CHAPTER 4

RESULTS

4.1. Successive solvent extraction of the plant leaves

The percentage yield of extracts of CA leaves in successive solvent extraction using petroleum ether, acetone, ethyl acetate, ethanol, methanol and aqueous are showed in Table.4.1. Ethanolic extract gave the highest yield (12.15%) followed by aqueous extract (10.79%), acetone extract (9.46%), methanol extract (3.63%), ethyl acetate extract (1.59%) and the petroleum ether extract gave the lowest yield (1.2%).

Table.4.1: Percentage yield of extracts.

Solvents	Colour of Extracts	Sample powder (g)	Extract (g)	% yield
Pet.E	green	30	1.20	1.2
AC	Deep green	28.74	2.72	9.46
E.A	Light orange	25.85	0.41	1.59
E	Deep brownish red	25.43	3.09	12.15
M	Brownish red	22.32	0.81	3.63
Aq	Red	21.50	2.32	10.79

Pet.E, petroleum ether; AC, acetone; E.A, ethyl acetate; M, Methanol; E, Ethanol; Aq, aqueous.

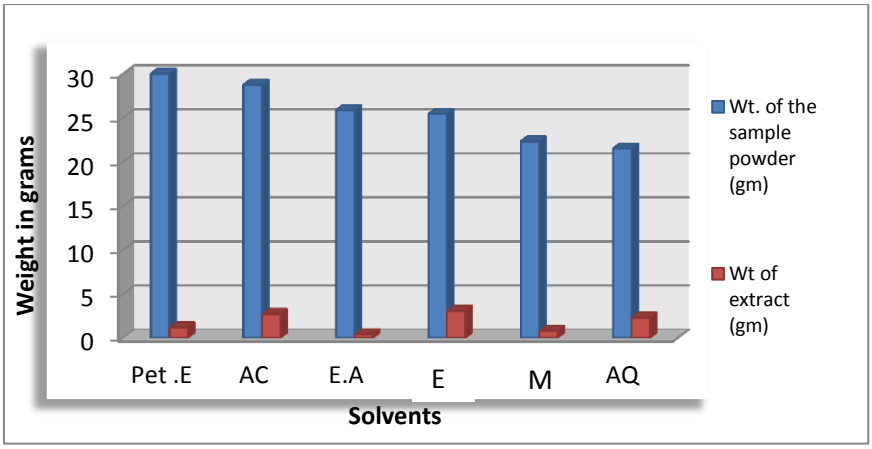


Fig.4.1: Graph showing yield of the extracts.

4.2. Preliminary phytochemical screening

The result of preliminary phytochemical screening of CAEE (CA Ethanol Extract), CAME (CA Methanol Extract) and CAAE (CA Aqueous Extract) are presented in Table.4.2. The results revealed the presence of some active compounds which are proved to have medicinal importance in literature. Some of the bioactive compounds

that are present in CAEE/CAME/CAAE are alkaloids, carbohydrates, resins (absent in CAME), flavonoids, terpenoids (absent in CAAE), diterpenes, phenols, tannins (absent in CAAE), proteins and amino acids, Cardiac glycosides (absent in CAEE and CAAE) and steroids. Phenolic compounds were found present in CAEE and CAME in significant amount. Conversely, phenols were found in low concentration in CAAE.

