiple biological effects, including antioxidant activity.

The calibration curve of concentration versus absorbance was generated for Gallic acid at different concentrations (2, 4, 6, 8, 10µg/ml) which was used as a standard. The amount of phenol present can be determined by linear regression analysis. The total Phenolic content of the MECL was determined by Folin-Ciocalteau reagent and it was expressed as milligram of Gallic acid equivalent (GAE) per g of extract.

Total Flavonoid content estimation [19-21]

Flavonoids are widely distributed in nature. It consists of one Benzene–gamma – pyrone structure.

They have ability to complex with metal ions and act as an antioxidants and bind to proteins such as structural proteins and enzymes. The different classes within the groups are distinguished by additional oxygen containing heterocyclic rings and hydroxyl groups which includes Flavones, Flavanones, Flavonols, Isoflavones, Catechin, Anthocyanidins, Leuco anthocynadins, Chalcones and Aurones.

The Aluminum chloride colorimetric technique was used for estimation of total Flavonoid content. The intensity of the colour is proportional to the amount of Flavonoids and can be estimated as Quercetin equivalent at wavelength of 415nm. The amount of Flavonoids present was determined by linear regression analysis. The total Flavonoid content in MECL was expressed as mg of Quercetin equivalents per g of extract.

Estimation of Tannins [16]

Tannins are naturally occurring polyphenolic compounds of varying structure. Tannins are having antioxidant and antimicrobial activities and also used as antiseptic and astringents. They are divided into two main groups namely hydrolysable and condensed. Hydrolysable tannins contain a polyhydric alcohol and Condensed tannins are mostly Flavonols.

Principle:

The tannins are estimated by Folin-Denis Method. This is based on the non stoichiometric oxidation of the molecules containing a phenolic hydroxyl group. Tannins reduce phosphotungsto molybdic acid in alkaline solution to produce a highly colored blue solution. The intensity is directly proportional to the amount of tannins and measured by using spectrophotometer at 700nm.

Procedure:

About 0.2ml of Methanolic extract of *C. lanatus* was pipetted into test tubes. To this, 0.5ml of Folin-Denis reagent and 0.8ml of distilled water was added. The tubes were kept aside for 15min. To this, 1ml of Sodium carbonate solution was added and the remaining volume was made up with 7.5ml of distilled water. Then the tubes were shaken and the absorbance was recorded at 700nm after 30min. Tannic acid, used as a standard was taken at different concentration i.e 2, 4, 8, 12, 16, 20µg/ml in different test tubes and the procedure adopted above was followed. The calibration curve for Tannic acid was plotted using concentration versus absorbance. A linear regression equation was calculated and the equation was used to calculate the amount of total Tannins as Tannic acid equivalent. The amount of Tannin content is expressed in mg/g of extract.

ESTIMATION OF VITAMINS

Estimation of Vitamin B_1 [22, 23]

Principle:

The reaction of Thiamine hydrochloride with Potassium obtained is ionic 1:1 in which Thiamine is present as a cation and $Cr_2O_7^{2-}$ as an anion. The chemical formula of the salt, ($C_{12}H_{18}N_4OS$) (Cr_2O_7), has been established by UV/Vis spectra absorbance of about 360nm.dichromate ($K_2Cr_2O_7$) has been studied in alcoholic solution at room temperature.

Sample Preparation:

10g of powdered plant material was homogenized with Ethanolic Sodium hydroxide, filtered and used as a sample.

Procedure:

Thiamine was weighed and dissolved in water to get stock solution of 2mg/ml. Further dilutions were made to get the concentrations ranging from 100-500µg/ml. To 10ml of sample, 10ml of Potassium dichromate solution was added and the colour produced was measured at 360nm. A calibration curve was constructed by plotting concentration versus absorbance of Thiamine. The above procedure was repeated for the plant extract and the absorbance was measured at 360nm. The amount of Vitamin B_1 can be determined by linear regression analysis and it was expressed as mg/g of extract.

Estimation of Vitamin B_2 [23, 24]

Principle:

Riboflavin was treated with Potassium permanganate ($KMnO_4$) and Hydrogen peroxide (H_2O_2). Mixing of Hydrogen peroxide solution, where upon the permanganate colour is destroyed, excess oxygen is expelled and then Sodium sulphate was added and a yellow colour was obtained. The absorbance of the colour was measured at 550nm by UV/Vis spectrophotometer.

Sample Preparation:

10g of powdered plant material was extracted with 50% Ethanol solution and shaken for 1hr. The plant extract was filtered and used as a sample.

Procedure:

Riboflavin was weighed and dissolved in water to get stock solution of 20mg/ml. Further dilutions were made to get the concentrations ranging from 2-10mg/ml. To 15ml of sample and 10ml of 0.5% Potassium permanganate and 1ml of 30% Hydrogen peroxide were added and allowed to stand over a hot water bath for about 30min. 2ml of 40% Sodium sulphate was added. This was made up to 5ml. The absorbance of the chromogen was measured at 550nm in a UV visible spectrophotometer. A calibration curve was constructed by plotting concentration versus absorbance of Riboflavin. The above procedure was repeated for the plant extract and the

absorbance was measured at 550nm. The amount of Vitamin B_2 present can be determined by linear regression analysis and it was expressed as mg/g of extract.

Estimation of Vitamin C [25-30]

Vitamin C is also an important physiological antioxidant and has been shown to regenerate other antioxidants within the body, including α-Tocopherol (Vitamin E). Vitamin C might help to prevent or delay the development of certain cancers, cardiovascular disease, and other diseases in which oxidative stress plays a causal role. In addition to its biosynthetic and antioxidant functions, vitamin C plays an important role in immune function and improves the absorption of non-heme iron, the form of iron present in plant-based foods.

Principle:

The keto group of Ascorbic acid undergoes a condensation reaction with 2, 4 Dinitro phenyl hydrazine to form a hydrazone which is orange yellow and has an absorbance of about 520nm.

Procedure:

Ascorbic acid was weighed and dissolved in water to get stock solution of 1mg/ml. Further dilutions were made to get the concentrations ranging from 40-200µg/ml. To 1ml of sample 0.5ml of Dinitro phenyl hydrazine solution was added and incubated for 3hr at 37ºC. After 3hr, 2.5ml of 85% Sulphuric acid was added and the absorbance was measured after 30min at 520nm. A calibration curve was constructed by plotting concentration versus absorbance of Ascorbic acid. The procedure was repeated for the plant extract as above and the absorbance was measured at 520nm after 3hr. The amount of Vitamin C can be determined by linear regression analysis and it was expressed as mg/g of extract.

Thin layer chromatography of MECL:

Stationary phase - Silica gel G

Solvent System I: Toluene: Ethyl acetate: Methanol (7:2:1)

Solvent System II: Chloroform: Methanol (9:1)

Detection: Visual and UV light at 254 & 366nm

The Rf value of the spots obtained by standard formula.

High performance thin layer chromatography of MECL:

Stationary Phase:

Aluminium sheet pre-coated with silica gel Merck GF254, 0.2mm layer thickness was used as stationary phase.

Mobile phase:

Toluene: Ethyl acetate: Methanol (7:2:1) was used as mobile phase for development of

chromatogram.

Detection wavelength:

The developed plates were examined at wavelength 254nm and 366nm in Densitometry TLC scanner 3. The TLC visualization, 3D display of the finger print profile and peak display at 254nm and 366nm.The Rf values and area under curve for each peak was determined.

