CHAPTER-IV

RESULTS

4.1 Yield of *E.operculata* Roxb., leaves extract

The fine powder of *E.operculata* leaves was sequentially extracted with solvents of increasing polarity: petroleum ether, ethyl acetate, methanol, ethanol and water. Then it was filtered and concentrated using rotary evaporator. The percentage yields obtained from each extract are as follows in Table 4.

Extractant	Percentage yield (%)
Petroleum ether	5.4
Ethyl acetate	0.8
Ethanol	24.4
Methanol	8.4
Aqueous	22.54

Table 4: Percentage yield of different solvents.

4.2 In vitro screening of *E.operculata* leaves extracts for α-amylase and α-glucosidase enzyme inhibitory activities

E.operculata petroleum ether, ethyl acetate, ethanol, methanol and aqueous extract of leaves were screened for their inhibitory activities against α-amylase and α-glucosidase.

4.2.1 Alpha amylase inhibition

In vitro α-amylase inhibitory studies demonstrated that all crude *E.operculata* leaves extracts (petroleum ether, ethyl acetate, ethanol, methanol and aqueous) screened for α-amylase inhibitory activity inhibited porcine pancreatic alpha amylase. The crude ethanol and aqueous extract shows maximum inhibitory activity against all the extracts with 96.39 and 92.62 percent inhibition respectively. Petroleum ether extract shows the minimum inhibitory activity with 33.62% inhibition. (Table 5)

conc of *E.operculata* (µg/ml)	% inhibition of pet ether	% inhibition of ethyl acetate	% inhbition of methanol	% inhibiion of ethanol	% inhibition of aqueous	% inhibition of acarbose
10	3.28	7.9	14.9	20.28	14.89	15.18
20	7.8	12.07	36.48	48.21	43.62	46.13
40	12.74	21.09	47.52	69.58	68.11	61.34
60	24.16	32.16	58.28	83.37	78.74	78.23
80	29.18	43.8	70.8	92.68	86.08	84.26
100	33.62	57.9	79.32	96.39	92.62	94.67

Table 5: Alpha amylase inhibitory activities of different extract.

The concentration of the extracts required for 50% inhibition (IC_{50}) for all the extracts were determined from corresponding dose response curves of percentage inhibition versus inhibitor concentration and compared with those of acarbose, a known inhibitor of α-amylase. Ethanol extract of *E.operculata* leaves appeared to be better inhibitors of porcine pancreatic α-amylase with IC_{50} 28.32µg/ml in comparision with acarbose IC_{50} 34.83µg/ml as shown in Table 6 and Figure 4.

Extract	**IC50**
pet ether	143.54
ethyl acetate	89.63
Methanol	49.87
Ethanol	28.32
Aqueous	38.82
Acarbose	34.83

Table 6: IC_{50} valuesof *E.operculata* crude and acarbose against procine pancreatic α-amylase.

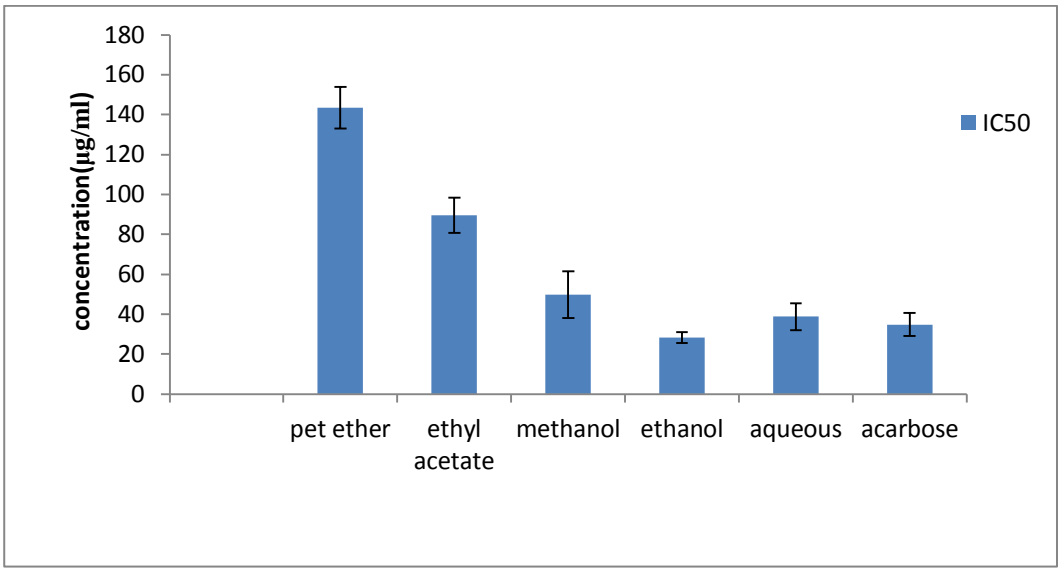


Fig 4. IC_{50} values of *E.operculata* crude extracts and acarbose against procine pancreatic alpha amylase.

4.2.2 Alpha glucosidase inhibition

In vitro α-glucosidase inhibitory studies demonstrated that all crude *E.operculata* leaves extracts (petroleum ether, ethyl acetate, ethanol, methanol and aqueous) screened for α-glucosidase inhibitory activity shows positive results against yeast alpha glycosidase. The crude ethanol and aqueous extract shows maximum inhibitory activity against all the extracts with 82.47 and 89.14 percent inhibition respectively. Petroleum ether extract showed the minimum inhibitory activity with 36.13% inhibition as shown in Table 7.

conc of *E.operculata* (µg/ml)	% inhibition of pet ether	% inhibition of ethyl acetate	% inhbition of methanol	% inhibiion of ethanol	% inhibition of aqueous	% inhibition of acarbose
10	4.37	5.19	17.39	26.1	33.08	31.19
20	7.29	12.33	31.06	39.74	41.16	43.06
40	13.19	23.67	44.16	47.5	49.06	61.18
60	24.73	31.74	52.36	49.64	60.22	73.9
80	27.34	37.06	60.83	60.98	74.86	82.27
100	36.13	43.18	71.28	82.47	89.14	97

Table 7: Alpha glucosidase inhibitory activities of different extract.

The concentration of the extracts required for 50% inhibition (IC_{50}) for all the extracts were determined from corresponding dose response curves of percentage inhibition versus inhibitor concentration and compared with those of acarbose, a known inhibitor of α-glucosidase. Aqueous extract of *E.operculata* leaves appeared to be better inhibitors of yeast α-glucosidase than the remaining extracts with IC_{50} value 38.61µg/ml. but it is found to be less potent then the standard acarbose showing IC_{50} 30.57µg/ml as shown in Figure 5.

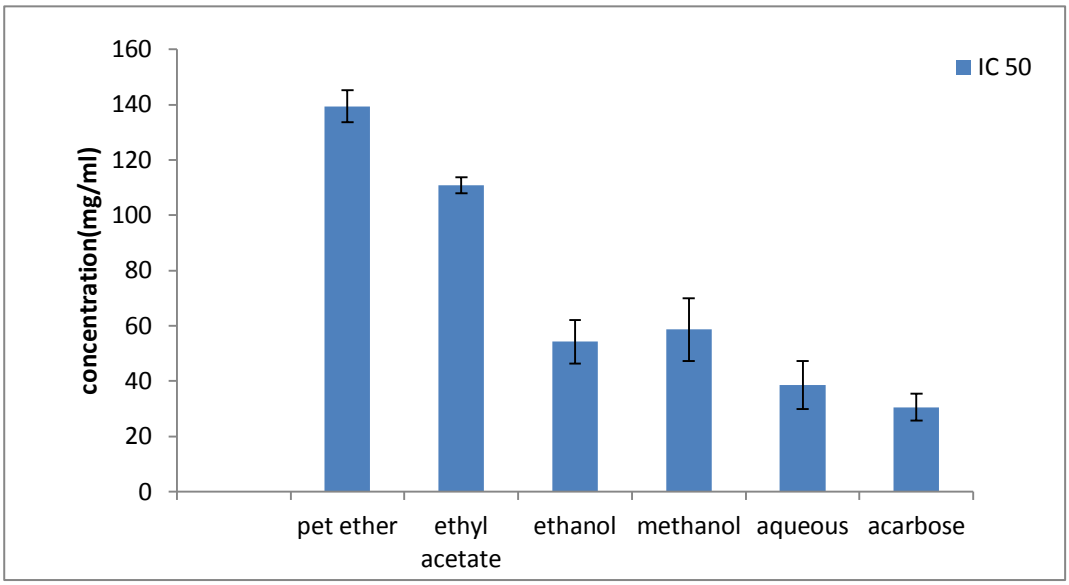


Figure 5: IC_{50} values of *E.operculata* crude extracts and acarbose against yeast alpha glucosidase.

4.3 In vivo antidiabetic effects of *E.operculata* leave extracts

In order to determine whether the observed in vitro effects of *E.operculata* leave extracts are applicable in vivo and to study the possible hypoglycemic mechanism of action, the following effects of *E.operculata* ethanol and aqueous leave extracts were studied in normal and STZ induced diabetic mice.

i) Acute oral toxicity test of ethanol and aqueous extracts

ii) Effects of *E.operculata* on postprandial glucose level after an oral administration of glucose.

iii) Long term treatment effects of *E.operculata* on fasting blood glucose levels, plasma triglycerides, serum lipid profile and cholesterol.

4.3.1 Acute oral toxicity test

To establish the safety of the extracts, a dose dependent ethanol and aqueous extract up to 3000mg/kg b.w were administered to normal mice of both sexes. No significant toxic signs or death of animal was observed during the 7 days of observation. None of the animal showed clinical toxic signs such as anorexia, depression, lethargy, convulsion, ataxia, and diarrhea and also, no mortality happened throughout the examination.

4.3.2 Effect of *E.operculata* ethanol and aqueous extract on oral glucose tolerance test

Table 8 and Figure 6,7,8. shows the effect of *E.operculata* ethanol and aqueous extract on postprandial blood glucose levels in normal and STZ induced diabetic mice when fed simultaneously with glucose (2.5mg/kg b.w). The blood glucose levels of the entire group increased sharply 30 minutes after an oral administration of glucose and then decreased steadily thereafter.

Extract	0 min	30 min	60 min	120 min
NC	126±3.9	278.66±10.6	244.66±5	160.83±5
E.operculata ETNM	137.5±1.2	250.66±5.2	150.33±5.7	138.5±3.6
E.operculata AqTNM	123.9±1.2	261±4.5	157.5±3.6	144.5±5.2
GTNM	134.83±2	228.33±4.7	143.5±4.23	128.33±6.3
DC	348±1.4	479±1.1	483±3.7	474±1.1
E.operculata ETDM	336±1.7	428±3.7	418±1.3	379±1.4
E.operculata AqTDM	334±1.7	412±1.1	406±4.7	387±1.1
GTDM	339±2.1	394±3.4	377±2.6	362±4.2

Table 8: Effect of *E.operculata* extract and glibenclamide on postprandial blood glucose level. (NC= Normal control, ETNM= Ethanol treated normal mice, AqTNM= Aqueous treated normal mice, GTNM= Glibenclamide treated normal mice, DC= Diabetic control, ETDM= Ethanol treated diabetic mice, AqTDM= Aqueous treated diabetic mice, GTDM= Glibenclamide treated diabetic mice).

In case of normal untreated mice rise in blood glucose level falls from 66.68 % after 30 minutes to 27.48 % at 120 minutes. Whereas in case of diabetic control group there is no significant difference in blood glucose level.

In extract treated groups of both normal and diabetic mice, there is a strong and significant opposing effect on the rise of serum glucose level when fed simultaneously with glucose. After 120 minutes AqTNM, ETNM, AqTDM, ETDM shows an inhibition of 72.9%, 76.06%, 86.30%, and 88.65% from 40.75%, 42.03%, 81.07%, &78.50 % respectively. The suppression of postprandial rise in normal mice by *E.operculata* ethanol extract was almost similar to that caused by glibenclamide, whereas the suppression in diabetic mice was significantly lower than that caused by standard.

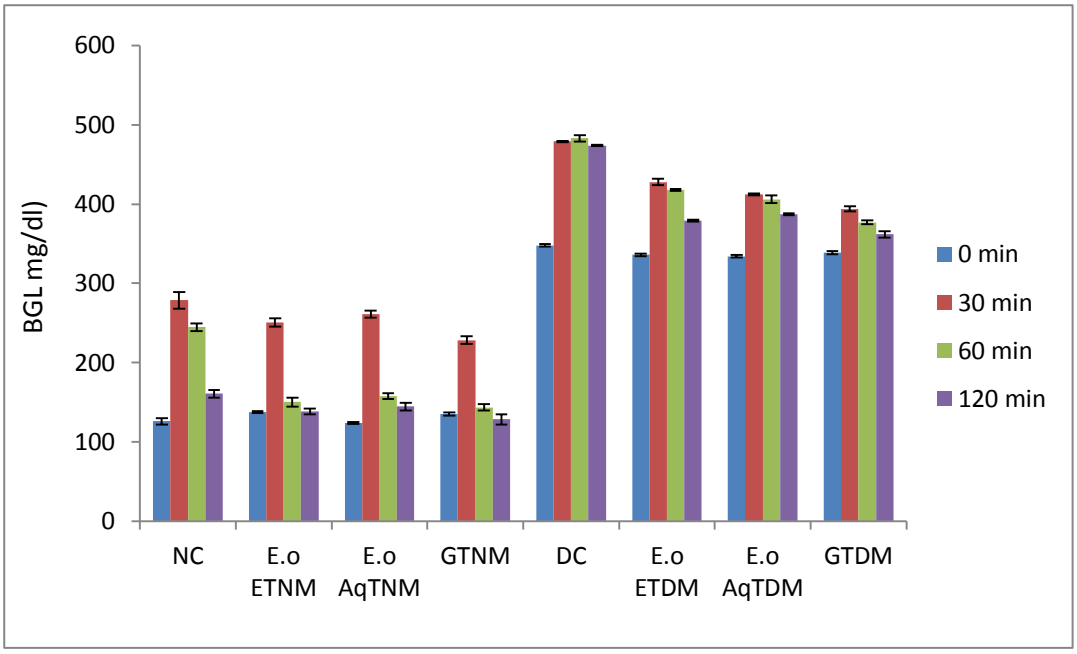


Fig6: Effect of *E.operculata* extract and glibenclamide on postprandial blood glucose level.