Table.4.2: Result of preliminary phytochemical screening of CAEE, CAME and CAAE

Constituents	Test	CAEE	CAME	CAAE
Alkaloids	a. Meyer's test	+	+	-
	b. Wagner's test	+	+	+
	c. Hager's test		+	-
	d. Dragendroff's test	-	+	-
Carbohydrates	a. Molisch's test	+	+	+
	b. Benedict's test	+	+	+
	c. Fehling's test	+	+	+
Resins	Acetone H_2O test	+	-	+
Flavonoids	a. Alkaline reagent test	+	+	-
	b. Lead acetate test	+	+	+
	c.Zinc HCl reduction test	-	-	-
	d. Shinoda test	-	-	-
Terpenoids	Salkowski's test	+	+	-
Anthraquinones	Anthraquinone test	-	-	-
Diterpenes	Copper acetate test	+	+	+
Glycosides	a.Modified Borntrager's test	-	-	-
	b. Legal's test	-	-	-
Phenols	Ferric chloride test	++	+ +	+
Tannins	Gelatin test	+	+	-
Proteins and amino acids	a. Xanthoproteic test	+	+	-
	b. Ninhydrin test	+	+	+
	c. Biuret test	+	-	-
Cardiac glycosides	Killer Kilani test	-	+	-
Steroids	Libermann- Burchand test	+	+	+

+, present; ++, present at high concentration; -, absent.

4.3. Quantification of total polyphenols. Folin-Ciocalteu method (Slinkard and singleton, 1977)

The total phenolic content of CAEE, CAME and CAAE was spectrometrically determined according to Folin Ciocalteu method. Gallic acid was used as a standard compound and the total phenols were expressed as mg/g gallic acid equivalent using the standard curve equation: y=0.029x – 0.148, R^2 = 0.990, where y is absorbance at 760 nm and x is the total phenolic content in the different extracts expressed in mg/g. the standard curve equation was determined from the gallic acid standard curve.

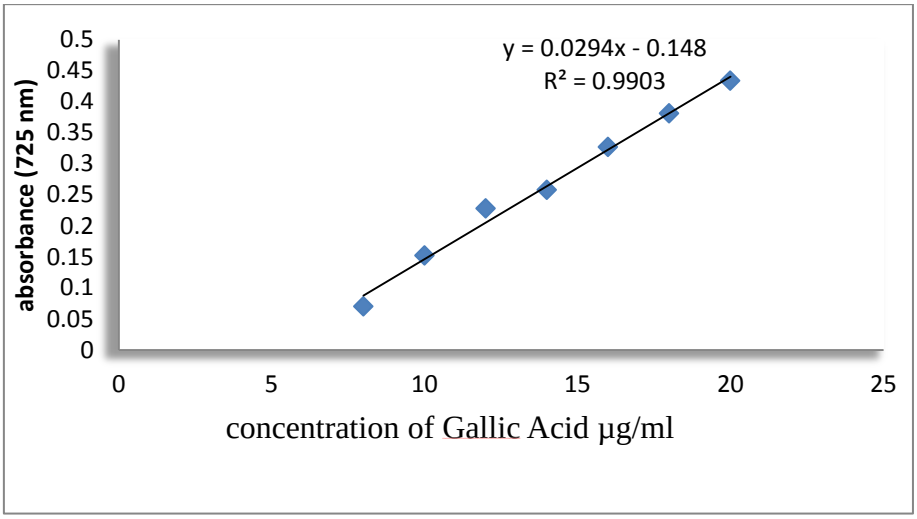


Fig. 4.2: Gallic acid standard curve.

Total Phenolic Content (TPC) of CAEE, CAME and CAAE were determined and are displayed in Table.4.3. TPC of the extracts were expressed in terms of mg GAE per gram of extract. The phenolic contents of the extracts ranged from 26.46 to 35.08 mg GAE/g dry extract). TPC of CAEE, CAME and CAAE are found to be 31.56, 35.08 and 26.46 (mg GAE/g dry extract). The result indicated that CAME contained highest TPC followed by CAEE and CAAE.

Table.4.3: Total Phenolic Content (TPC) of CAEE, CAME and CAAE.

Test sample	TPC (Total Phenolic Content) mgGAE/g of extract
CAEE	31.56±0.45
CAME	35.08±0.52
CAAE	26.46±0.55

Each value in the table is represented as Mean ± S.D. n=3. Showed a significant difference at $p<0.001$.

4.4. Antioxidant activity assessment

In the present work, the antioxidant activity of the CAEE, CAME and CAAE extracts were determined using four different assays: DPPH, reducing power assay (RPA), Ferric reducing/ antioxidant power (FRAP) assay and Nitric oxide radical scavenging assay (NRSA).