In- vitro ANTIDIABETIC ACTIVITY

Non enzymatic glycosylation of Hemoglobin assay [31-33]

Glycated hemoglobin (HbA1c) is a form of hemoglobin that is measured primarily to identify an average plasma glucose concentration over prolonged period of time. The amount of glycated Hemoglobin should not be more than 12%. In Diabetes mellitus, higher amounts of glycated Hemoglobin, indicating poor control of blood glucose levels, have been associated with cardiovascular disease, nephropathy and retinopathy.

Procedure:

The MECL and α-Tocopherol was dissolved separately in DMSO to get a stock solution of 1mg/ml. To the various concentrations of the stock solution (200-1000µg/ml), 1ml of glucose solution, 1ml of Hemoglobin solution and 1ml of Gentamycin (20 mg/100 ml) in 0.01M Phosphate buffer (pH 7.4) was added. The mixture was then incubated in dark at room temperature for 72 hr. α-Tocopherol was used as a standard drug. Control was prepared in the similar manner except the addition of extract. The degree of glycosylation of Hemoglobin in the presence of different concentration of extracts and their absorbance were measured at 520nm using UV Visible spectrophotometer.

The percentage inhibition was calculated using the following formula;

$$\% \text{ Inhibition} = \text{Abs sample} - \text{Abs control} / \text{Abs sample} \times 100$$

Glucose uptake by yeast cells [34-36]

It is reported that in yeast cells glucose transport is extremely complex and it is generally agreed that glucose is transported in yeast by facilitated diffusion process. Facilitated carriers are specific carriers that transport solutes down the concentration gradient. This means that effective transport is only attained if there is removal of intracellular glucose.

Preparation of yeast suspension:

1g of the commercial Baker's yeast was washed by repeated centrifugation at 3,000 RPM for 5min in distilled water until the supernatant fluids were clear and a 10% (v/v) suspension was prepared in distilled water.

Procedure:

To the various concentrations (40–200µg/ml) of the MECL was added to 1ml of glucose solution (5 & 10mM) and incubated together for 10min at 37°C. The reaction was started by adding 100µl of yeast suspension, vortexed and further incubated at 37°C for 60min. After 60min, the tubes were centrifuged at 2,500RPM for 5min. To 100µl of glucose oxidase solution, 500µl of supernatant solution was added and kept for half an hour at room temperature. Then the percentage increase in glucose uptake by the yeast cells was measured at 520nm using a UV visible spectrophotometer. Distilled water was used as a blank. Acarbose was taken as standard drug. The reaction without extract was used as control. The percentage increase in glucose uptake by yeast cells was calculated using the following formula,

Increase in glucose uptake (%) = Abs sample – Abs control/ Abs sample × 100

Inhibition of alpha amylase enzyme assay [37-40]

Pancreatic α-amylase belongs to the class of α 1, 4- gluconohydrolases and is one of the important target enzymes for the conventional treatment of diabetes. It catalyzes the initial step in hydrolysis of starch to maltose and maltotriose which are then acted upon by α-glucosidases, broken down into glucose and it enters the blood stream. Naturally available α-amylase inhibitors from medicinally important plants are shown to be very effective in managing post prandial hyperglycemia which is a major concern in type 2 diabetes.

Pancreatic α-amylase is a key enzyme in the digestive system and catalyzes the initial step in the hydrolysis of starch, which is a principal source of glucose in the diet. α-amylase inhibitors are agents which inhibit the amylase activity that results in the delay of carbohydrate digestion and prolong overall carbohydrate digestion time causing reduction in the rate of glucose absorption and consequently reducing the post prandial plasma glucose rise.

Preparation of 0.1M Sodium acetate buffer:

An accurately weighed 820.3mg of Sodium acetate and 18.7mg of Sodium chloride was dissolved in 100ml of distilled water.

Preparation of Iodine – Iodide indicator:

An accurately weighed 635mg of Iodine and 1g of Potassium iodide was dissolved in 250ml of distilled water.

The MECL and Acarbose were dissolved in Sodium acetate buffer to get a stock solution of 1mg/ml. To various concentrations of the stock solution (200-1000µg/ml), 1ml of 1% w/v of soluble starch solution, 1ml of α-amylase enzyme & 2ml of 0.1M Sodium phosphate buffer (pH 7.4) was added. Then this solution was incubated for 1hr at 37°C. After incubation, 0.1ml of

Iodine-iodide indicator was added. The intensity of the colour was measured at 565nm using UV Visible spectrophotometer. 0.1M Sodium acetate buffer was used as a blank. The reaction without extract was used as a control. Acarbose was used as a standard drug. Inhibition of enzyme activity was calculated by using the following formula:

% Inhibition of enzyme activity = Abs sample – Abs control / Abs sample ×100

Inhibition of α- glucosidase enzyme assay [41-44]

α-glucosidase inhibitors are used to establish greater glycemic control over hyperglycemia in Diabetes type 2 particularly with regard to postprandial hyperglycemia. They may be used as monotherapy in conjunction with an appropriate diabetic diet and exercise, or they may be used in conjunction with other anti-diabetic drugs. α-glucosidase inhibitors may also be useful in patients with type 2 Diabetes.

To the various concentrations of the (200-1000µg/ml) of MECL and Acarbose add 0.1ml of α-glucosidase enzyme (1U/ml), 1ml of 0.2M Tris buffer (pH 8) was added. Then the mixture was incubated for 60min at 35°C. Then the reaction was terminated by heat it for 2min in boiling water bath. The amount of liberated glucose is measured by glucose oxidation method at 540nm using UV Visible spectrophotometer. Acarbose was used as a standard drug. Control was prepared in similar way except extract. Distilled water was used as a blank. The percentage inhibition was calculated using the formula.

% Inhibition = Abs sample – Abs control / Abs sample× 100

RESULTS AND DISCUSSION

Preliminary phytochemical screening of *C.lanatus* leaves (Tab 1)

Table 1: Preliminary phytochemical screening of *Citrullus lanatus* leaf

S. No.	Test	Extract	
		Aqueous extract	**Methanolic extract**
1.	**Test for Carbohydrates**		
	a. Molisch's test	+	+
	c. Benedict's test	+	+
	b. Fehling's test	+	+
2.	**Test for Alkaloids**		
	a. Mayer's reagent	+	+
	b. Dragendroff's reagent	+	+
	c. Hager's reagent	+	+
	d. Wagner's reagent	+	+
3.	**Test for Phytosterols**		
	a. Salkowski's test	+	+
	b. Libermann- burchard's test	+	+
4.	**Test for Glycosides**		

	a. Anthraquinone glycosides	-	-
	i) Borntrager's test	-	-
	ii) Modified Borntrager's test	-	-
5.	**Test for Proteins**		
	a. Millon's test	+	+
	b. Biuret test	+	+
	Amino acids		
	a. Ninhydrin test	+	+
6.	**Test for Mucilage**	-	-
7.	**Test for Flavonoids**		
	a. Shinoda test	+	+
	b. Alkali test	+	+
	c. Acid test	+	+
8.	**Test for Terpenoids**	+	+
9.	**Test for Phenolic compounds**		
	a. 5% Ferric chloride solution	+	+
	b. Lead acetate solution	+	+
	c. Bromine water	+	+
	d. Acetic acid solution	+	+
	e. Dilute iodine solution	+	+
	f. Tannic acid	+	+
10.	**Test for Tannins**		
	$FeCl_3$ test	+	+
11.	**Test for Saponins**		
	Foam test	+	+
12.	**Test for Volatile oils**	-	-

Quantitative estimation of phytoconstituents

Estimation of Phenolic content

Phenolic content of MECL and calibration curve of Gallic acid was presented (Tab 2 & Fig. 1)