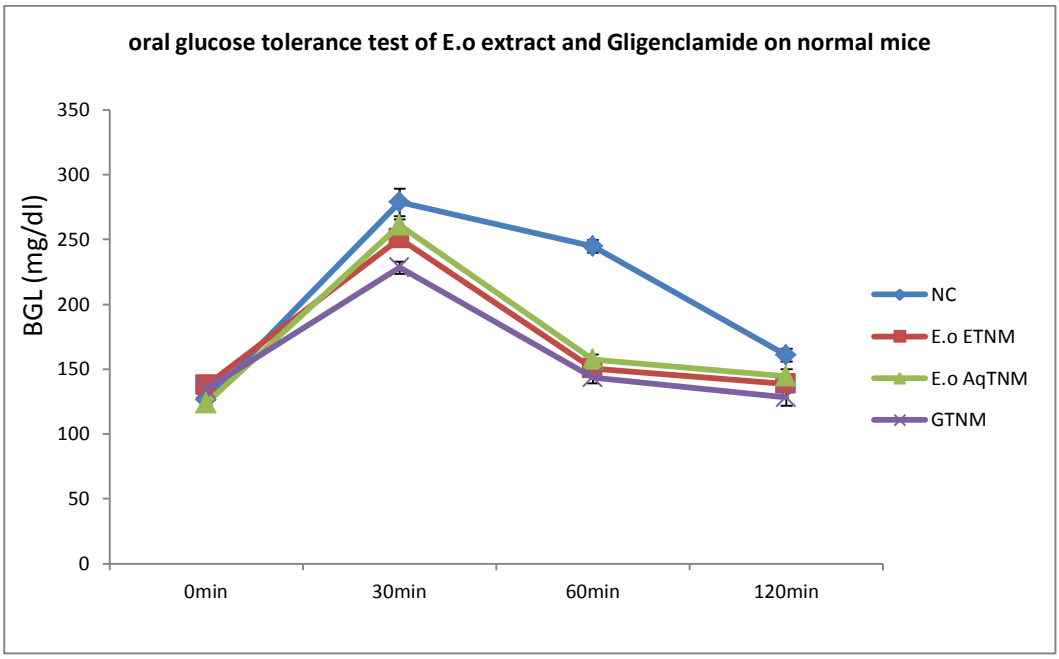


Fig 7: Effect of *E.operculata* extract and glibenclamide on postprandial blood glucose level in normal mice.

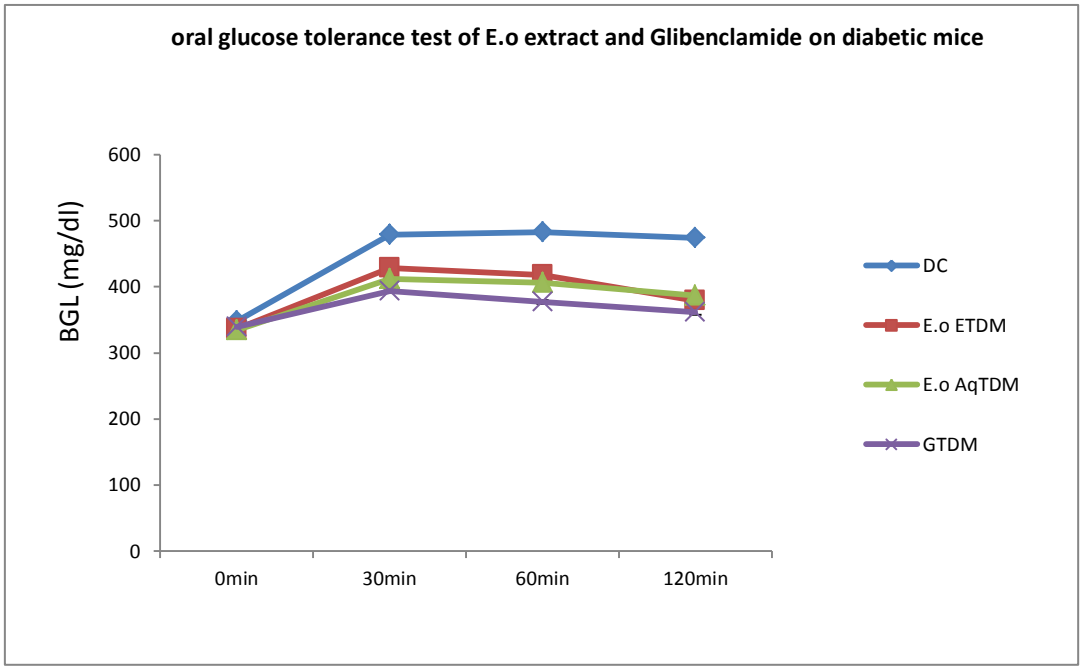


Fig 8. Effect of *E.operculata* extract and glibenclamide on postprandial blood glucose level in diabetic mice.

4.3.3 In vivo long term treatment effects of *E.operculata* ethanol and aqueous extract

4.3.3.1 Effects on fasting blood glucose level

Induction of diabetes in the experimental mice was confirmed by the presence of high blood glucose level. In this study, the level of glucose for diabetic control group 2 was significantly ($P<0.05$) increased from 296.66±15.1 from day 1 to 548.50±19.73 on 21st day. Glibenclamide treated diabetic mice of standard group, group 7 showed significant reductions ($p<0.001$) in blood glucose level starting from first week after the initiation of treatment to the end of the experiment i.e from 167.83±10.88 to 113.33±1.54. Administration of *E.operculata* aqueous and ethanol at dosages of 250 and 500mg/kg b.w to diabetic mice tends to bring down the values of glucose level to near normal. Among these two doses of both extract, the dose of 250mg/kg b.w showed better result with a fall of glucose level from 308.66±27.78 to 157.33±17.14 in aqueous extract and 444.00±17 to 204.33±19.75 in ethanol extract from the starting of treatment to the end of experiment. Whereas group treated with ETDM and AqTDM 250 groups were more significant ($p<0.001$) than GTDM group. Therefore the dose of 250mg/kg b.w of *E.operculata* Aq and *E.operculata* E were selected for further analysis.

Groups of mice	**Variables (mean±sem)**			
	Blood glucose level before treatment	**After 1stweek of treatment**	**After 2ndweek of treatment**	**After3rd week of treatment**
NC	135.16±3.15	137.50±2.47	140.50±7.4	109.83±6.7
DC	296.66±15.1	454.83±32.3	516.83±26.35	548.50±19.7
AqTDM 250mg/kg b.w	308.66±27.7	163.16±16.7**	160.0±15.56***	157.3±17.1***b
AqTDM 500mg/kg b.w	325.50±15.3	181.16±16.3***	130.83±8.8***	127.5±6.4**
ETDM 250mg/kg b.w	440.00±17.6	338.5±11***a***b***	189.66±20.7 b***	204.33±19.7***a*b***

ETDM 500mg/kg b.w	360.16±23	136.83±20.1***	126.16±19.5**	126.33±11.2**
GTDM	363.33±11.6	167.83±10.8***	130.83±7.4***	113.33±1.54***a*

Table 9: Effect of *E.operculata* extract in both normal diabetic mice. ***P<0.001, **p<0.01 as compared to diabetic control, a*p<0.05 when compared to AqTDM250mg/kg b.w., b***p<.001 when compared to GTDM. (NC= Normal control, ETNM= Ethanol treated normal mice, AqTNM= Aqueous treated normal mice, GTNM= Glibenclamide treated normal mice, DC= Diabetic control, ETDM= Ethanol treated diabetic mice, AqTDM= Aqueous treated diabetic mice, GTDM= Glibenclamide treated diabetic mice).

4.3.3.2 Effects on urine glucose

The normal control mice showed absence of sugar in urine. The urine sugar levels of different groups of diabetic animals treated with standard drug, glibenclamide and *E.operculata* aqueous and ethanol for 21days decreased towards the normal level. Whereas, in case of diabetic control group the urine sugar level keeps on rising consistently.

Groups of mice	**Before treatment**	**After 1st week**	**After 2nd week**	**After 3rd week**
Normal control	Nil	Nil	Nil	Nil
Diabetic control	+++	+++	++++	++++
AqTDM 250mg/kg b.w	+++	++	++	+
AqTDM 500mg/kg b.w	+++	++	++	+
ETDM 250mg/kg b.w	+++	++	+	+
ETDM 500mg/kg b.w	+++	++	++	+
GTDM	+++	++	+	+

Table 10: Effect of *E.operculata* extract and glibenclamide on urine glucose level. Keys (+) =mild, (++)= moderate, (+++)=higher, (++++)=severe.

4.3.3.3 Effects on body weight

Diabetes is characterized by weight loss and it was also seen in this study. STZ administration brought marked reduction in body weight of diabetic mice. This reduction was found to be statistically significant ($P<0.05$) when compared with normal control group. These reduced body weight were found to increase significantly ($p<0.001$) upto 28.86±0.4 and 29.50±0.5 from 27.83±0.6 and 29.09±0.3 for group treated with Aq250mg/kg b.w and E500mg/kg b.w respectively as shown in Table 11.

Groups of mice	Body weight before treatment Mean±sem (g)	Body weight after 1 week Mean±sem (g)	Body weigkt after 2nd week Mean±sem (g)	Body weight after 3rd week Mean±sem (g)
NC	27.35±0.84	27.11±0.72	26.99±0.58	27.60±0.7
DC	29.66±1.1	28.40±1	27.90±1	24.37±0.48
AqTDM 250mg/kg b.w	27.83±0.6	26.93±0.6	25.81±0.55	28.86±0.4***
AqTDM 500mg/k	26.00±0.56	24.86±0.55**	26.19±0.56	26.96±1*
ETDM 250mg/k g b.w	24.97±0.31	25.30±0.43*	25.44±0.41*	25.34±0.12
ETDM 500mg/k g b.w	29.09±0.3	28.82±0.3	28.96±0.24	29.50±0.21***
GTDM	27.16±0.25	26.95±0.33	26.91±0.21	25.73±0.27

Table 11: Effect of *E.operculata* on body weight (gm). *p<0.05, **p<0.01 ***p<0.001 compared to diabetic control.(NC= Normal control, ETNM= Ethanol treated normal mice, AqTNM= Aqueous treated normal mice, GTNM= Glibenclamide treated normal mice, DC= Diabetic control, ETDM= Ethanol treated diabetic mice, AqTDM= Aqueous treated diabetic mice, GTDM= Glibenclamide treated diabetic mice).

4.3.3.4 Effects on serum lipid profile

E.operculata extracts both aqueous and ethanol exhibited significant reduction ($p<0.001$) in all tested lipid parameters.

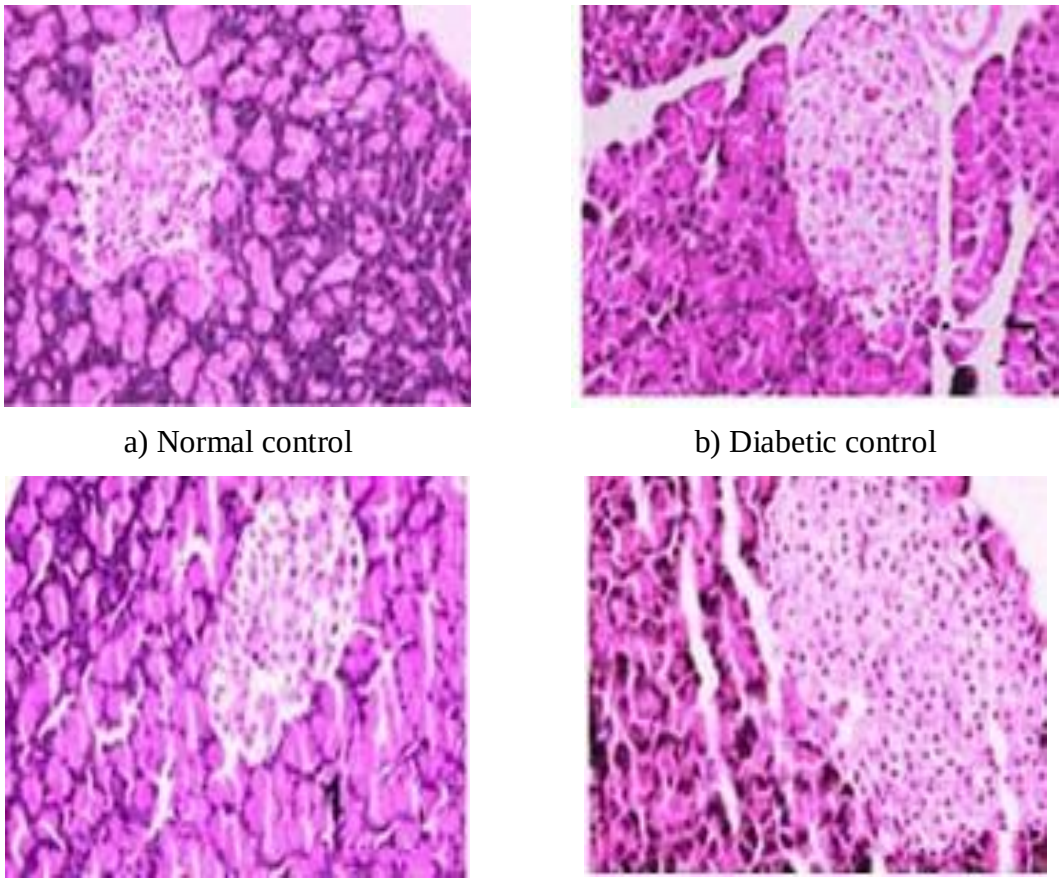
Groups		STG (mg/dl)	STC (mg/dl)	HDLc (mg/dl)	LDLc (mg/dl)	VLDLc (mg/dl)
NC	Mean ±SEM	92.16±0.47	81±0.44	37.16±0.6	25.4±0.3	18.43±0.1
DC	Mean ± SEM	133.33±0.8	156±0.63	16.83±0.6	112.5±0.2	26.66±0.17
AqTDM250mg/kg b.w	Mean ± SEM	114.5±0.70**	130±1.52**	31.16±0.6**	75.93±1**	22.9±0.1**
ETDM250mg/kg b.w	Mean ± SEM	119***	134.5±2**	24.16±0.9**	86.53±1.4**	23.80**
GTDM	Mean ± SEM	102.16±1.88***	112.8±2.3**	25.33±1.5**	67.06±1.4**	20.43±0.4**

Table 12: Effect of *E.operculata* on lipid profile. ***p<0.001, **p<0.01 compared with diabetic mice. (NC= Normal control, DC= Diabetic control, ETDM= Ethanol treated diabetic mice, AqTDM= Aqueous treated diabetic mice, GTDM= Glibenclamide treated diabetic mice).

A marked increase in total cholesterol and decrease in HDLc were observed in untreated diabetic mice. *E.operculata* administration decreased serum triglycerides (STG), total cholesterol (STC), LDLc and VLDLc levels and increased HDLc level. The markers of gyslipidemia such as STC/HDLc and LDLc/HDLc ratios were found to be significantly elevated in the diabetic group.