4.4.1. DPPH free radical scavenging assay

DPPH radical scavenging activities of CAEE, CAME and CAAE was investigated and the results are presented in Fig.4.3. CAME showed the highest DPPH radical scavenging activity, followed by CAEE, then CAAE. However none of the extracts were upto the level of the control ascorbic acid (AA). In the dose-response curve [Fig. 4.3(a)] the DPPH radical scavenging activity of all the extracts and the standard AA are compared. The graph demonstrated a clear significant increased in the percentage (%) inhibition of the extracts with the extract concentration. Fig.4.3(b) presents the IC_{50} values for DPPH radical scavenging activity of the extracts. The IC_{50} of the extracts, which is defined as the efficient concentration of antioxidant/extract necessary to decrease the initial DPPH radicals concentration by 50%, for DPPH scavenging were determined from the percent inhibition versus log (extract concentration) curve. For CAEE, CAME and CAAE, IC_{50} values are, 4.2µg/ml ($p<0.005$ significant against AA), 2.4µg/ml ($p<0.005$ significant against AA), 7.06µg/ml ($p<0.001$ significant against AA) respectively and the IC_{50} of positive control ascorbic acid is 0.85µg/ml. The lowest IC_{50} indicates the strongest ability of the extracts to act as DPPH radical scavengers. Out of the extracts CAME showed the lowest IC_{50}. Fig.4.3(c), (d), (e) and (f) are the percent inhibition versus log (extract concentration) curve.

4.4.2. Reducing power assay (RPA)

The reducing power of CAEE, CAME and CAAE, as a function of their concentration is shown in Fig.4.4. The reducing activity of the extracts of CA was compared with the reducing power of the standard ascorbic acid. Higher absorbance indicates higher reducing power. In the concentration range investigation of RPA, the reducing power of the extracts increased linearly with concentration. At 20, 40, 60, 80 and 100µg/ml, the reducing power of CAEE is 0.17, 0.39, 0.59, 0.82, 0.98 respectively; CAME is 0.25, 0.41, 0.65, 0.88, 1.01 respectively; CAAE is 0.13, 0.3, 0.48, 0.76, 0.87 respectively; and AA is 0.87; 0.26, 0.46, 0.71, 0.94, 1.2 respectively.