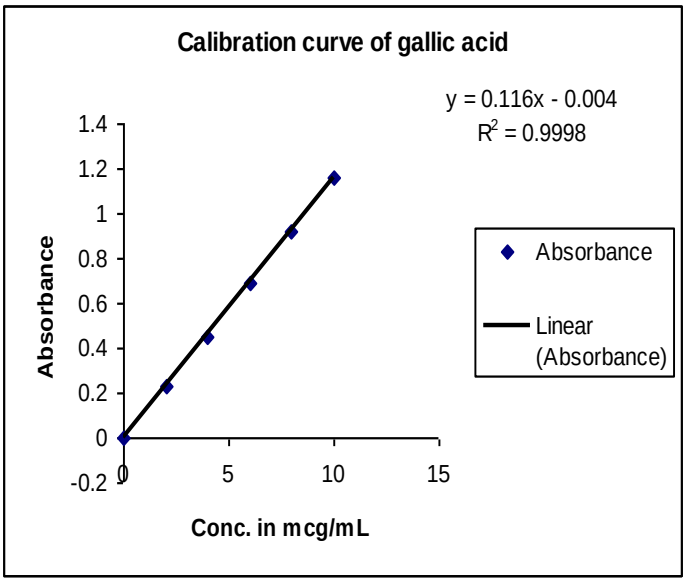


Figure. 1: Calibration curve of Gallic acid for estimation of total Phenolic content

Table 2: Total Phenolic content of MECL in terms of Gallic acid equivalents

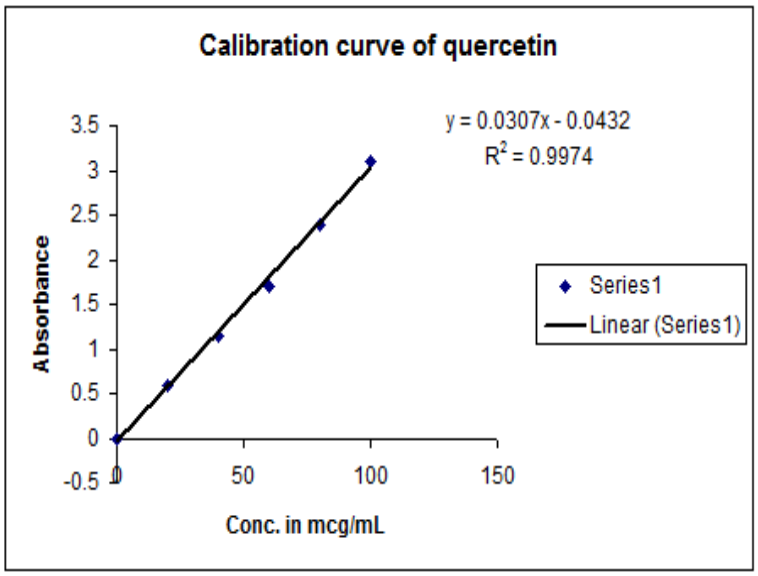
S. No.	Conc. of Gallic acid in µg/ml	Absorbance at 760nm	Conc. of extract in µg/ml	Absorbance at 760nm*	Amount of total Phenolic content in terms mg GAE/g of extract*
1	2	0.229 ± 0.010	50	0.256±0.004	43.90±0.304
2	4	0.452 ± 0.006	100	0.578±0.004	50.20±0.373
3	6	0.695 ± 0.005	**Average 47.05 ± 0.338**		
4	8	0.918 ± 0.031			
5	10	1.162 ± 0.028			

The linear regression equation was found to be y=0.116x-0.004 while the correlation coefficient was found to be 0.9998. The amount of Phenolic content present in the extract in terms mg GAE/g of extract was found to be 47.05±0.338 by using the above linear regression equation.

Polyphenols are naturally occurring compounds largely found in the herbals and medicinal plants. Phenolic compound may protect cell constituents against oxidative damage and, therefore, limit the risk of various degenerative diseases associated with oxidative stress[45]. Consumption of plant polyphenols offer protection against development of diabetes, cancers, cardiovascular diseases and neurodegenerative diseases [46, 47].

Estimation of total Flavonoids

Total Flavonoid estimation MECL & Calibration curve of Quercetin was presented (Table 3 & Figure. 2).



*** mean of three readings ±SEM**

Figure. 2: Calibration curve of Quercetin

Table 3: Total Flavonoid content of MECL in terms of Quercetin equivalents

S. No.	Conc. of Quercetin in µg/ml	Absorbance at 415nm	Conc. of Methanolic extract in µg/ml	Absorbance at 415nm*	Amount of total Flavonoid content in terms mg Quercetin equivalent/ g of extract*
1	20	0.589 ± 0.01	50	0.090±0.001	86.55±0.21
2	40	1.151 ± 0.04	100	0.243±0.003	93.44±0.39
3	60	1.710 ± 0.09	**Average 89.99±0.30**		
4	80	2.390 ± 0.03			
5	100	3.112 ± 0.03			

*** mean of three readings ±SEM**

The linear regression equation was found to be y=0.0307x-0.0432 while the correlation was found to be 0.9974. The amount of Flavonoid content present in the extract in terms mg Quercetin equivalent/g of extract was found to be 89.99 ± 0.30 by using the above linear regression equation. More than 4,000 varieties of flavonoids have been identified, many of which are responsible for the therapeutic activity in humans. Quercetin, Myricetin, Catechin etc., are some most common flavonoids[48]. Quercetin is known to possess strong anti diabetic activity. Recently reported Quercetin has ability to protect the alterations in diabetic patients during oxidative stress. Quercetin significantly protected the lipid peroxidation and offer antioxidant effect in Diabetes[49].

Total Tannin estimation

Total Tannin estimation of MECL & calibration curve of Tannic acid was presented (Table 4 & Figure 3).

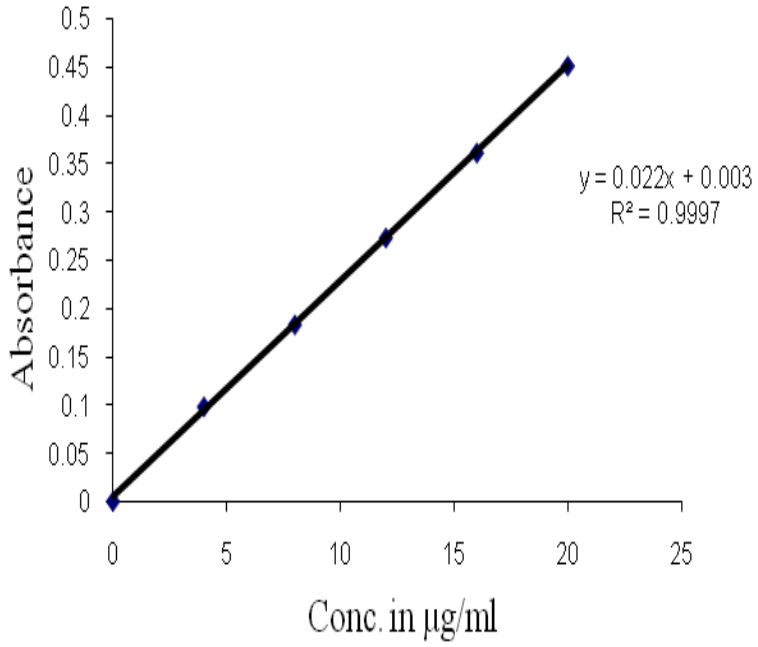


Figure. 3: Calibration curve of Tannic acid

Table 4: Total Tannin content in MECL in terms of Tannic acid equivalents

S. No.	Conc. of Tannic acid in µg/ml	Absorbance at 760nm	Conc. of Methanolic extract in µg/ml	Absorbance at 760nm*	Amount of total Tannin content in terms mg Tannic acid/g of extract*
1	4	0.098 ± 0.020	10	0.060±0.03	260.60±1.51
2	8	0.183 ± 0.010	20	0.131±0.07	292.42±2.00
3	12	0.203 ± 0.010	**Average**		
4	16	0.361 ± 0.200	**276.51±1.75**		
5	20	0.451 ± 0.100			

*** mean of three readings ±SEM**

The linear regression equation was found to be y = 0.022x + 0.003 while the correlation was found to be 0.9997. The amount of Tannin content present in the MECL in terms of mg Tannic acid/g of extract was found to be 276.51 ± 1.75 by using the above linear regression equation.