4.3.3.5 Histopathology of pancreas

Photomicrographs of the pancreas in control animals showed normal pancreatic parenchymal cell and islet (Fig 9). In diabetic control, pancreas showed moderate hyperplasia of islet cells, more number of voculation, and severe congestion in pancreatic parenchyma, number of islet was reduced and mild infiltration of inflammatory cells. In diabetic animals treated with *E.operculata* extract, pancreas section showed hyperplasia of islet was recovered, mild congestion of pancreatic parenchyma and number of islet increased. In diabetic animal treated with glibenclamide showed increased number of pancreatic islet and no fibrosis or inflammation was noted.



a) Normal control b) Diabetic control

c) *E.operculata* AqTDM d) *E.operculata* ETDM

Fig 9: Histopathological studies of mouse pancreas.

4.3.3.6 Electron microscopical studies of mouse pancreas

Beta cells of control mice (Fig 10 A and B) possessed normal architecture with a spherical or oval nucleus and the organelles; mitochondria, granular endoplasmic reticulum are in an active state also contained increased number of mature secretory granules with an electrodense core and immature granules with a less electrodense core. Whereas the beta cells of STZ induced diabetic mice (Fig 11 C and D) showed severe alteration with vacuolization and lysis area in the entire cytoplasm with discerned nuclear membrane and pores and least number of secretory granules, almost absent.

The protective action of *E.operculata* was more evident from the electron microscopical structure of mouse pancreas treated with *E.operculata* extract for 21 days (Fig 12 E, F and G) with numerous numbers of secretory granules in the cytoplasm both mature and immature. Mitochondria and of hypertrophied cytoplasmic

organelles such as Golgi complex and endoplasmic reticulum were present and spread uniformly in cytoplasm.

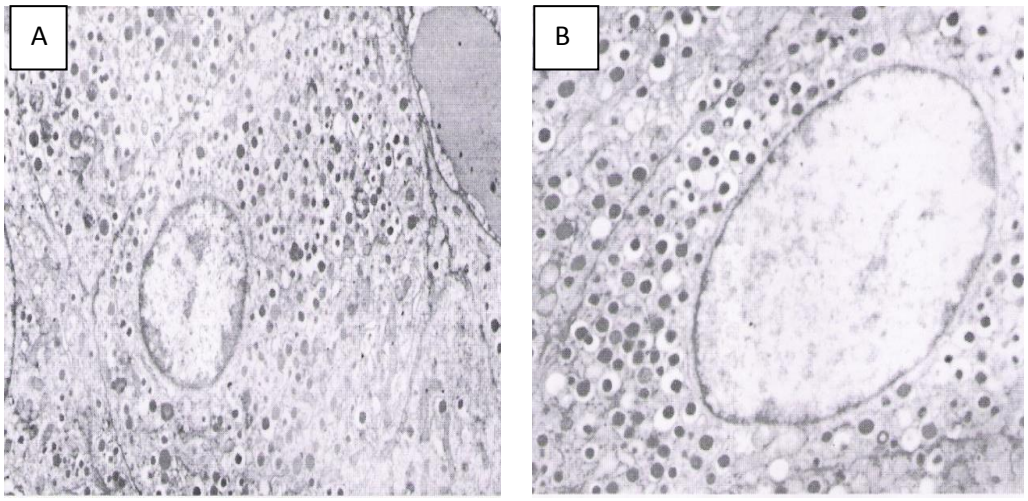


Fig 10: A and B showing Beta cells from the pancreas of normal control mice.

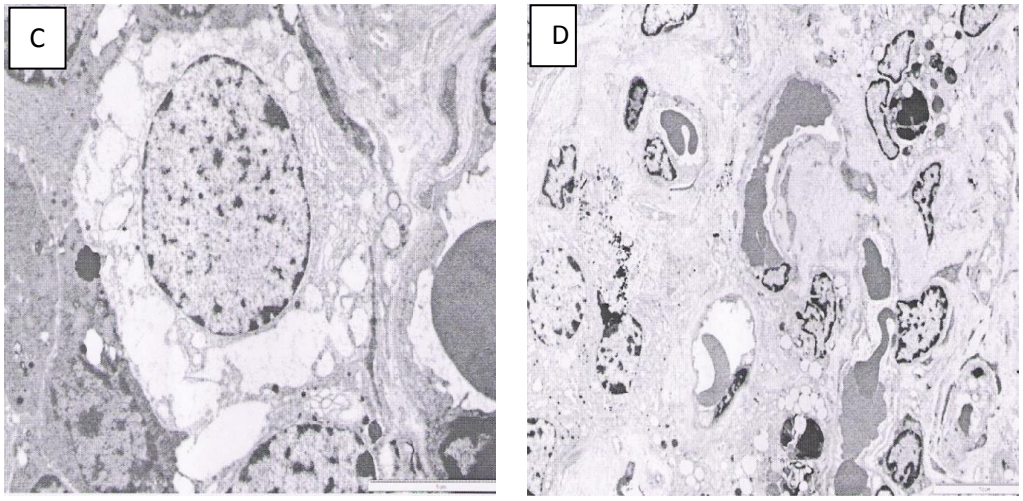


Fig 11: C and D Beta cells from the pancreas of streptozotocin induced diabetic mice.

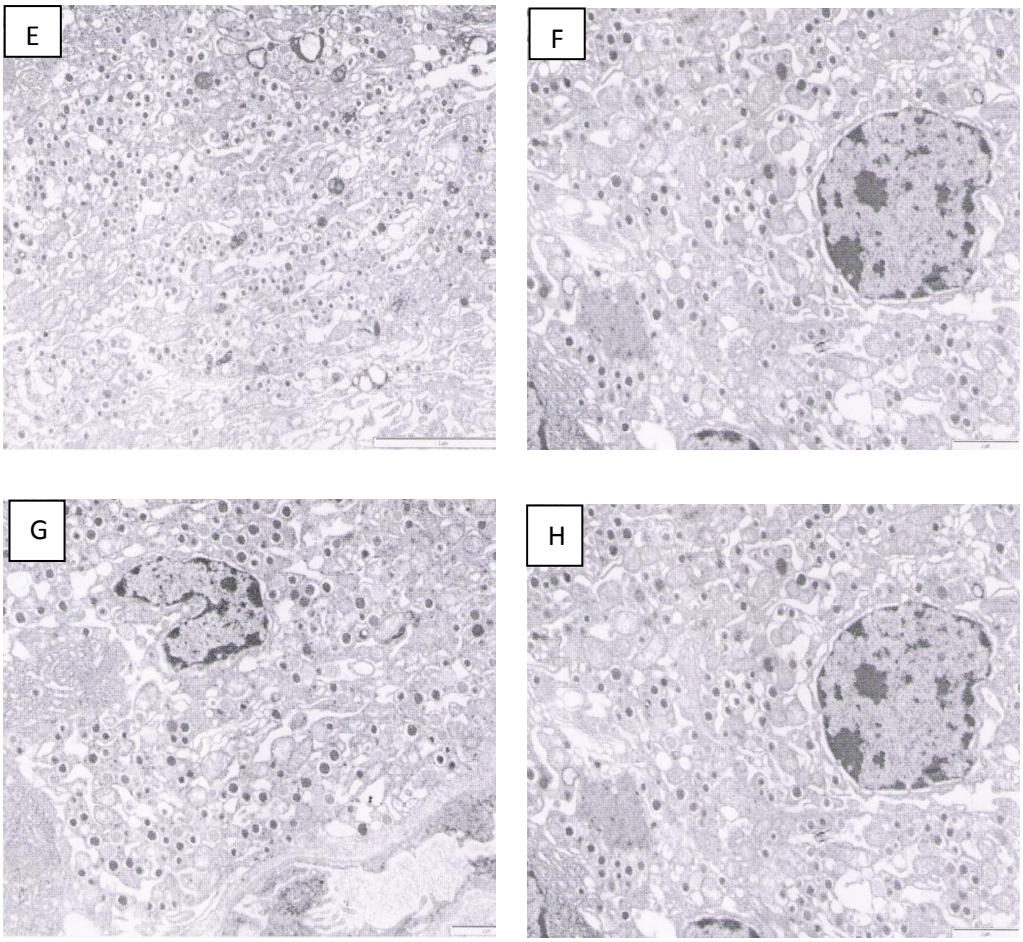


Fig 12: E, F and G. Beta cells from the pancreas of Ethanol treated diabetic mice. H showing pancreas of Glibenclamide treated diabetic mice.

4.4 Estimation of liver glycogen content

There was significant increase in liver glycogen level to 458±21.3mg/100gm ($p< 0.001$) on day 21 in glibenclamide treated diabetic group. Similarly *E.operculata* treatment significantly ($p<0.001$) increased the glycogen content to 386.32± 6.4mg/100gm for AqTDM and 436.14±8.24mg/100gm for ETDM. In diabetic control group the glycogen content decrease upto 243.67±12.6mg/100gm as shown in Table 13 and Fig 13.

Group	liver glycogen content (mg/100gm)
NC	527± 9.1
DC	243± 12.6
E.operculata ETDM	386± 6.4
E.operculata AqTDM	436±8.24
GTDM	458±21.3

Table 13: Liver glycogen content.

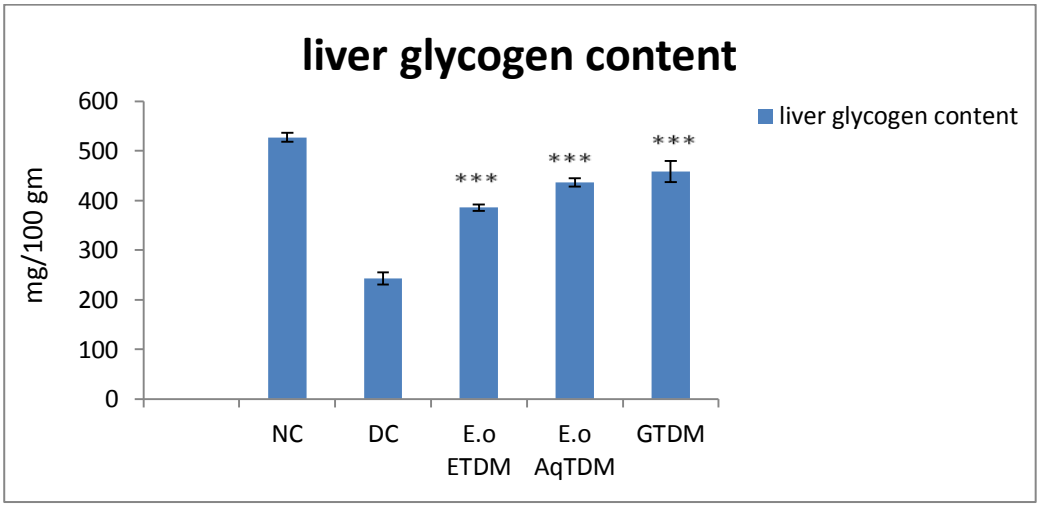


Fig 13: Liver glycogen content. (NC= Normal control, DC= Diabetic control, ETDM= Ethanol treated diabetic mice, AqTDM= Aqueous treated diabetic mice, GTDM= Glibenclamide treated diabetic mice).

4.5 Estimation of glycosylated hemoglobin

Table 14 and Fig 14 illustrated the changes in glycosylated haemoglobin of normal control, diabetic control and extract and glibenclamide treated mice. In diabetic control mice, the glycosylation of haemogloibin was almost triple as compared to normal control. Whereas, in case of diabetic mice treated with *E.operculata* extract the glycosylatin of haemoglobin decreased significantly ($P<0.05$). And these results were found to be comparable to glibenclamide treated mice.

Group	Glycosylated Hg
NC	4.05±0.4
DC	13.47±1.8
E.operculata ETDM	5.74±1.6
E.operculata AqTDM	6.18±1.2
GTDM	5.04±2.1

Table 14: Changes in glycosylated haemoglobin of normal control, diabetic control, extract and glibenclamide treated mice.

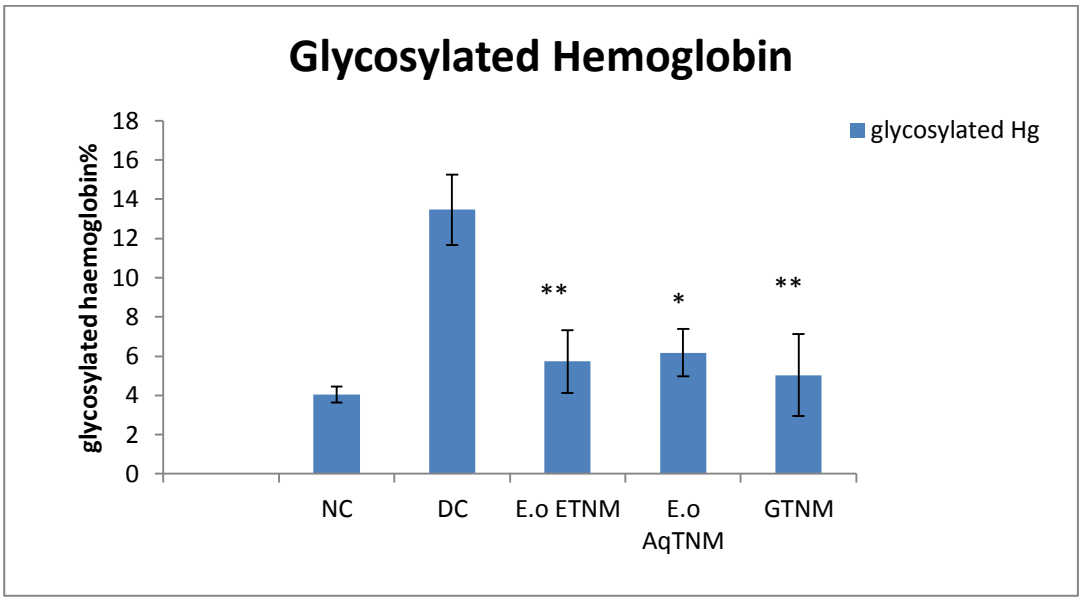


Fig 14: Changes in glycosylated haemoglobin of normal control, diabetic control, extract and glibenclamide treated mice.

4.6 In vitro Antioxidant activity of *E.operculata* extract

4.6.1 DPPH radical scavenging activity (RSA) assay

The extract showed substantial antioxidant activity in a dose dependent manner similar to that of ascorbic acid which was used as a control standard antioxidant.

sample	IC_{50} (µg/ml)
Ethanol extract	52.96
Aqueous extract	41.73
Ascorbic acid	26.28

Table 15: IC_{50} of *E.operculata* extract and ascorbic acid.