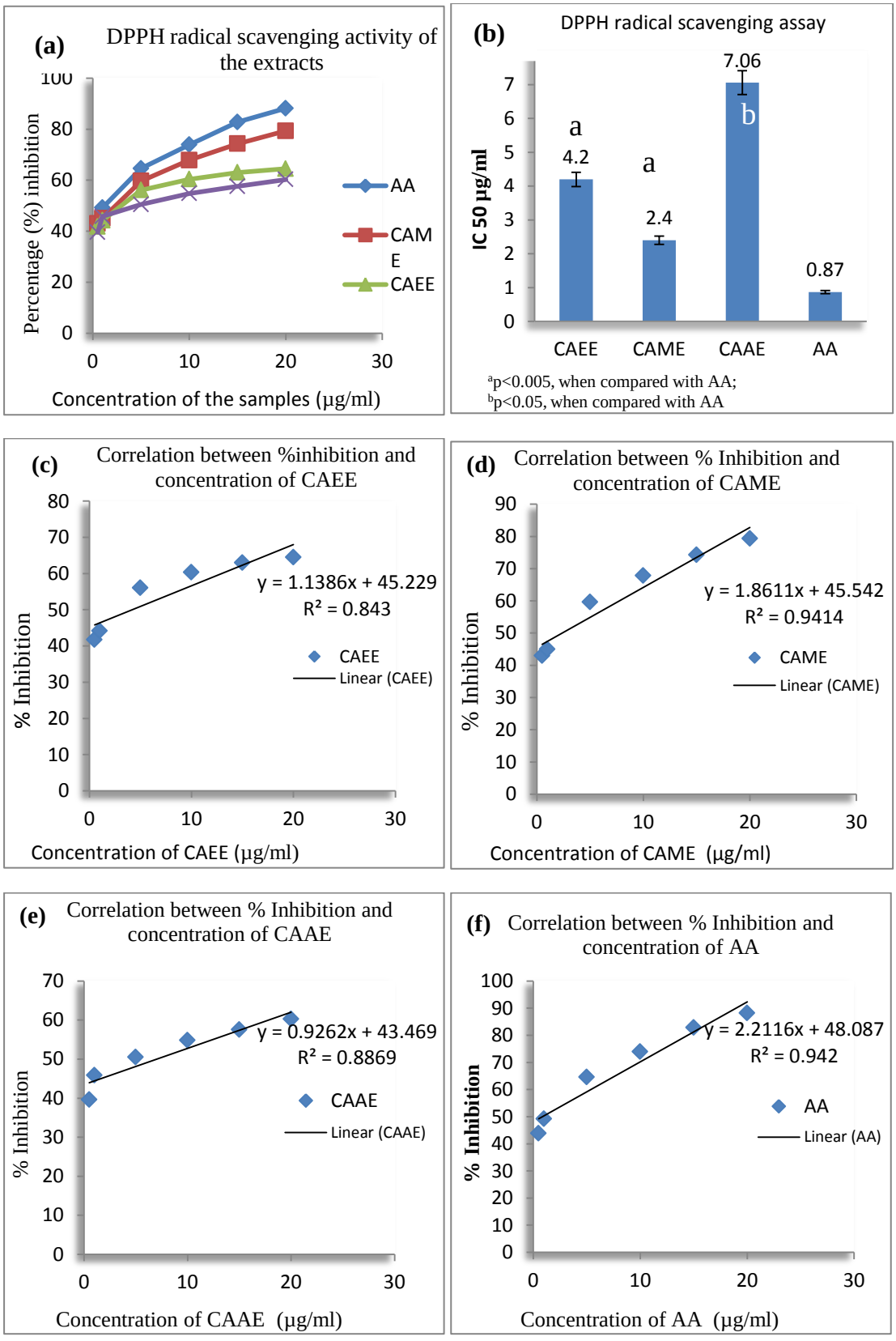
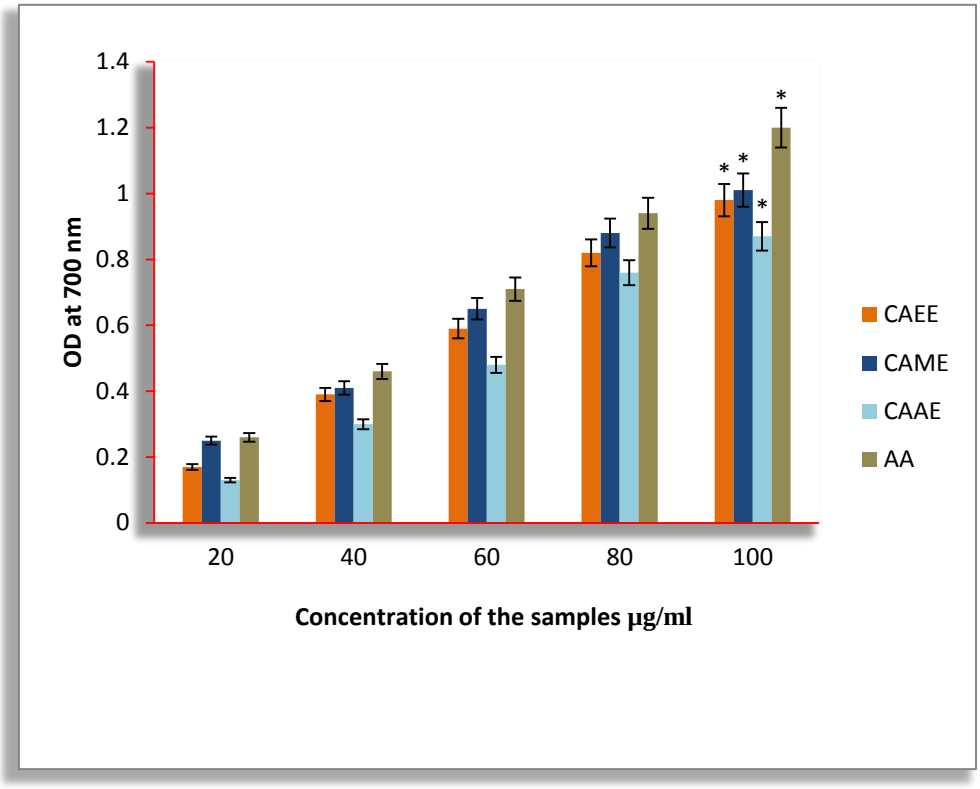