There is a clear link between hyperglycemia and active oxygen/nitrogen species in diabetes[50]. Accumulation of Reactive Oxygen Species (ROS) due to oxidative stress which easily oxidize vital cellular components such as lipids, proteins and DNA. Plant derived anti-oxidant (Tannins) treatment has been reported to reduce the development of diabetic complications such as retinopathy, cataract formation, neuropathy, vascular complication and nephropathy[51].

ESTIMATION OF VITAMINS

Estimation of Vitamin B_1

Vitamin B_1 content of MECL and calibration curve of Thiamine was presented (Table 5 & Figure 4).

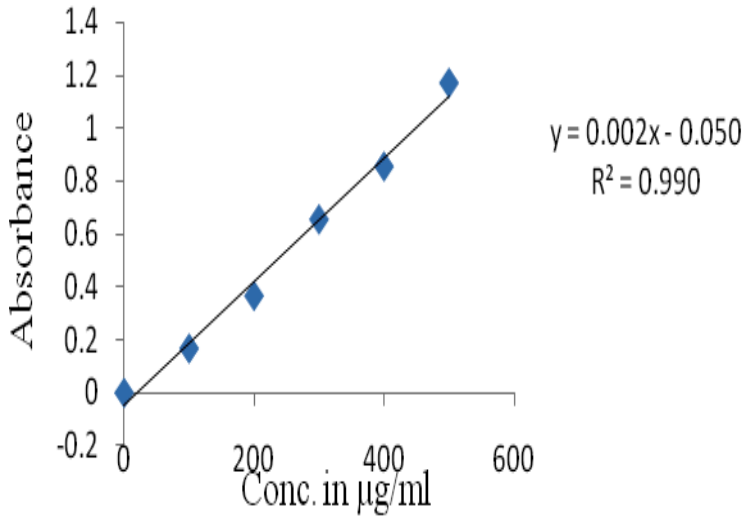


Figure. 4: Calibration curve of Standard Thiamine

Table 5: Estimation of Vitamin B_1 of MECL

S. No.	Conc. of Thiamine in µg/ml	Absorbance at 360nm	Conc. of Methanolic extract in µg/ml	Absorbance at 360nm*	Amt of Vitamin B_1 present mg/ g of extract*
1	100	0.168 ± 0.001	100	0.286 ± 0.004	54.82 ± 0.006
2	200	0.367 ± 0.002	200	0.321 ± 0.007	55.02 ± 0.006
3	300	0.656 ± 0.002	300	0.456 ± 0.003	59.00 ± 0.002
4	400	0.856 ± 0.001	**Average**		
5	500	1.172 ± 0.003	**56.28 ± 0.004**		

***mean of three readings ± SEM**

The linear regression equation was found to be y = 0.002x - 0.050 and a correlation coefficient of 0.990. The amount of Vitamin B_1 content present in the MECL was found to be 56.28 ± 0.004 mg/g by using the above linear regression equation.

Thiamine supplementation prevented the development of early-stage nephropathy in diabetic rats and reversed increased urinary albumin excretion in patients with type2 diabetes[52]. Dysfunction of β-cells and impaired glucose tolerance in Vitamin B_1 deficiency and provides a link of impaired glucose tolerance with dietary Thiamine indicates that Thiamine therapy may plays a future role in prevention of Type 2 Diabetes[53]. Vit B_1 acts as a coenzyme for Pyruvate dehydrogenase, Transketolase (TK) and α-Ketoglutarate dehydrogenase complexes which play a fundamental role for intracellular glucose metabolism. Thiamine used as potential and inexpensive approach to the prevention and/or treatment of diabetic vascular complications [54].

Estimation of Vitamic B_2

Vitamin B_2 content of MECL & Calibration curve for standard Riboflavin was presented (Table 6 & Figure. 5)

Table 6: Estimation of Vitamin B_2 of MECL

S. No.	Conc. of Riboflavin in mg/ml	Absorbance at 360nm	Conc. of Methanolic ext. in mg/ml	Absorbance at 360nm*	Amt of Vitamin B_2 present mg/ g of extract*
1	2	0.161 ± 0.006	2	0.047 ± 0.004	31.99 ± 0.011
2	4	0.377 ± 0.012	4	0.111 ± 0.007	32.50 ± 0.006
3	6	0.555 ± 0.002	6	0.203 ± 0.003	37.29 ± 0.010
4	8	0.766 ± 0.005	**Average**		
5	10	0.958 ± 0.004	**34.00 ± 0.009**		

***mean of three readings ± SEM**

The linear regression equation was found to be y = 0.0969x - 0.015 and a correlation coefficient of 0.9989. The amount of Vitamin B_2 content present in the MECL was found to be 34.00 ± 0.009 mg/g by using the above linear regression equation.

Riboflavin coenzymes are involved in the regulatory functions of some hormones that are connected with carbohydrate metabolism [55].

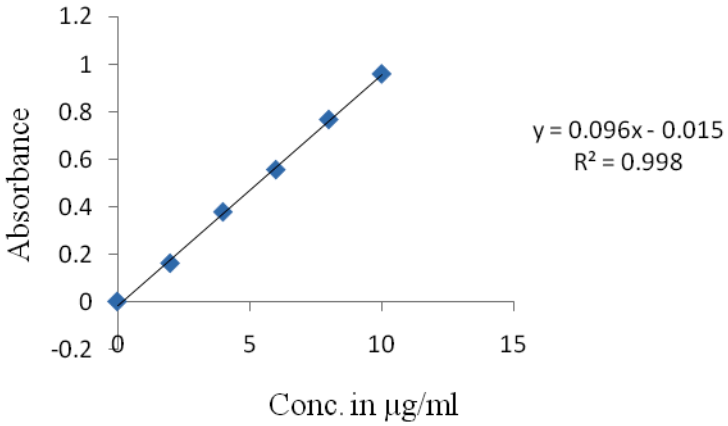


Figure. 5: Calibration curve of Standard Riboflavin

Estimation of Vitamin C

Vitamin C content of MECL & calibration curve of standard Ascorbic acid was presented (Table 7 & Figure 6).

Table 7: Estimation of Vitamin C of MECL

S. No.	Conc. of Ascorbic acid in µg/ml	Absorbance at 520nm	Conc. of Methanolic ext in µg/ml	Absorbance at 520nm	Amt of Vitamin C present / g of extract
1	40	0.135 ± 0.000	100	0.076 ± 0.004	237.03 ± 0.006
2	80	0.265 ± 0.015	200	0.137 ± 0.007	253.70 ± 0.006
3	120	0.346 ± 0.010			
4	160	0.468 ± 0.011	**Average**		
5	200	0.525 ± 0.010	**245.37 ± 0.006**		

***mean of three readings ± SEM**

The linear regression equation was found to be y = 0.0027x + 0.012 and a correlation coefficient of 0.9982. The amount of Vitamin C content present in the MECL was found to be 245.37 ± 0.006mg/g by using the above linear regression equation.

Oxidative stress may be linked to tissue damage and the development of regenerative disorders. Oxidative stress may be a common pathway linking diverse mechanisms for the pathogenesis of complications in diabetes. Vitamin C act as antioxidant to scavenge free radicals which responsible for pathogenesis of Diabetes [56].