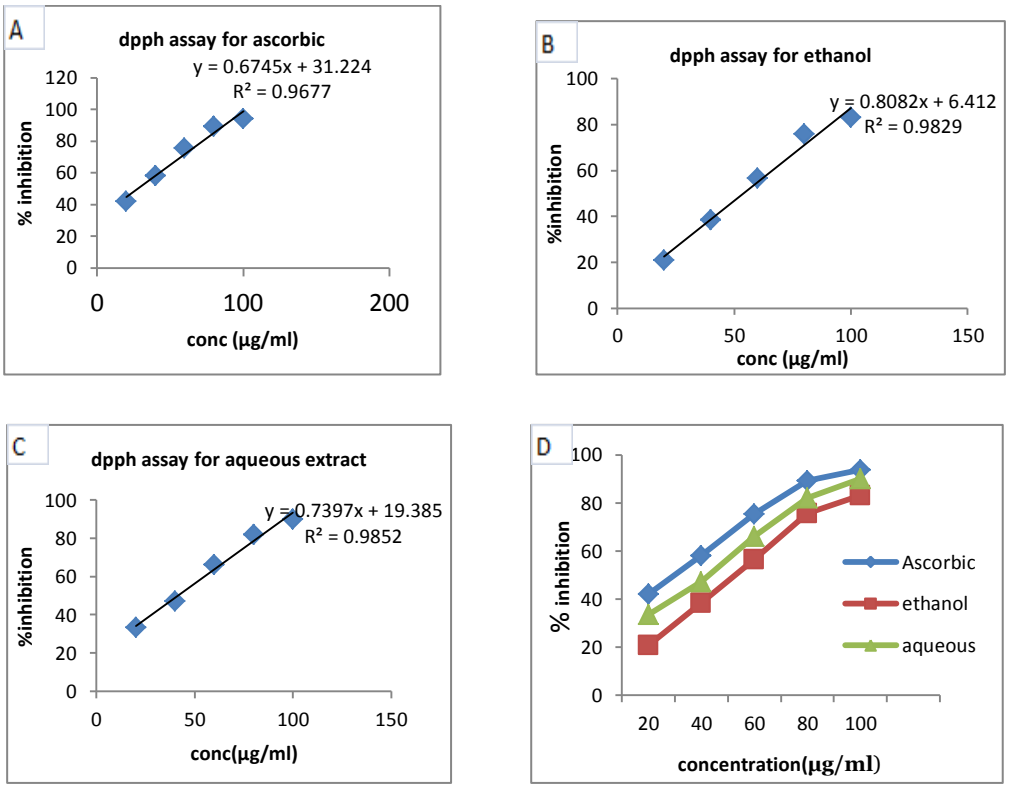


Fig 15: Effect of *E.operculata*leave extracts on DPPH assay. Fig 15(A, B, C) determine the correlation between %inhibition and concentration of extracts and ascorbic. Fig 15(D) shows DPPH scavenging activity.

Figure 15 (A,B,C) illustrated the linear regression correlation of %inhibition and concentration of the sample, showing positive relation with correlation coefficient (R^2) 0.967,0.982,0.985 bearing IC_{50} values 26.28±1.37 µg/ml, 52.96±0.94 µg/ml, 41.73±0.4µg/ml for ascorbic acid, ethanol and aqueous respectively (Table 15). Fig 15 (D) illustrated decreases in the concentration of DPPH radical due to the scavenging activity with increases in % inhibition.

4.6.2 Reducing power assay

Reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity. It is a measure of reductive ability of antioxidants and it is evaluated by the transformation of Fe^{3+} to Fe^{2+} in the presence of extracts. In this assay, the yellow color of the test solution changes to various shades of green and blue depending on the reducing power of each compound. Fig 16 shows the reducing power of standard ascorbic acid, ethanol and aqueous extracts of *E.operculata* at different concentrations and it reveals that the reducing power of the entire test increased with increase concentration.

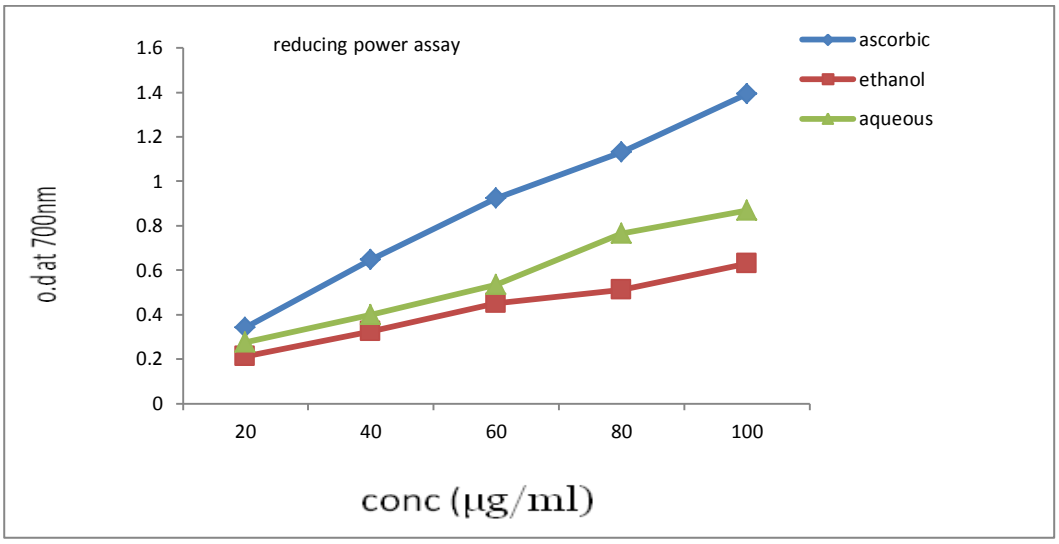


Fig 16: Reducing ability of the standard ascorbic acid and the extracts at various concentrations.

4.7 Phytochemical screening

Phytochemical screening was performed using the suitable tests to assess the type of phytochemicals present in the crude extract of ethanol and aqueous. And from this screening it was observed that phytoconstituents like carbohydrate, resins, flavonoid, terpenoids, phenol, tannins and cardiac glycoside are present in both extracts. Whereas, traces of alkaloids and protein are found in aqueous extract only.

4.8 Total flavonoid and phenolic content

The evaluation of phenolic and flavonoids contents of *E.operculata* extract shows that phenolic content of aqueous extract is 0.71% higher than that of ethanol extract whereas it's the reverse in flavonoid, ethanol contains 1.109% in comparison to 0.78 % of aqueous extract.

4.9 Bioassay guided isolation and characterization of antidiabetic component(s)

As ethanol extract of *E.operculata* showed more promising antidiabetic properties while comparing with aqueous extract, the characterization of compounds were conducted only on ethanol extract.

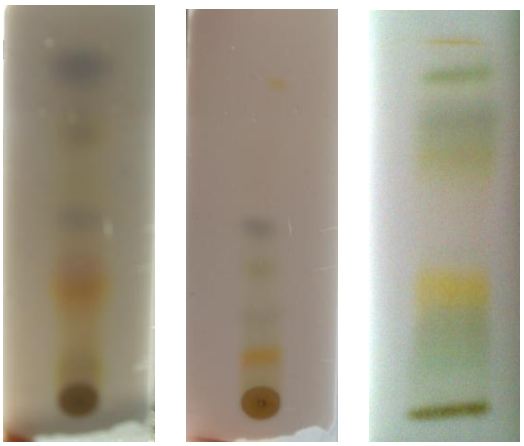
Sl.No.	Constituents	Test	*E.operculataperculata*	
			***E.operculata*-ethanol**	***E.operculata*-Aqueous**
1	Alkaloids	a.Meyer's test b.Wagner's test c.Hager's test	- - -	- - +
2	Carbohydrates	a.Molisch's test b.Benedict's test c.Fehling's test	+ + +	+ + +
3	Resins	Acetone H_2O test	+	+
4	Flavonoids	a.Alkaline reagent test b.Lead acetate test c.ZincHCl reduction test d.Shinoda test	- + + +	- + - +
5	Terpenoids	Salkowski's test	+	+
6	Anthraquinones	-	-	-
7	Diterpenes	Copper acetate test	-	-
8	Glycosides	a.ModifiedBorntrager's test b.Legal's test	+ -	- -
9	Phenols	Ferric chloride test	+	+
10	Tannins	Gelatin test	+	+
11	Proteins and amino acids	a.Xanthoproteic test b.Ninhydrin test	- -	- +
12	Cardiac glycosides	Killer Kilani test	+	+

Table 16. Preliminary screening of secondary metabolites from aqueous and ethanol extract of *E.operculata*.

4.9.1 Determination of suitable solvent system for column chromatography

Various combinations of polar and non-polar solvents were tested for suitability as mobile phase for column chromatography using analytical TLC plates for

E.operculata ethanol extract. Of the solvent system tested, the chloroform: methanol (5.5:4.5, 6.5:3.5) solvent system gives the best resolution showing maximum bands in Fig 17.



A B C

Fig 17: A) chloroform: methanol (5.5:4.5), B) chloroform: methanol (6.5:3.5), C) ethyl acetate: methanol.

4.9.2 Fractionation and characterization of antidiabetic component(s)

The crude ethanol extract subjected to column chromatography leads to the collection of 22 sub fractions (250ml). The fractions were combined on the basis of TLC leading to 6 main fractions. Fraction 3 was further subjected to column chromatography as it showed positive result on enzyme assay. Fraction 3 was chromatographed over a silica column eluted with chloroform: methanol mixtures of increasing polarity. Sub fractions of main fraction 3 were further subjected to repeated column chromatography and each fraction was tested for antidiabetic activity. The fraction which showed high activities of both the parameters was chosen for further fractionation and TLC analysis. The process was repeated till the sub-fractions showed purity in TLC profile and finally 4 sub fractions were collected as compound 1 to 4. Of the four compound isolated the Rf value of compound 1 and 4 were almost similar with standard quercetin and kaempferitrin.

Ethanol extract (active)

↓

Column chromatography

↓

22 fractions were collected

↓

Based on the TLC plates, fractions were pooled together and 6 major fractions were obtained

↓

Fraction 3 shows maximum activity

↓

4 sub-fractions were collected

↓

Compound 1 to 4

Fig 18: Schematic diagram for isolation of compounds

Plate 4: TLC fractions collected from column.

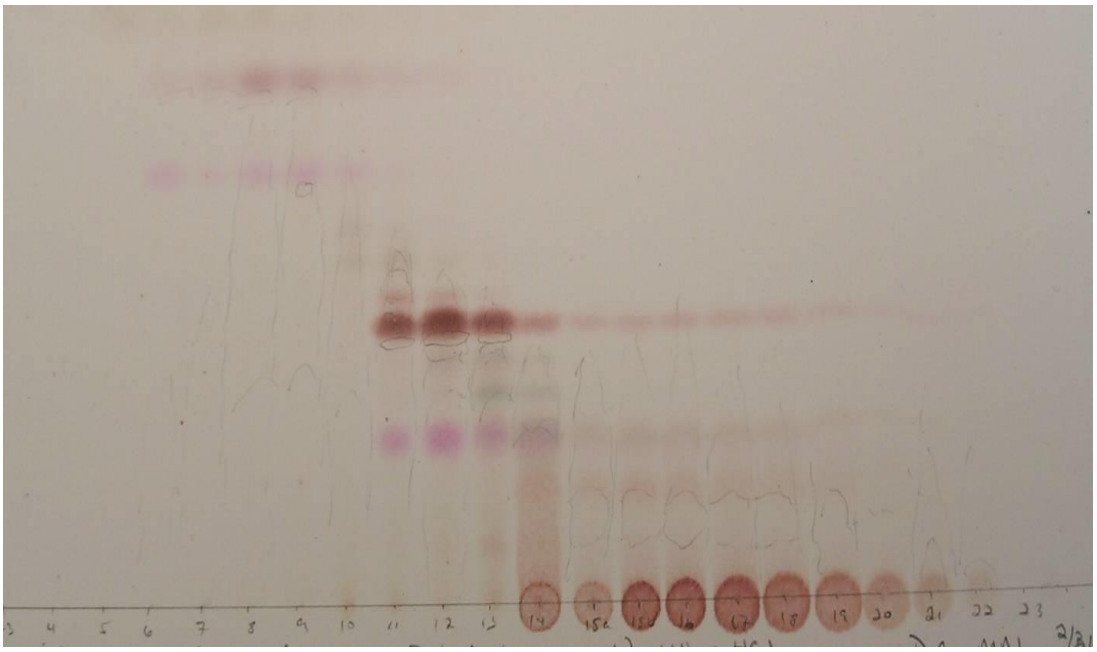


Fig 19: TLC of the first 22 main fractions collected from column.

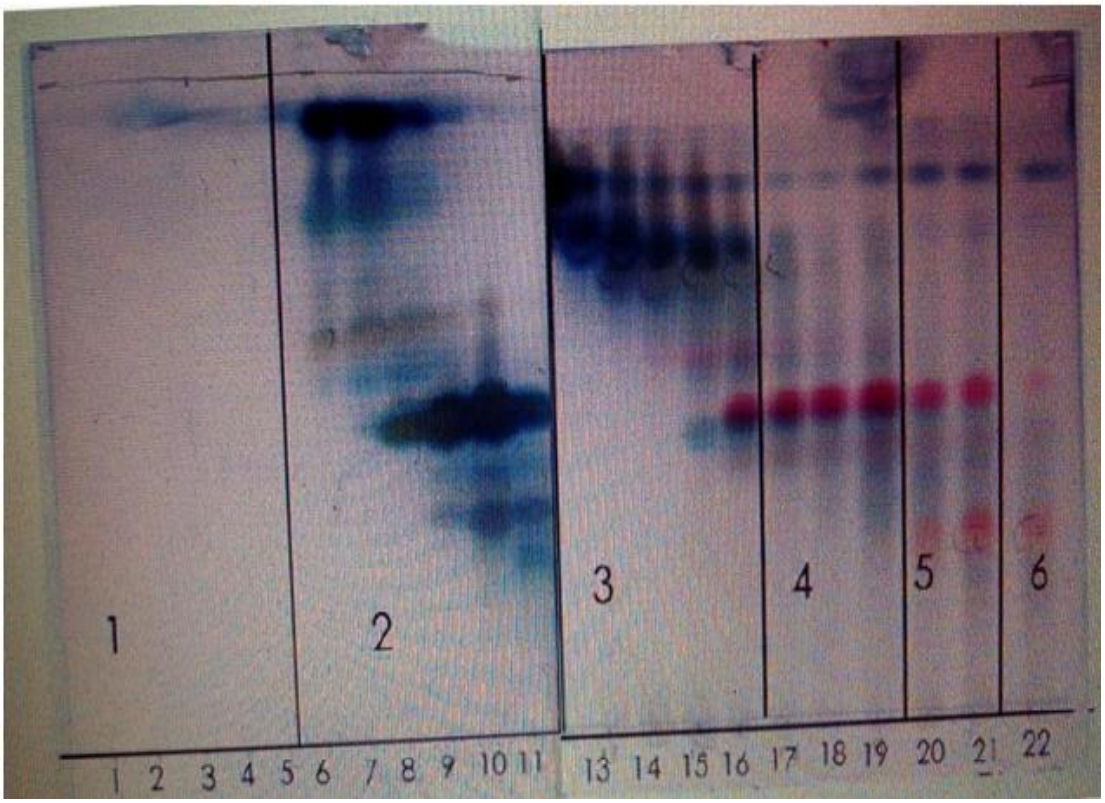
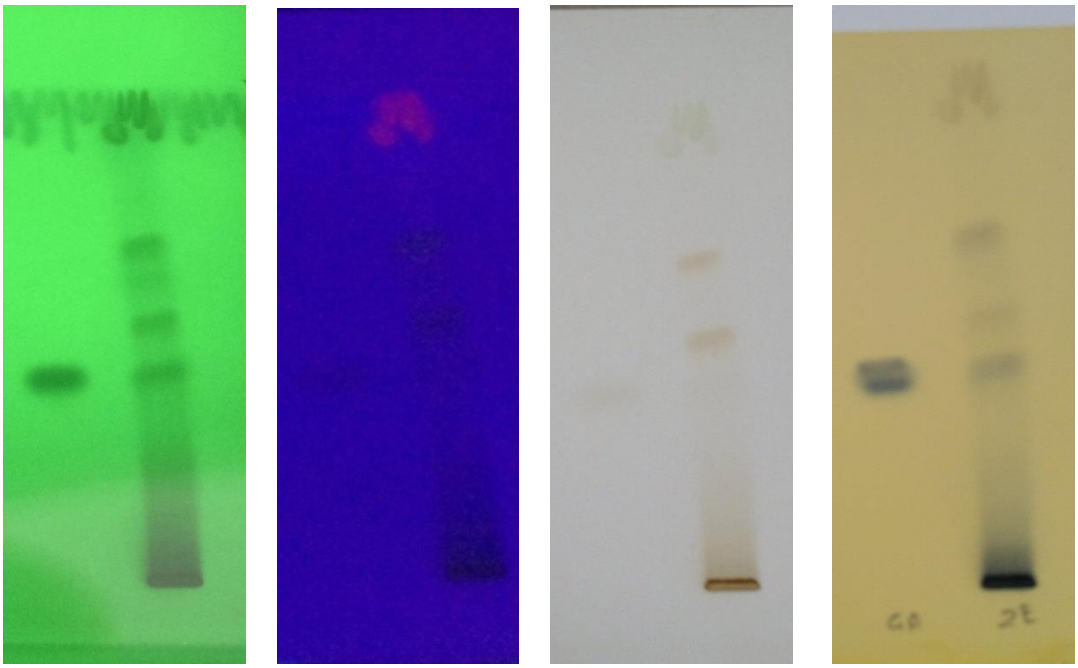
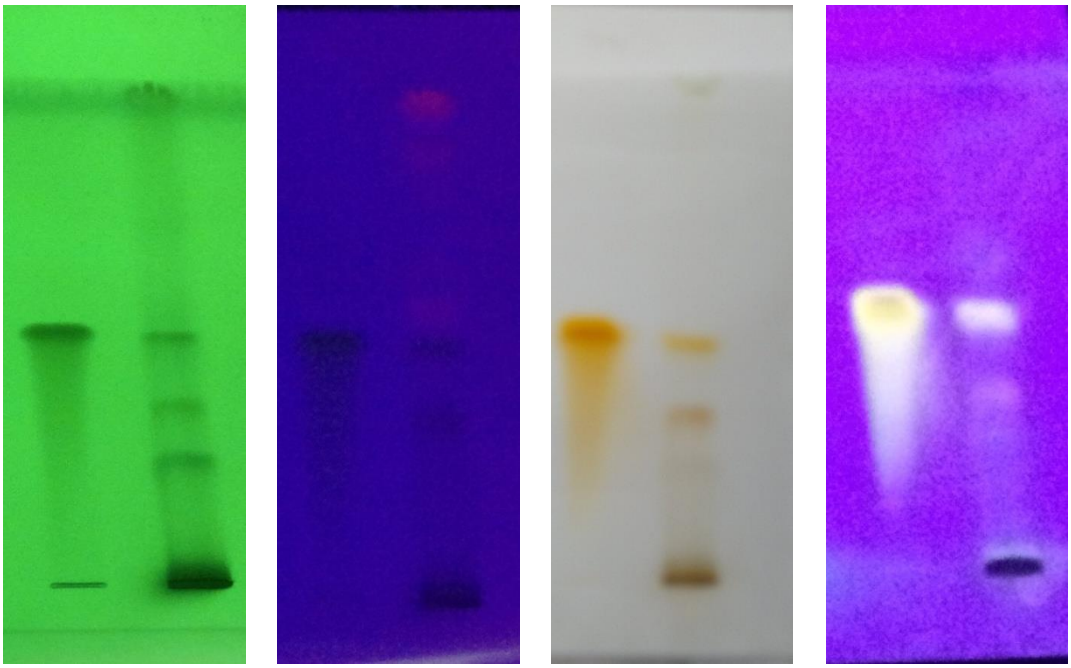


Fig 20: Combination of fractions into 6 main fractions based on TLC.



A) At 254nm B) At 366nm C) At visible light D) After derivation with NP/PEG at 366nm

Fig 21: TLC of final fraction with standard kaempferitin (Rf-0.37).



A) At 254nm B) At 366nm C) At visible light D) After derivation with 10% $FeCl_3$

Figure 22: TLC of final fraction with standard querceti (Rf-0.47).

4.9.3 Characterization of compounds

4.9.3.1 Quercetin-3-o-β-D-glucopyranosyl (1-4)-α-L-rhamnopyranoside

Compound 1 was purified with a column eluted with chloroform and methanol. HRESIMS showed a molecular peak at m/z 607.1411 $[M^{-}H]^{-}$ corresponding to the molecular formula $C_{27}H_{30}O_{16}$. The ^{1}H NMR showed five signals in the aromatic hydrogen region, two broad singlets at δH 6.21/6.40, assigned to H-6/H-8, two doublets at δH 6.88 (d,1H, J 8.0 HZ, H-5') and 7.58 (d, 1H, J 8.0HZ, H-6') as well as one broad singlet at δH, 7.71 assigned to H-2'. This spectrum also showed signals that characterize the β-D glucoside unit. A broad singlet at δH 5.22 (H-1''') assigned to an aromatic di-equatorial hydrogen, which, associated to the doublet at δH 1.25 (d, 3H, J 6.0 H-3, H-6''') suggests the presence of α-L-rhamnose. The ^{13}C, DEPT and HSQC spectra of compound 1 confirmed the purposed structure showing 27 carbon signals being 1 methyl, 1 methylene, 15 methyne and 10 non-hydrogenated carbons. Oxymethine carbon signals, confirmed the presence of glucose and rhamnose in the molecule of 1. HMBC and COSY spectra showed important correlations between hydrogen's and carbons, which indicated that rhamnose is linked at C-4 of glucose. Therefore analysis of the obtained data along with previous reports was found reliable with the structure quercetin-3-o-β-D-glucopyranosyl (1-4)-α-L-rhamnopyranoside.

4.9.3.2 Kaempferol-3-o-β-D-glucopyranosyl (1-4)-α-L-rhamnopyranoside

The ^{1}H NMR spectrum of compound 2 is almost similar with that of compound 1, with difference in the substitution of kaempferol, due to the presence of two doublets integrated to two hydrogen each and thus assigned to H-3'/ H-5' and H-2'/ H-6', respectively. The presence of kaempferol aglycone moiety is also confirmed through 13C NMR spectra, including DEPT by showing one carbonyl carbon signal at δC 179.4 (C-4) and aromatic carbon signals at range δC 166.95. This information in addition with literature data confirmed the identification of compound 2 as kaempferol-3-o-β-D-glycopyranosyl (1-4)-α-L-rhamnopyranoside.

4.9.3.3 Quercetin-3-o-β-D-glucopyranosyl-(1-6)-α-L-rhamnopyranoside

Yellow amorphous solid; HRESIMS: 609.1616 $[M-H]^-$ (calculated to $C_{27}H_{29}O_{16}$: 609.1455) and 301.0851 [M-glucose unit]$^-$; ^{1}H NMR (DMSO-D_6, 400 MHz) δ 6.21 (d, *J* 2.0 Hz, H-6), 6.40 (d, *J* 2.0 Hz, H-8), 7.66 (d, *J* 2.0 Hz, H-2'), 6.86 (d, *J* 8.5 Hz, H-5'), 7.60 (dd, *J* 8.5 and 2.0 Hz, H-6'), 5.11 (d, *J* 7.5 Hz, H-1''), 4.52 (d, *J* 1.5 Hz, H1'''), 1.18 (d, *J* 6.0 Hz, H-6'''), 3.20-3.90 (H-2'' to H-6'', H-2''' to H-5'''); ^{13}C NMR (DMSO-D_6, 100 MHz) δ 158.5 (C-2), 135.9 (C-3), 179.5 (C-4), 163.0 (C-5), 100.0 (C-6), 166.1 (C-7), 94.9 (C-8), 159.0 (C-9), 105.6 (C-10), 123.0 (C-1'), 117.9 (C-2'), 145.8 (C-3'), 150.0 (C-4'), 116.1 (C-5'), 123.6 (C-6'), 104.7 (C-1''), 75.7 (C-2''), 77.2 (C-3''), 71.4 (C-4''), 78.2 (C-5''), 68.6 (C-6''), 102.4 (C-1'''), 72.1 (C-2'''), 72.3 (C-3'''), 73.1 (C-4'''), 69.7 (C-5'''), 18.0 (C-6''').

4.9.9.4 Kaempferol-3-7-o-α-dirhamnoside

^{1}H NMR (DMSO-D_6 400 MHz) δ6.46 (d, *J* 2.4 Hz, H-6); 6.79 (d, *J* 2.0 Hz, H-8); 7.80 (d, *J* 8.8 Hz, H-2' and 6'); 6.93 (d, *J* 8.8 Hz, H-3' and 5'); 5.30 (d, *J* 1.6 Hz, 3-O-Rh-1"); 3.99 (dd, *J* 1.6 and 3.2 Hz, H-2"); 3.47 (dd *J* 3.2 and 8.8 Hz, H-3"); 3.15 (t, *J* 8.8 Hz, H-4"); 3.13 (m, H-5"); 0.81 (d, *J* 5.6 Hz, 3-O-Rh-CH_3); 5.56 (d, *J* 1.6 Hz, 7-O-Rh-1"'); 3.84 (dd, *J* 1.6 and 3.4 Hz, H-2"'), 3.64 (dd, *J* 3.4 and 9.2 Hz, H-3"'); 3.31 (t, *J* 9.2 Hz, H-4"'); 3.43 (m, H-5"'); 1.13 (d, *J* 6.4Hz, 7-O-Rh-CH_3); ^{13}C NMR (DMSO-D_6, 100 MHz): δ 157.8 (C-2), 134.5 (C-3), 178.0 (C-4), 160.9 (C-5), 99.4 (C-6), 161.7 (C-7), 94.6 (C-8), 156.1 (C-9), 105.8 (C-10), 120.7 (C-1'), 130.7 (C-2' and 6'), 115.4 (C-3' and 5'), 160.1 (C-6'), 101.9 (3-O-Rh-C-1"), 70.0 (C-2"), 70.3 (C-3"), 71.1 (C-4"), 70.7 (C-5"), 17.5 (3-O-Rh-CH_3), 98.4 (7-O-Rh-C-1"'), 69.8 (C-2"'), 70.2 (C-3"'), 71.6 (C-4"'), 70.0 (C-5"'), 17.9 (7-O-Rh-CH_3).