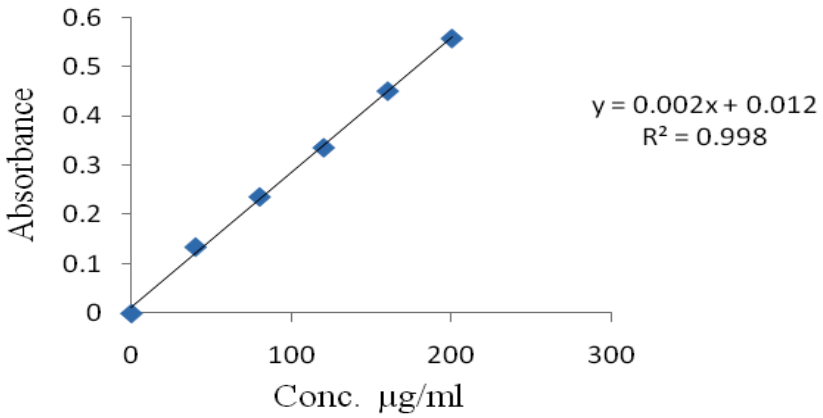


Figure. 6: Calibration curve of Ascorbic acid

TLC of MECL

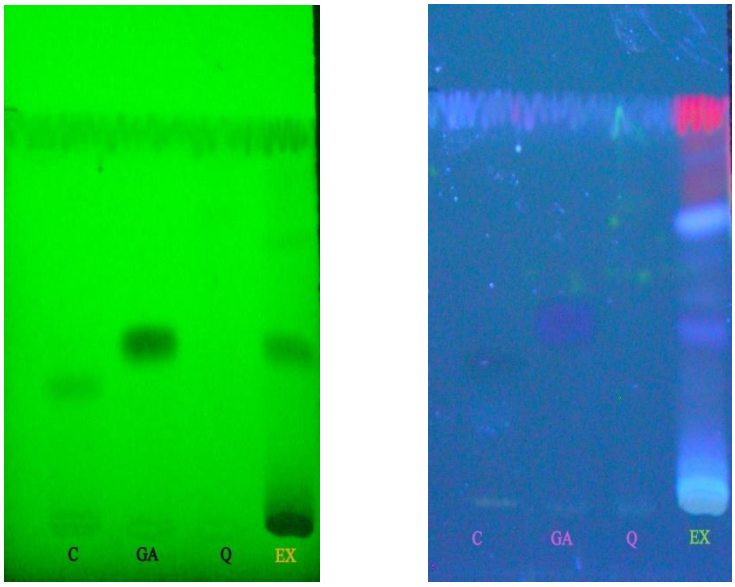
Table 8: Phytochemical evaluation of MECL by TLC studies

S. No	Solvent system	Detecting agent	No. of spots	Colour of spots	Rf values
1.	Toluene: Ethyl acetate: Methanol (7:2:1)	Under UV at 366nm	5	Orange	0.94
				Dark orange	0.84
				Dark red	0.72
				Light pink	0.45
				Light pink	0.37
		Under Visible light	6	Yellow	0.92
				Brown	0.82
				Dark green	0.72
				Yellowish green	0.58
				Yellowish green	0.47
				Yellow	0.35
2	Chloroform: Methanol(9:1)	Under UV at 366nm	4	Dark brown	0.90
				Dark orange	0.72
				Yellow	0.68
				Light pink	0.46
		Under Visible light	6	Dark green	0.92
				Yellow	0.74
				Yellow	0.70
				Light green	0.52
				Brown	0.46
				Brown	0.34

The extract showed 5 spots at 366nm and 6 spots at visible light. The Rf value of 0.72, 0.37 and 0.45 may be due to the presence of flavonoids, phenolic compounds and tannin. When viewed under UV at 366m and visible light after development in the mobile phase namely Chloroform: Methanol (9:1). The Rf value of 0.9 and 0.72 may be due to the presence of Cucurbitacin glucoside B and Cucurbitacin glucoside E. The extract also showed different Rf value under UV at 366nm and Visible light. The Rf may be due to the presence of different active principle might be responsible for the therapeutic activity.

High Performance Thin Layer Chromatography of MECL

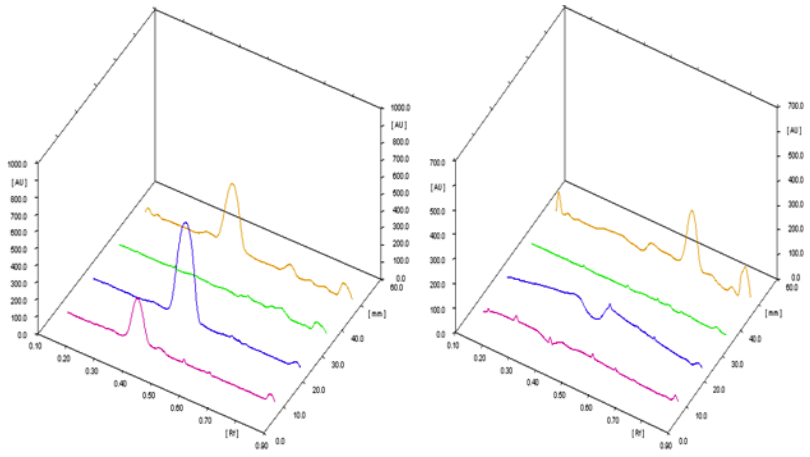
The visualization of the HPTLC plate of MECL at 254nm and 366nm was presented (Figure. 7). The photo of plate at 254nm showed the presence of 8 spots while at 366nm showed the presence 8 spots.



@254nm **@366nm**

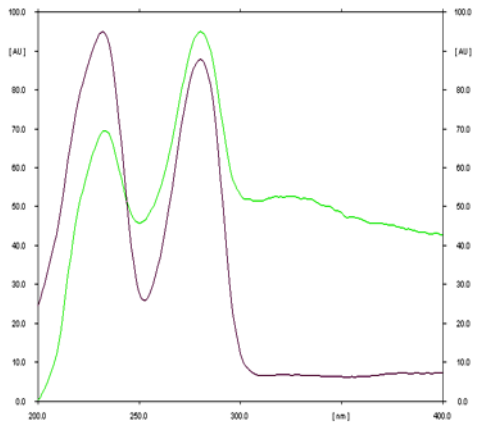
Figure.7: Visualization of TLC plate

The 3D display of the fingerprint profile and the peak display of MECL at 254nm and 366nm were presented (Figure 8, 9, 10). The display at 254nm showed the presence of 8 peaks while at 366nm showed the presence 8 peaks. The Rf values of the peaks along with the area under the curve for each peak at 254 and 366 nm was presented (Table 9).

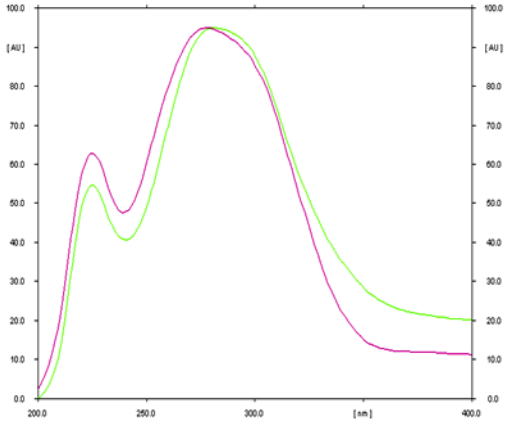


@254nm **@366nm**

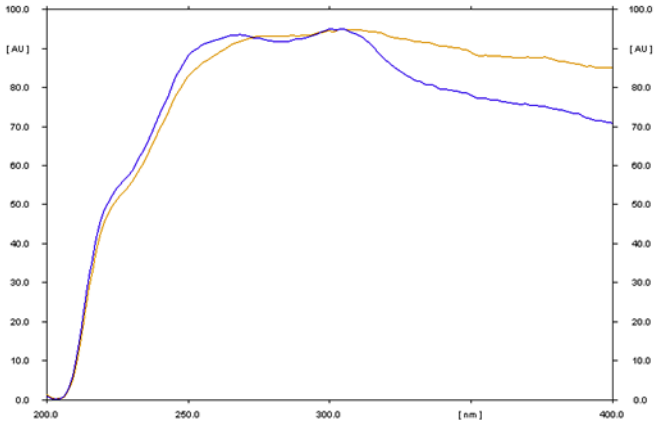
Figure. 8: 3D Display of the extract and standards at 254 & 366nm



For Catechin

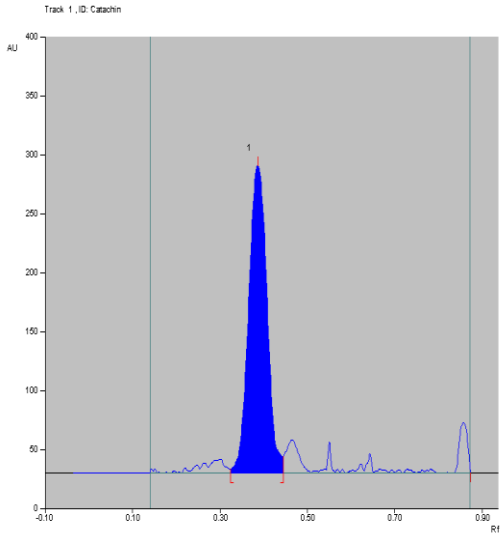


For Gallic acid

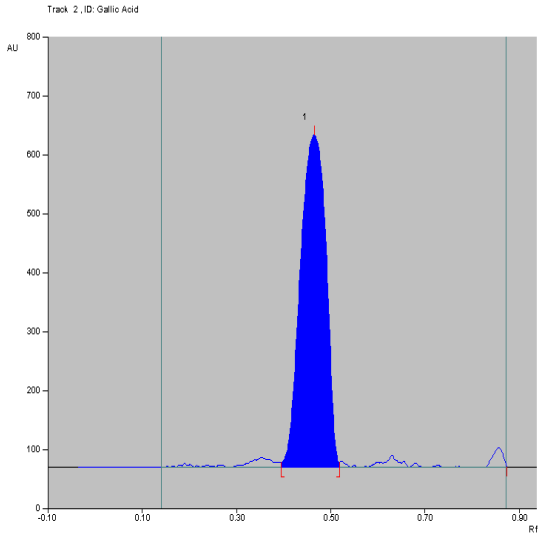


For Tannic acid

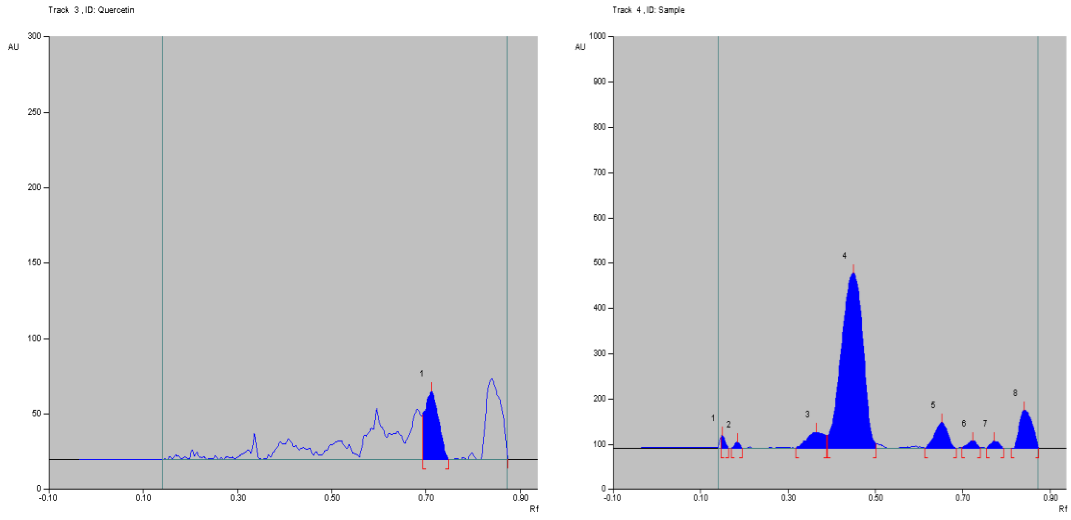
Figure. 9: Overlay Spectral Display



Track 1



Track 2



Track 3 **Track 4**

Track 1: Catechin, **Track 2**: Gallic acid,

Track 3: Quercetin, **Track 4**: MECL

Figure. 10: Peak Display

Table 9: Rf value of the spots and their area under curve at 254 and 366nm

S. No	Rf Value Track				Area (AU) Track			
	1	2	3	4	1	2	3	4
1	0.38	0.46	0.71	0.15	9798.0	27344.1	1187.6	248.6
2				0.18				156.4
3				0.36				1320.9
4				0.45				17028.3
5				0.65				1513.1
6				0.72				326.7
7				0.77				291.7
8				0.84				2203.4

@ 254nm

S. No	Rf Value 1	Area (AU) 2
1	0.15	758.8
2	0.18	135.7
3	0.39	1647.0
4	0.50	1881.2
5	0.65	9744.8
6	0.72	941.5
7	0.80	348.2
8	0.85	2984.6

@366nm

The HPTLC finger print profile of the MECL, Rf values compared with standard Quercetin, Gallic acid and Catechin. From that confirmed the presence of Quercetin, Gallic acid and Catechin in MECL.

***in vitro* Anti diabetic activity**

Non-enzymatic glycosylation of Hemoglobin assay

The results for *in vitro* non enzymatic glycosylation of Hemoglobin assay was presented (Tab 10) and the graphical representation of the same in Fig. 11. The percentage inhibition was found to be 72.398 ± 0.221 and 76.171 ± 0.213 for MECL and α-Tocopherol respectively. The IC_{50} value calculated using linear regression analysis was found to be 65.648µg/ml and 59.762µg/ml for MECL and α-Tocopherol respectively.

Table 10: *in-vitro* Non-enzymatic glycosylation of Hemoglobin method

S. No	Conc. in µg/ml	α-Tocopherol		MECL	
		Absorbance*	% inhibition	Absorbance*	% inhibition
1	20	0.159 ± 0.003	23.270 ± 1.674	0.147 ± 0.000	17.006 ± 0.496
2	40	0.187 ± 0.002	34.759 ± 1.016	0.169 ± 0.004	27.810 ± 1.847
3	60	0.258 ± 0.004	52.713 ± 0.813	0.233 ± 0.002	47.639 ± 0.589
4	80	0.386 ± 0.004	68.393 ± 0.342	0.346 ± 0.005	64.739 ± 0.623
5	100	0.512 ± 0.004	76.171 ± 0.213	0.442 ± 0.003	72.398 ± 0.221
IC_{50} Value		**59.762 µg/ml**		**65.648 µg/ml**	