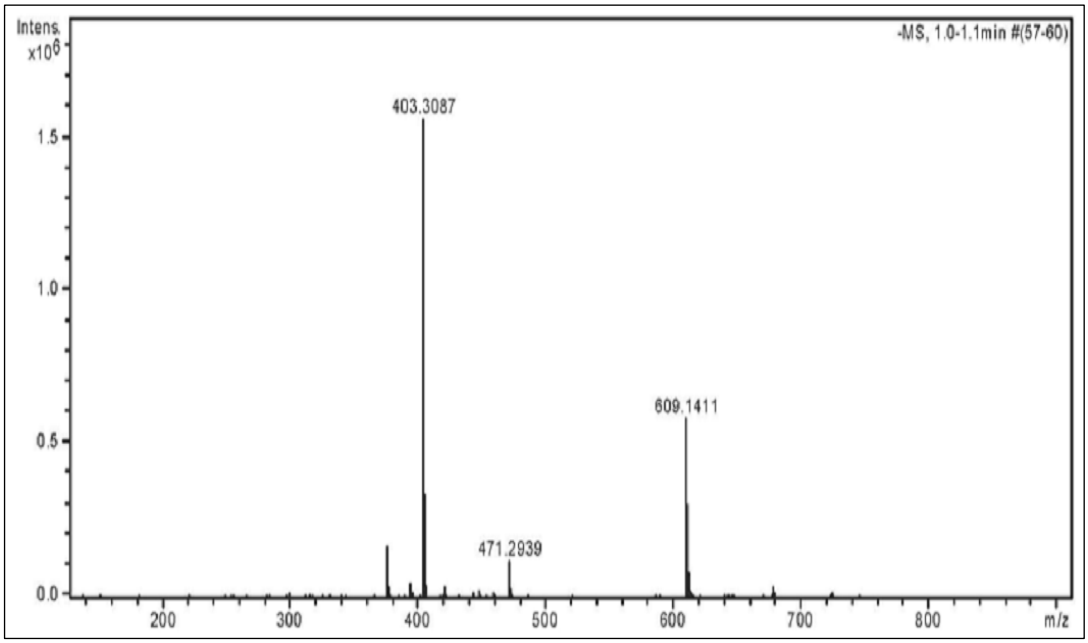


Fig 23: HRESIMS spectrum (negative mode) of compound 1

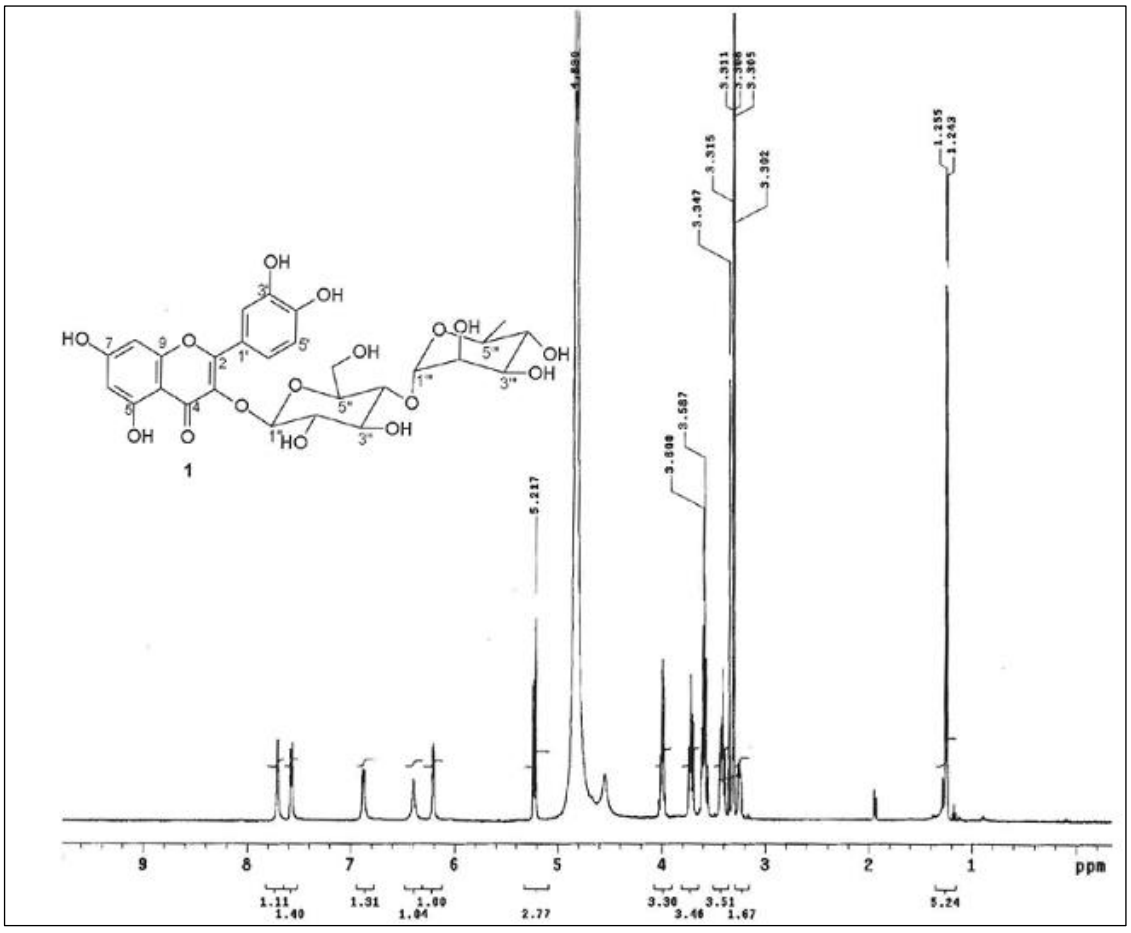


Fig 24: ^{1}H NMR spectrum of compound 1

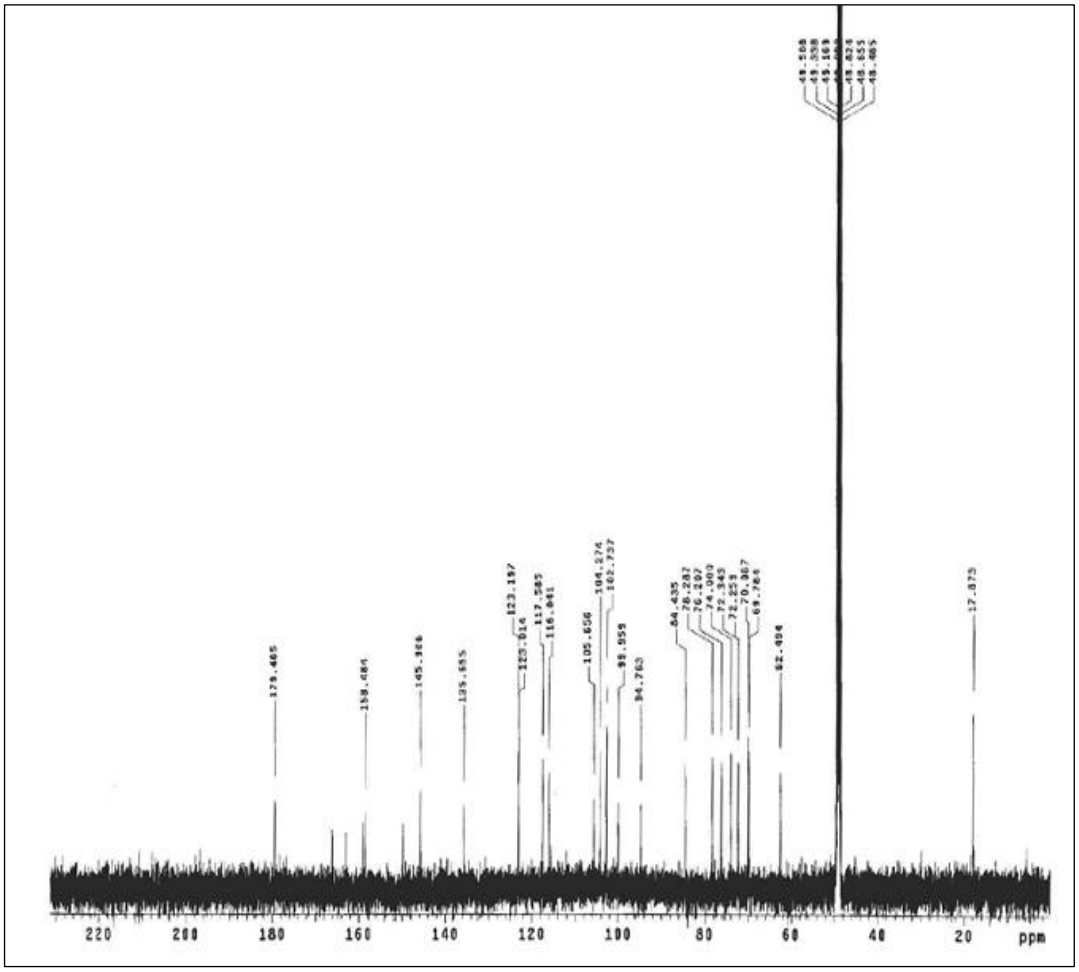


Fig 25: ^{13}C NMR spectrum of compound 1

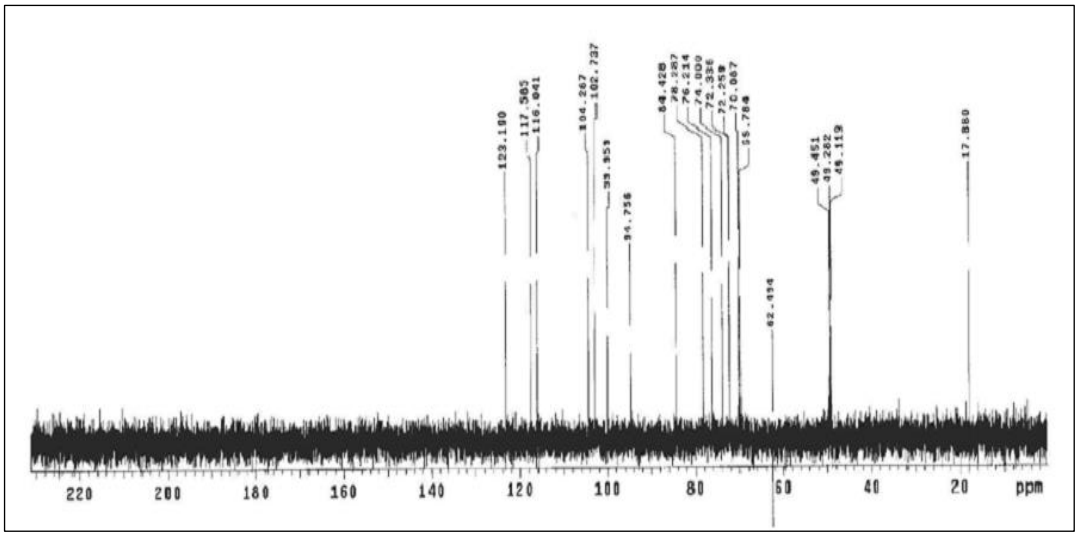


Fig 26: DEPT 135^{o} NMR experiment of compound 1

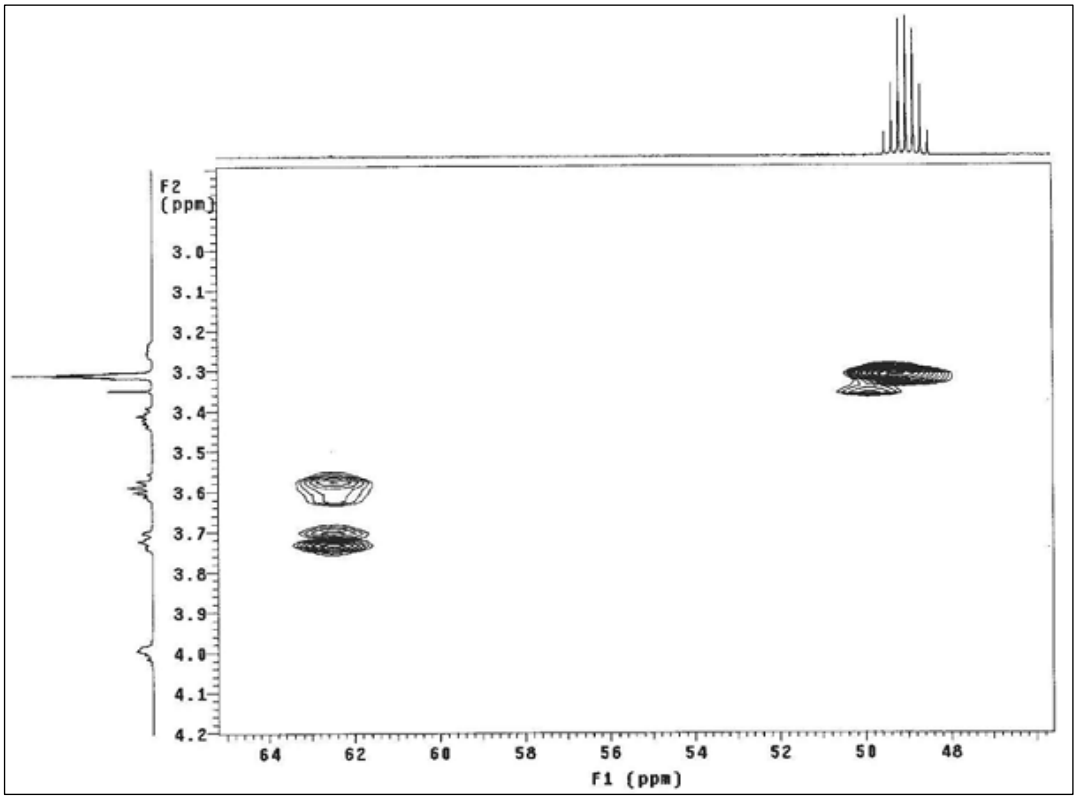


Fig 27: Expansion HMQC NMR experiment of compound 1

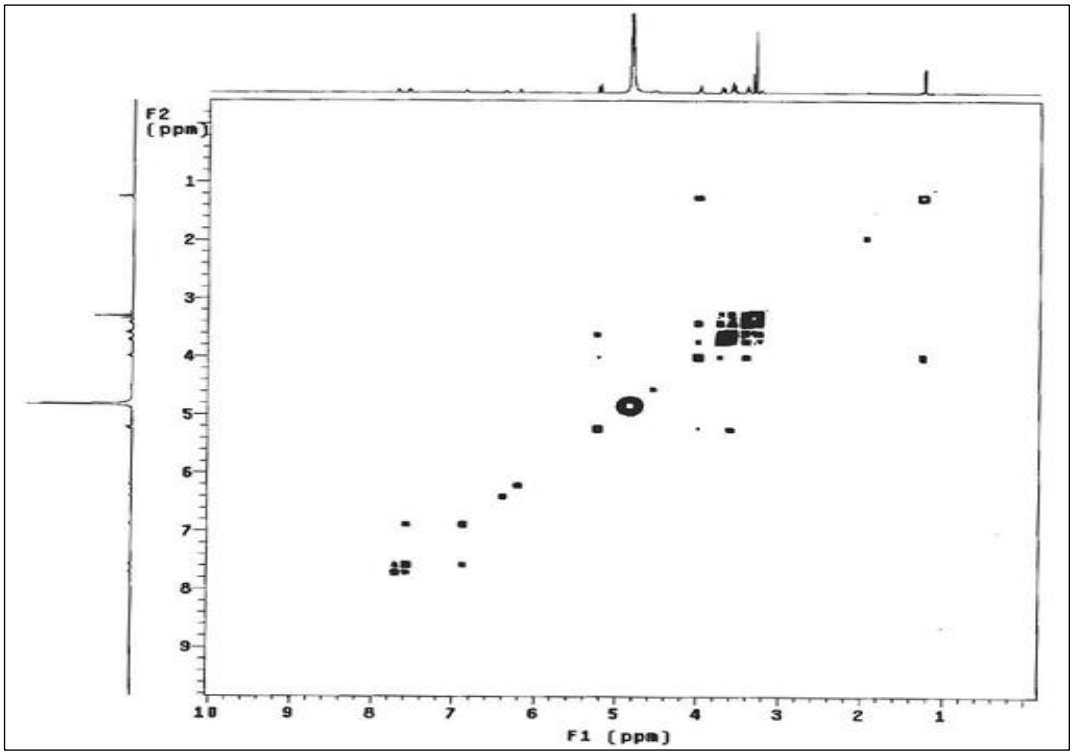


Fig 28: gCOSY NMR experiment of compound 1

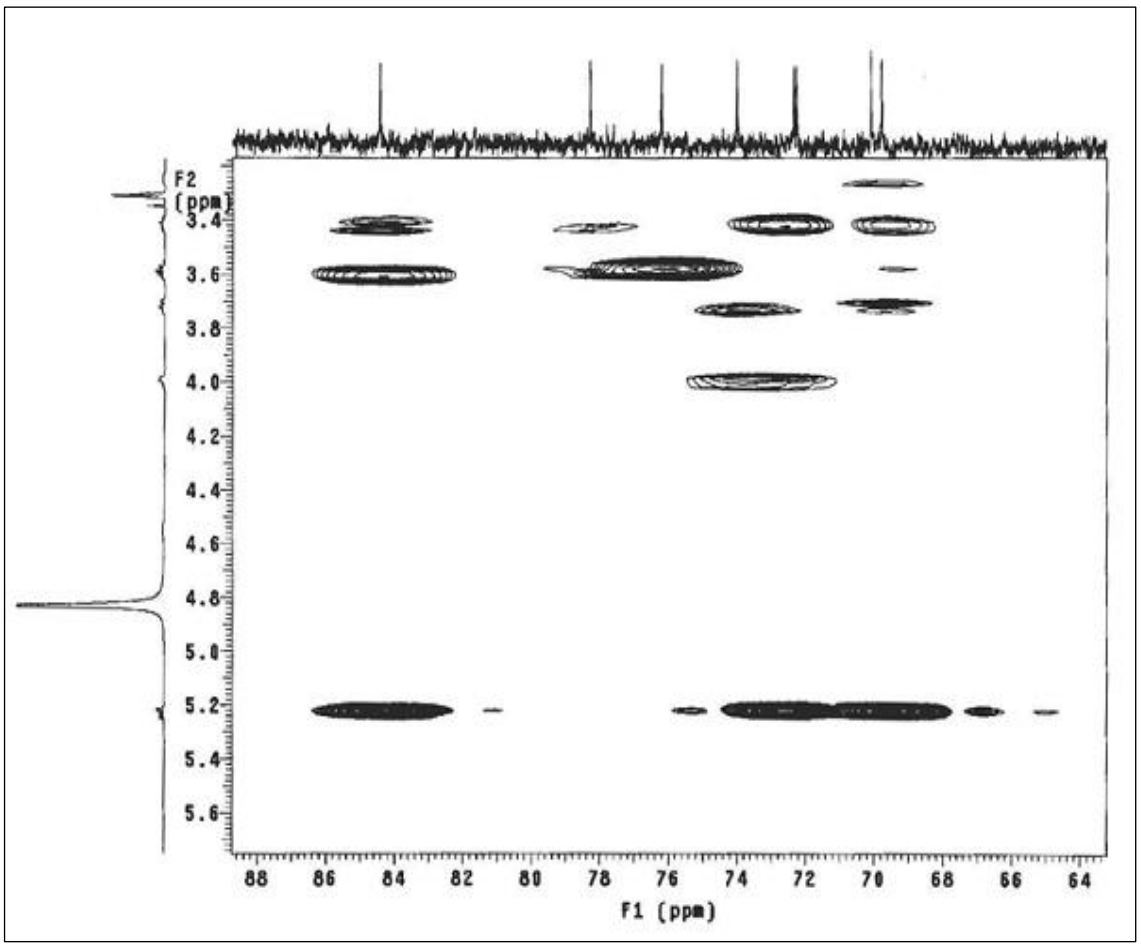


Fig 29: Expansion HMBC NMR experiment of compound 1

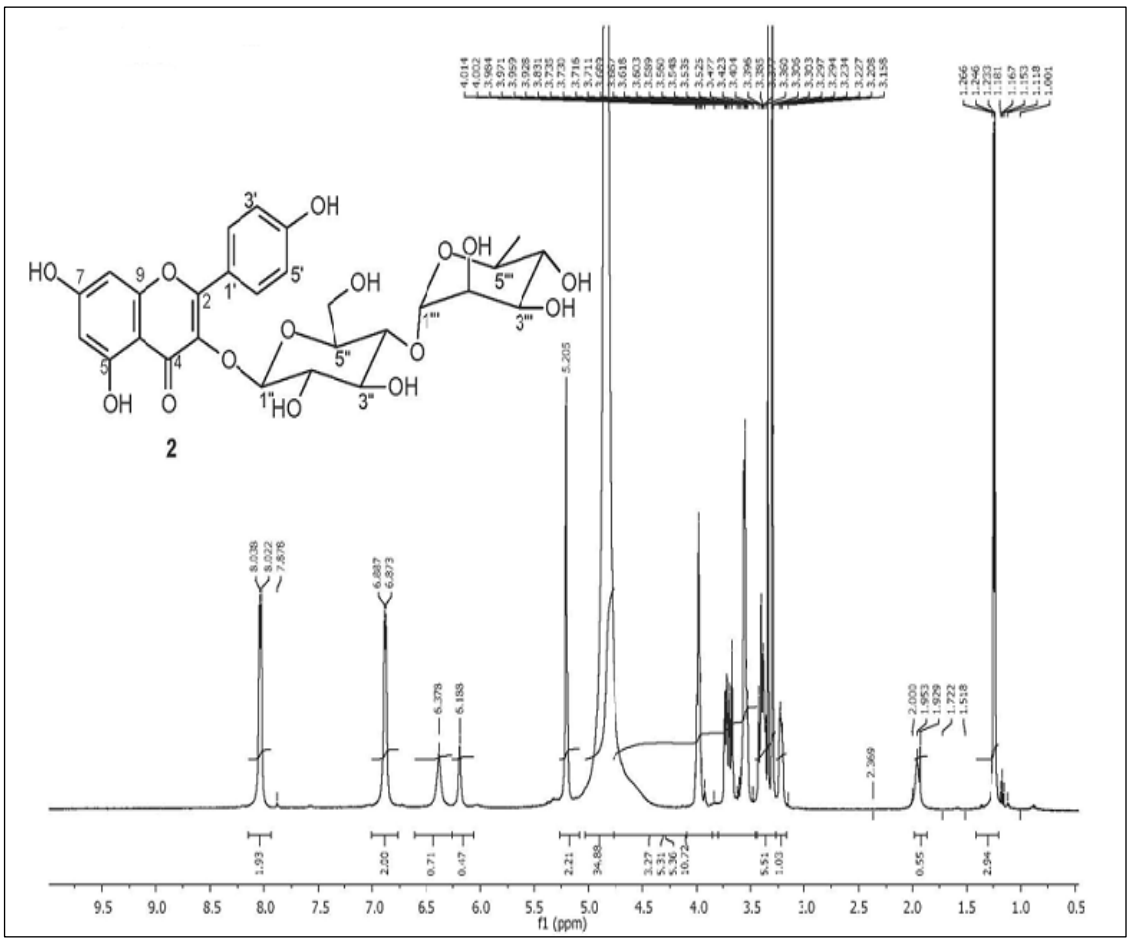


Fig 30: ^{1}H NMR spectrum of compound 2

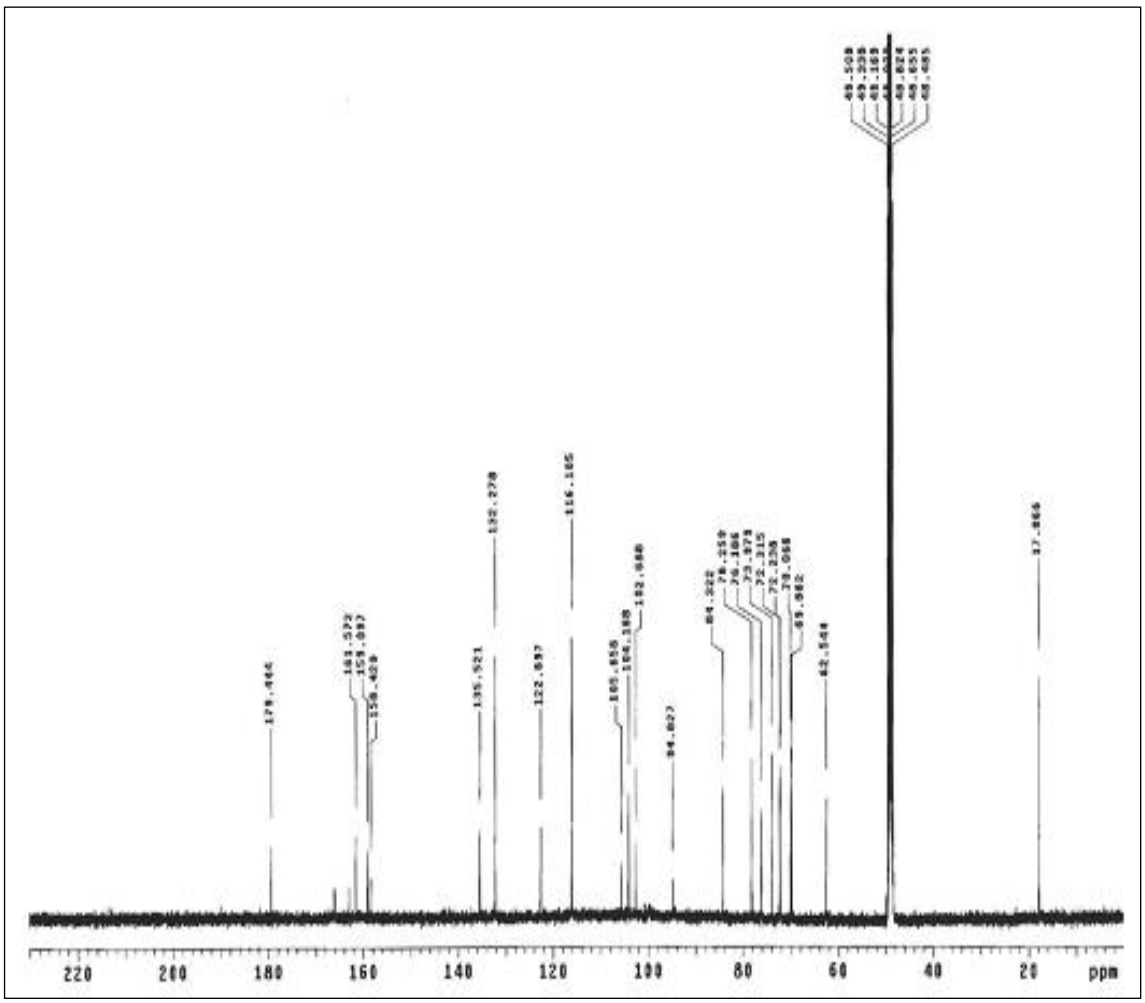


Fig 31: ^{13}C NMR spectrum of compound 2

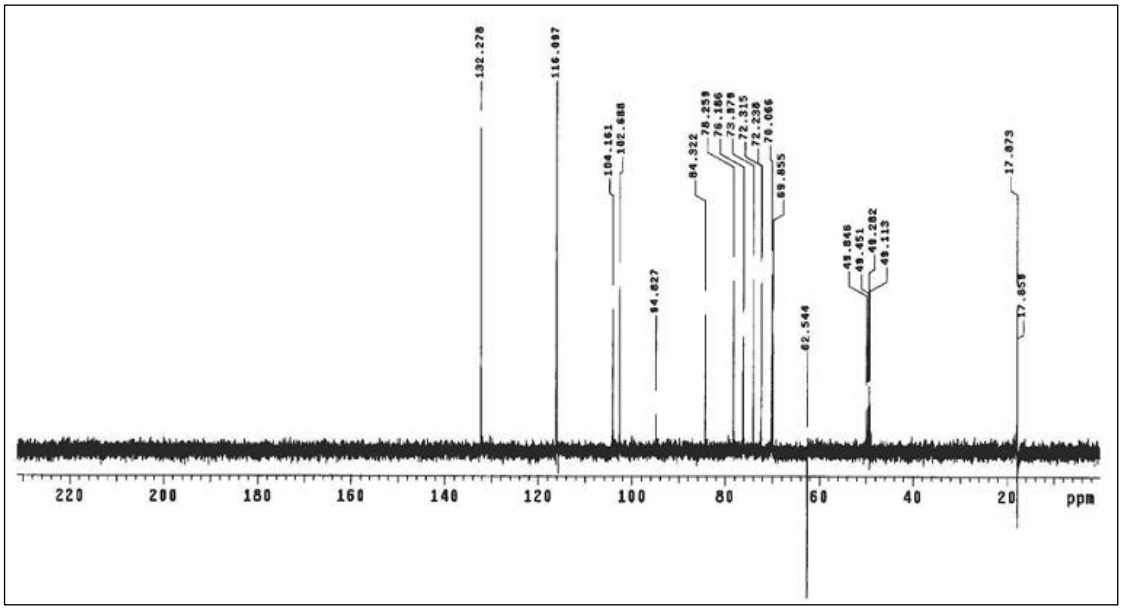


Fig 32: DEPT 135^{o} NMR experiment of compound 2

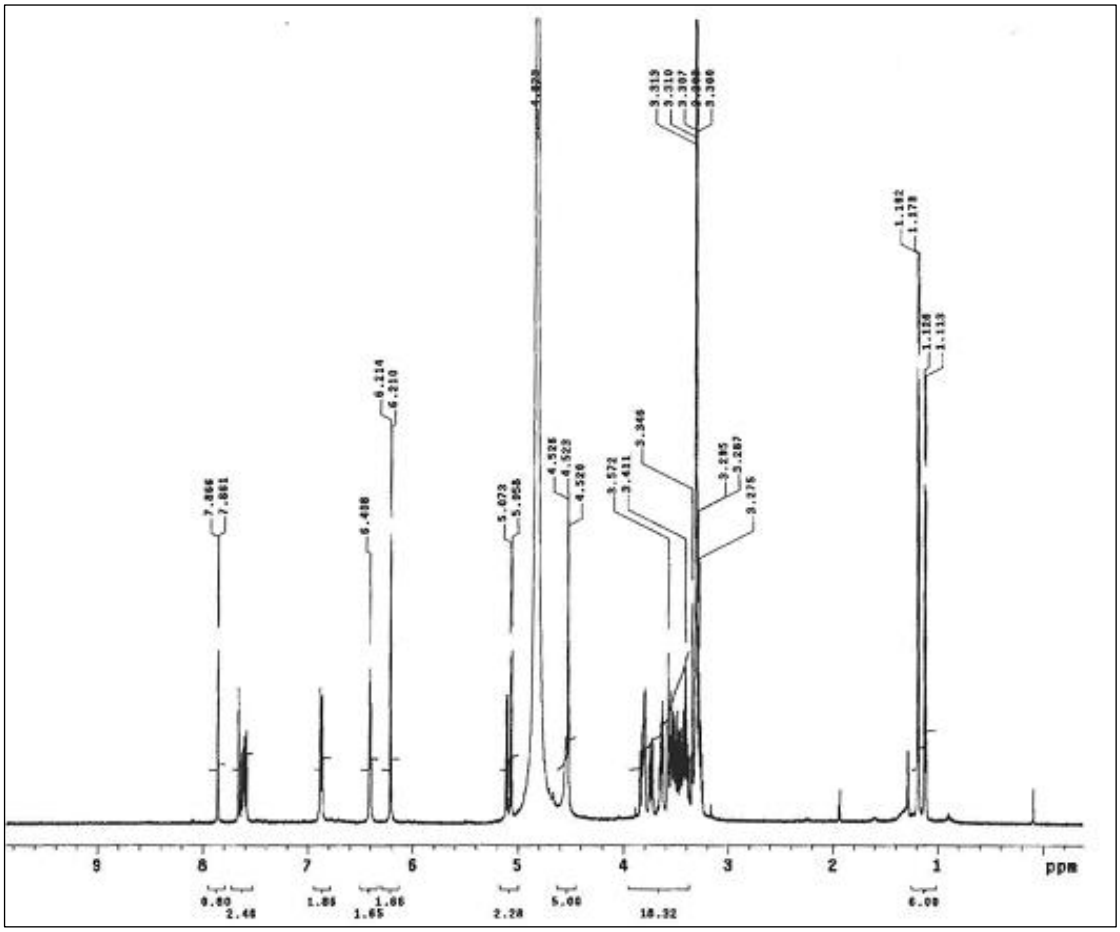