*** mean of three readings ±SEM**

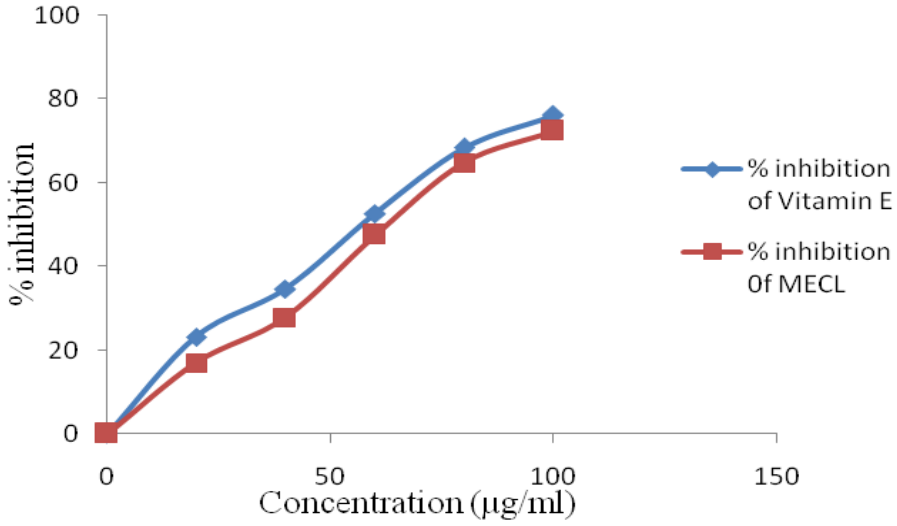


Figure. 11: *in-vitro* Non-enzymatic glycosylation of Hemoglobin method

Plant extracts play an important role the inhibition of the glycosylation end products. An increase in the glycosylation was observed on incubation of hemoglobin with the increasing concentration of the glucose over a period of 72hr. However, the plant extracts significantly inhibited the hemoglobin glycosylation which is indicated by the presence of increasing concentration of

hemoglobin. *Citrullus lanatus* exhibited significant inhibition of glycosylation as compared with the standard drug α-Tocopherol. The plant extracts also displayed the inhibition of hemoglobin glycosylation at different physiological concentrations of the glucose over the period of 72hr, indicating that the plant extracts decreases the formation of the glucose- hemoglobin complex and thus amount of free hemoglobin increases.

***in-vitro* glucose uptake in yeast cells**

The rate of glucose transport across cell membrane in yeast cells was presented (Fig. 12 & 13, Table 11 & 12).

Table 11: Percentage inhibition of Glucose uptake in 5mM glucose concentrations

S. No	Conc. µg/ml	Acarbose		MECL	
		Absorbance*	% inhibition	Absorbance*	% inhibition
1	40	0.102 ± 0.001	60.78 ± 0.220	0.099 ± 0.002	59.60 ± 0.386
2	80	0.117 ± 0.000	65.81 ± 0.222	0.106 ±0.002	62.26 ± 0.030
3	120	0.132 ± 0.001	69.70 ± 0.271	0.124 ± 0.003	67.74 ± 0.204
4	160	0.150 ± 0.002	73.33 ± 0.101	0.140 ± 0.001	71.43 ± 0.177
5	200	0.188 ±0.002	78.72 ± 0.248	0.152 ± 0.006	73.68 ± 0.362
IC_{50} Value		**74.083 µg/ml**		**80.218 µg/ml**	

*** mean of three readings ±SEM**

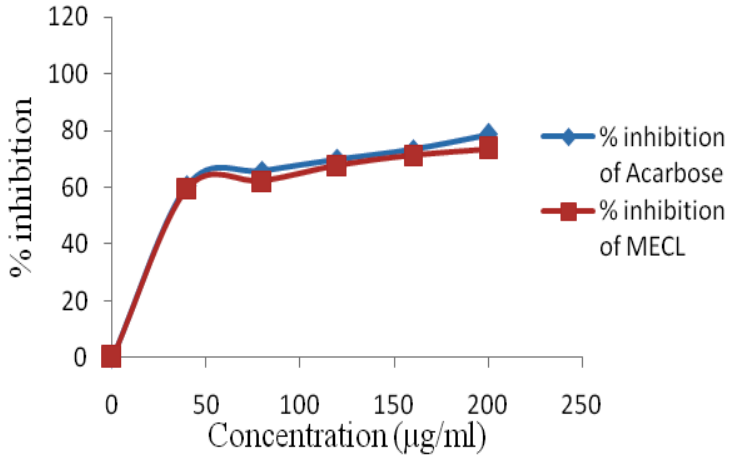


Figure. 12: % inhibition of Glucose uptake at 5mM concentration

Table 12: Percentage inhibition of Glucose uptake at 10mM concentration

S. No	Conc. µg/ml	Acarbose		MECL	
		Absorbance*	% inhibition	Absorbance*	% inhibition
1	40	0.200 ± 0.003	65.00 ± 0.242	0.170 ± 0.004	58.82 ± 0.257
2	80	0.214 ± 0.002	67.28 ± 0.163	0.220 ± 0.001	65.00 ± 0.282
3	120	0.249 ± 0.004	71.88 ± 0.178	0.248 ± 0.001	68.54 ± 0.081
4	160	0.303 ± 0.003	76.89 ± 0.206	0.311 ± 0.003	73.28 ± 0.077
5	200	0.379 ± 0.001	81.53 ± 0.181	0.335 ± 0.003	76.74 ± 0.214
IC_{50} Value		**67.408 µg/ml**		**77.031 µg/ml**	

*** mean of three readings ±SEM**

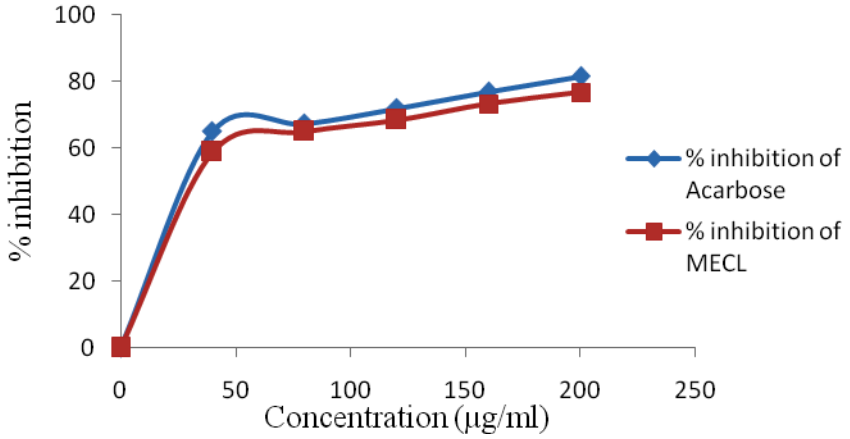


Figure. 13: % inhibition of Glucose uptake in 10mM glucose concentrations

The percentage inhibition was found to be 76.74 ± 0.214 and 81.53 ± 0.181 for MECL and Acarbose at 200µg/ml respectively. The IC_{50} value calculated using linear regression analysis was found to be 77.031µg/ml and 67.408µg/ml for MECL and Acarbose respectively.

Regulation of glucose level in the blood of the diabetic patient can prevent the various complications associated with the disease. The maintenance of plasma glucose concentration for a long term under a variety of dietary conditions is one of the most important and closely regulated processes observed in the mammalian species. The i*n vitro* assays of the present study indicated MECL possess good anti diabetic activity. In yeast, glucose transport takes place through facilitated diffusion. Type 2 Diabetes is characterized by the deficiency of Insulin causing increased amount of glucose in blood. After the treatment of the yeast cells with these leaf extracts, the glucose uptake was found to increase in a dose dependent manner. The Fig.12 & 13 depict the % increase in glucose uptake by the yeast cell at different glucose concentrations i.e. 5mM and 10mM respectively. The MECL exhibited significantly higher activity at all glucose concentrations showing the maximum increase in 10mM Glucose concentration. Results also indicated that *C.lanatus had* greater efficiency in increasing the glucose uptake by yeast cells as compared to standard drug Acarbose.