Fig 33: ^{1}H NMR spectrum of compound 3

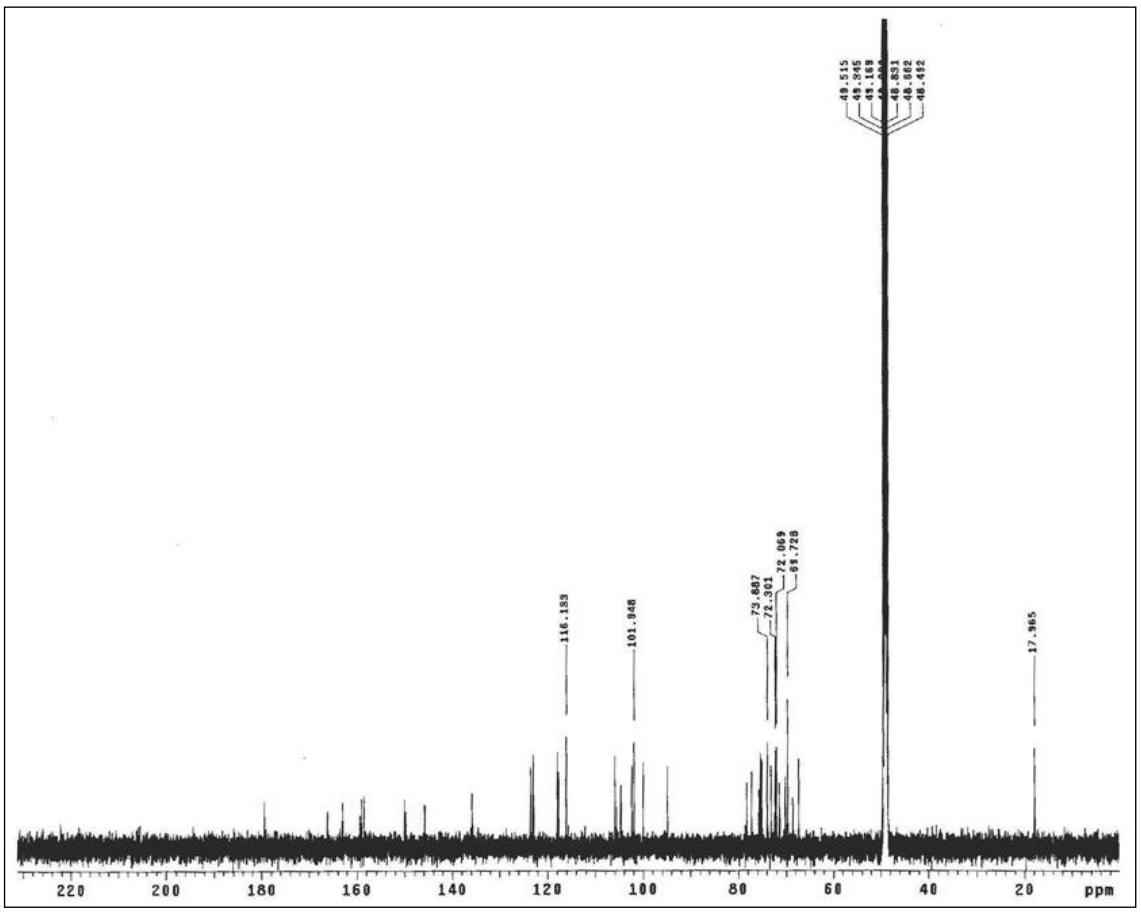


Fig 34: ^{13}C NMR spectrum of compound 3

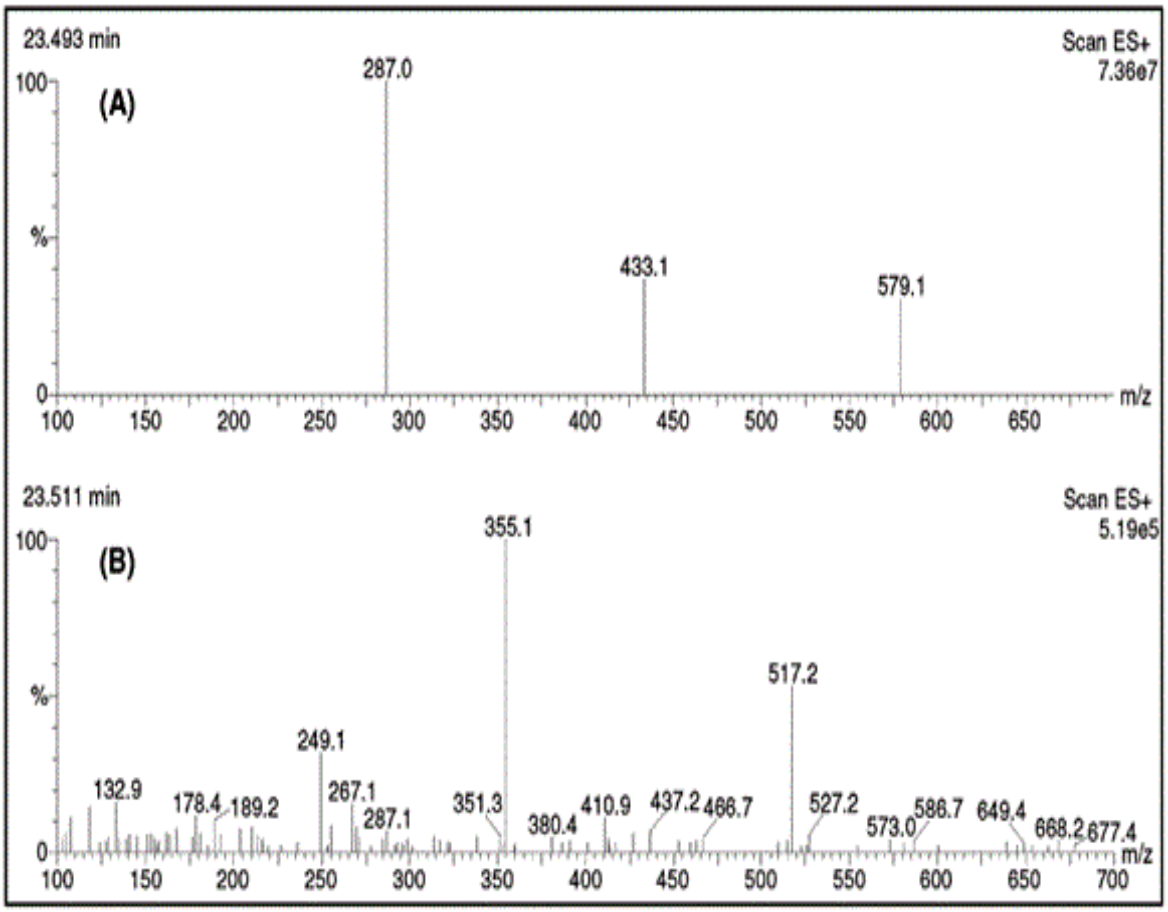


Fig 35: (A) Mass spectra of the reference kaempferitrin at retention time 23.493 min and (B) mass spectra of compound 4 at retention time 23.511 min.

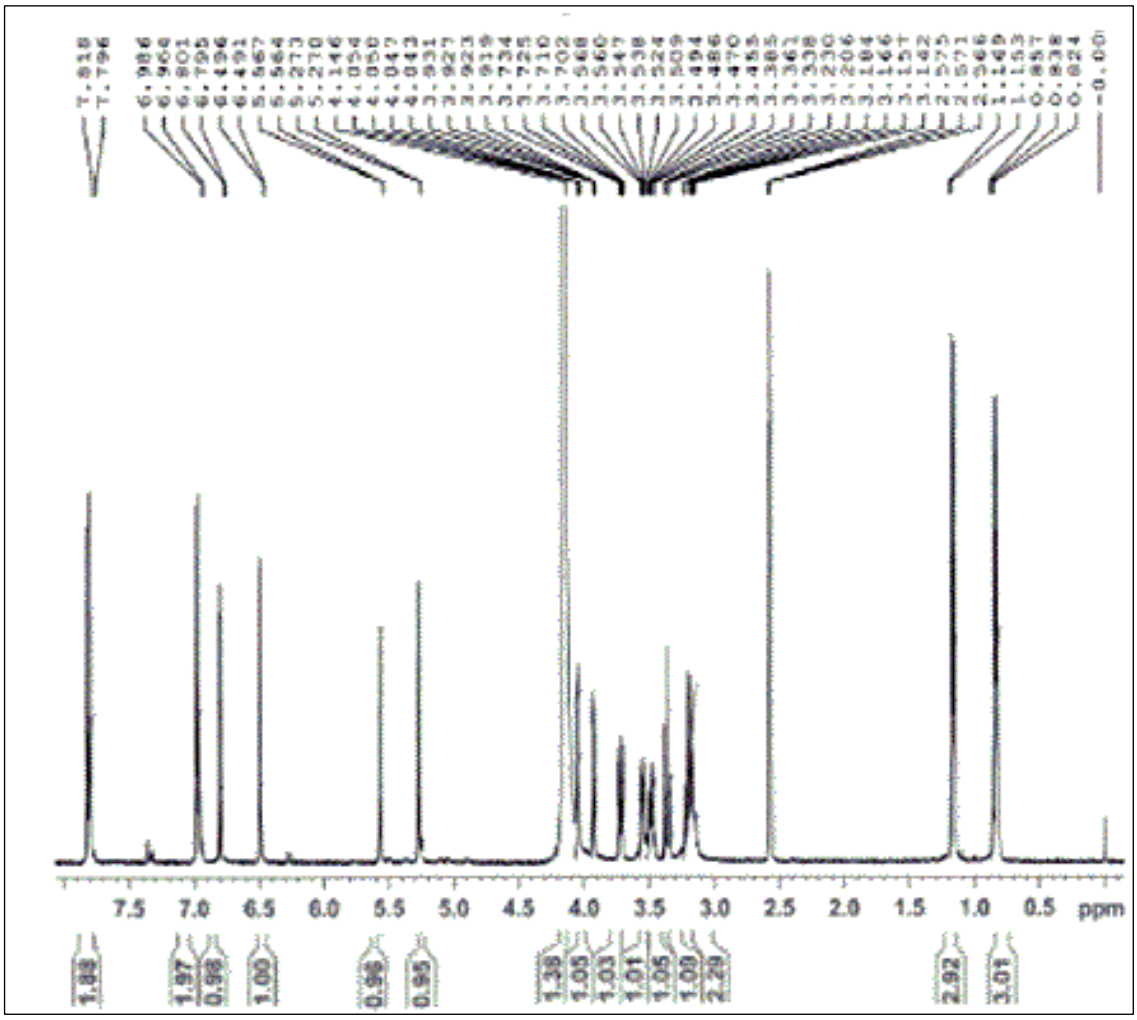


Fig 36: ^{1}H NMR of compound 4

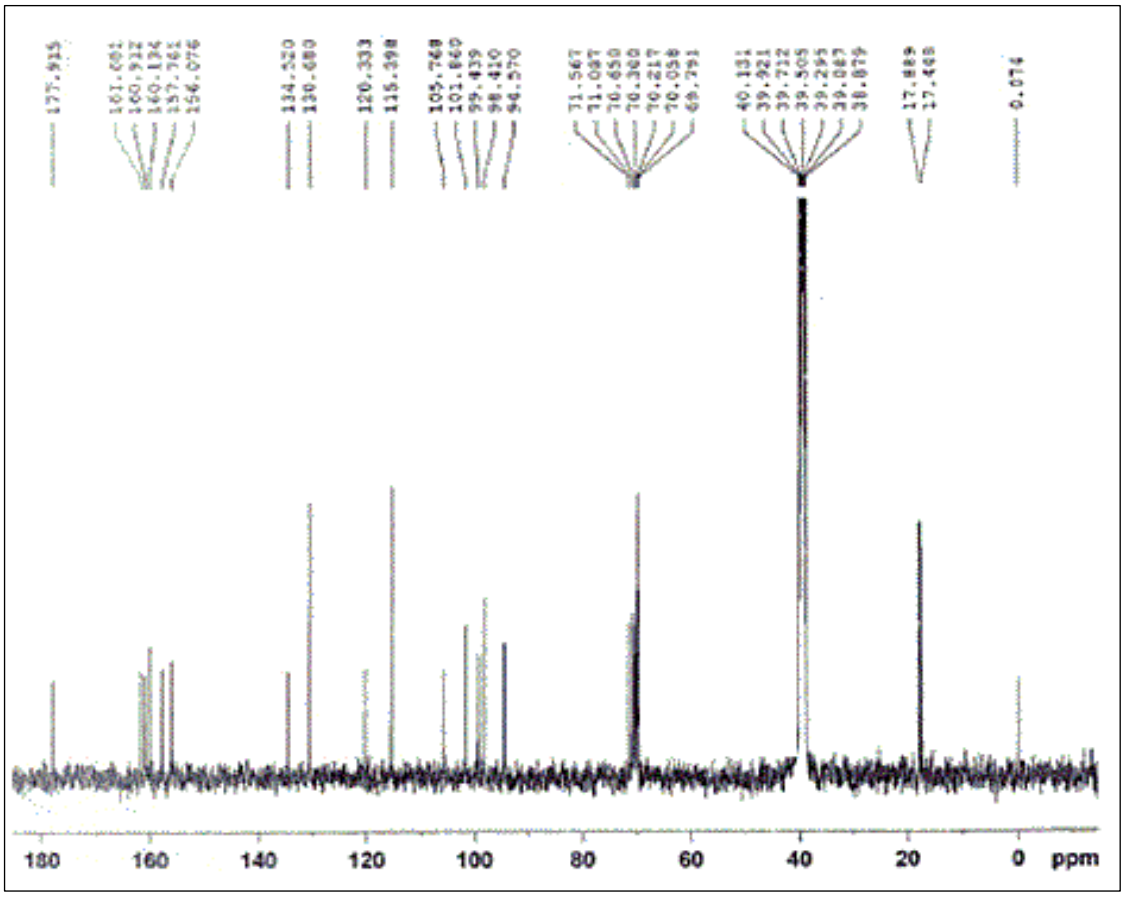


Fig 37: ^{13}C NMR of compound 4