***in-vitro* alpha amylase inhibition method**

The α-amylase inhibitor effectiveness of MECL was compared with standard drug Acarbose. The values were presented (Table 13) & graphical representation of the same in Figure. 14.

Table 13: *in-vitro* Alpha amylase inhibition

S. No	Conc. µg/ml	Acarbose		MECL	
		Absorbance*	% inhibition	Absorbance*	% inhibition
1	20	0.099 ± 0.001	44.44 ± 0.646	0.077 ± 0.003	28.57 ± 3.185
2	40	0.116 ± 0.004	52.58 ± 1.751	0.103 ± 0.002	46.60 ± 1.020
3	60	0.152 ± 0.003	63.81 ± 0.844	0.128 ± 0.004	57.03 ± 1.341

4	80	0.193 ± 0.002	71.50 ± 0.340	0.154 ± 0.003	64.28 ± 0.768
5	100	0.232 ± 0.006	76.29 ± 0.658	0.186 ± 0.002	69.44 ± 0.410
IC_{50} Value		**47.880 µg/ml**		**58.558 µg/ml**	

*** mean of three readings ±SEM**

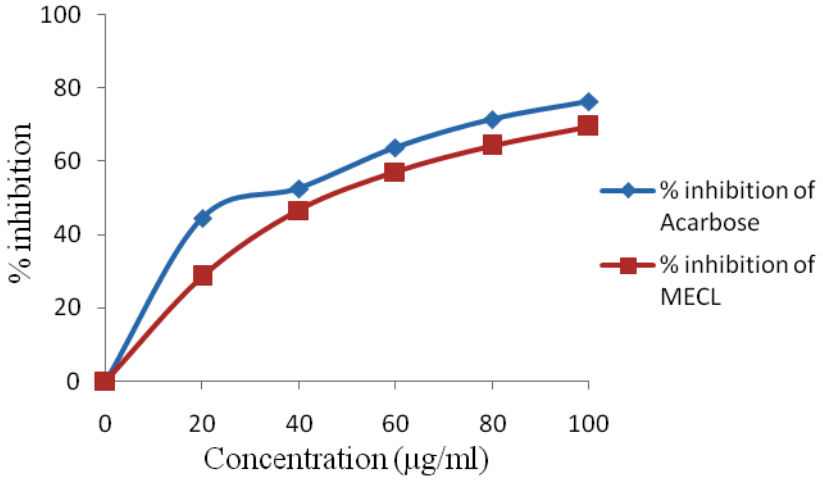


Figure. 14: *in-vitro* Alpha amylase inhibition method

The percentage inhibition was found to be 69.44 ± 0.410 and 76.29 ± 0.658 for MECL and Acarbose at 100µg/ml respectively. The IC_{50} value calculated using linear regression analysis was found to be 58.558µg/ml and 47.880µg/ml for MECL and Acarbose respectively

Alpha amylase is an enzyme that hydrolyses α-bonds of large α-linked polysaccharide such as glycogen and starch to yield glucose and maltose. α-amylase inhibitors bind to α- bond of polysaccharide and prevent break down of polysaccharide in mono and disaccharide. As the result, MECL showed significant activity as compared to Acarbose.

Drugs that inhibit carbohydrate hydrolyzing enzymes have been demonstrated to decrease postprandial hyperglycemia and improve impaired glucose metabolism without promoting insulin secretion of non insulin dependent diabetic patients. The results of *in vitro* studies showed that *Citrullus lanatus* inhibits α-amylase activity. Natural health products of vegetable origin were clearly indicated as a promising avenue for the prevention of chronic diseases [57].

***in-vitro* alpha glucosidase inhibition method**

The values for *in vitro* alpha glucosidase inhibition & graphical representation were presented in Table 14 and Figure.15.

The α-glucosidase inhibitor effectiveness of MECL was compared with Acarbose. IC_{50} of MECL and Acarbose were estimated as 627.270 &482.188µg/ml respectively.

Table 14: *in-vitro* α-glucosidase inhibition assay

S. No	Conc. µg/ml	Acarbose		MECL	
		Absorbance*	**% inhibition**	**Absorbance***	**% inhibition**

1	200	0.809 ± 0.004	24.46 ± 0.457	0.896 ± 0.002	16.34 ± 0.189
2	400	0.519 ± 0.002	51.54 ± 0.188	0.780 ± 0.001	27.17 ± 0.136
3	600	0.388 ± 0.001	63.77 ± 0.113	0.517 ± 0.002	51.73 ± 0.270
4	800	0.216 ± 0.001	79.83 ± 0.136	0.408 ± 0.002	61.90 ± 0.188
5	1000	0.106 ± 0/002	90.10 ± 0.243	0.204 ± 0.002	80.95 ± 0.273
IC_{50} Value		**482.188 µg/ml**		**627.270 µg/ml**	

***mean of three readings ±SEM**

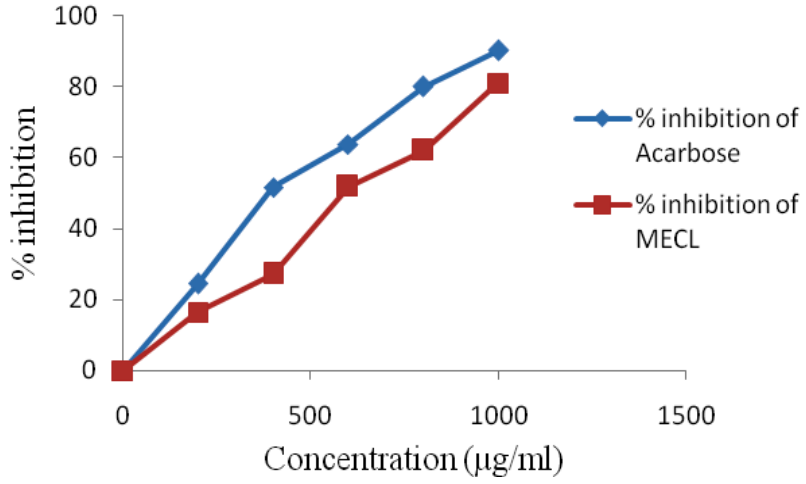


Figure. 15: *in-vitro* α-glucosidase inhibition assay

In *Diabetes mellitus*, control of postprandial plasma glucose level is critical in the early treatment. An inhibition of enzymes involved in the metabolism of carbohydrate is one of the therapeutic approaches for reducing postprandial hyperglycemia.

α-glucosidase is a key enzyme in carbohydrate digestion. It catalyzes the hydrolysis of 1, 4-α-glucosidic bonds within carbohydrates with release of α-glucose and promotes the increase of blood glucose level after meal. α-glucosidase inhibitors antagonize the activity of α-glucosidase, thereby delaying intestinal carbohydrate absorption and slowing the sharp rise in blood sugar levels that diabetic patients typically experience after meals. For this reason, α-glucosidase inhibitors, such as Acarbose and Voglibose, are clinically used as oral anti hyperglycemic agents. However, they often cause severe gastrointestinal side effects. Therefore, search for new α-glucosidase inhibitors from natural resources has become an attractive approach for the treatment of postprandial hyperglycemia. The extract showed better alpha-glucosidase inhibition property. α-glucosidase inhibitors have a potential for the treatment of diabetes because they reduce diet-induced hyperglycemia.

CONCLUSION

MECL possessed a significant *in vitro* anti diabetic activity may be due presence of Gallic acid, tannic acid, Catechin, Quercetin and Vitamin contents. Furthermore *in vivo* studies are required for providing scientific information on the plant.

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