Fig. 4.3: (a) Graph showing DPPH scavenging activity of the extracts; (b) Graph showing IC_{50} for DPPH scavenging activity of the extracts; (c), (d), (e) and (f), Percent inhibition of extracts versus log (extract concentration) curve.

The reducing power was found to be in order of CAME > CAEE > CAAE. At 100µg/ml of sample concentration the reducing power of CAEE was greater than CAAE by 11.22% and is lower than ascorbic acid by 18.33%. The reducing power of reference compound (Ascorbic acid) was found to be higher than all the tested extracts.



*p<0.05 significantly higher than the corresponding initial lowest dose

Fig.4.4: Reducing Power Assay (RPA)

4.4.3. Ferric Reducing Antioxidant Power (FRAP) assay

The reducing ability of the extracts determined by FRAP method is summarized in Fig.4.6. The standard calibration curve (Fig.4.5) of $FeSO_4.7H_2O$ was plotted, which was a linear between 50 and 200µM, with y = 0.002x - 0.118 and R^2 = 0.992. FRAP value (x value in the equation) of CAEE, CAME and CAAE were calculated by substituting the y (in the equation) with the respective absorbance of the extracts. The FRAP value was expressed as mmol FeII/mg of extract sample. The FRAP values of the three extracts were: 22.38mmol FeII/mg of extract for CAEE, 25.87mmol FeII/mg of extract for CAME and 18.32mmol Fe II/mg of extract for CAAE. There is a statistical significant difference (at p<0.05) between the FRAP value of the extracts. The FRAP value of CAME is greater than CAEE by 13.49% and CAAE by 29.18%.

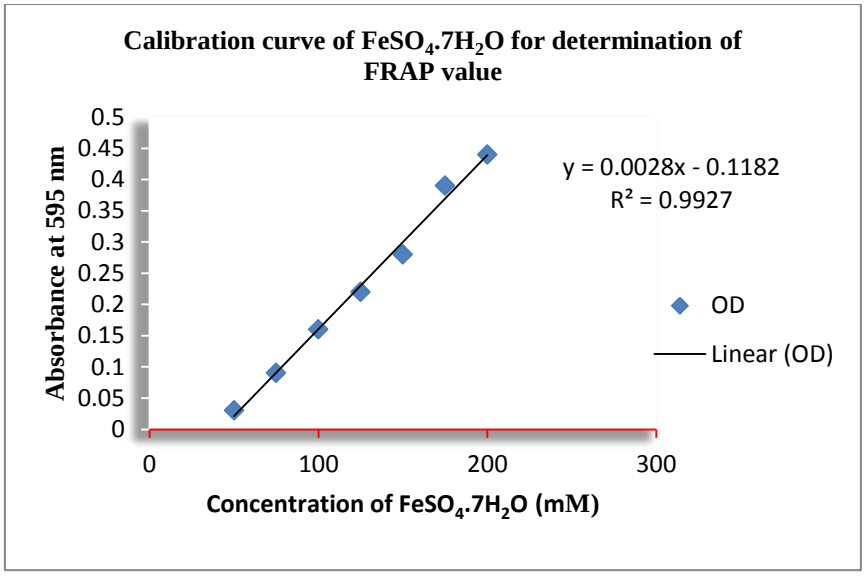
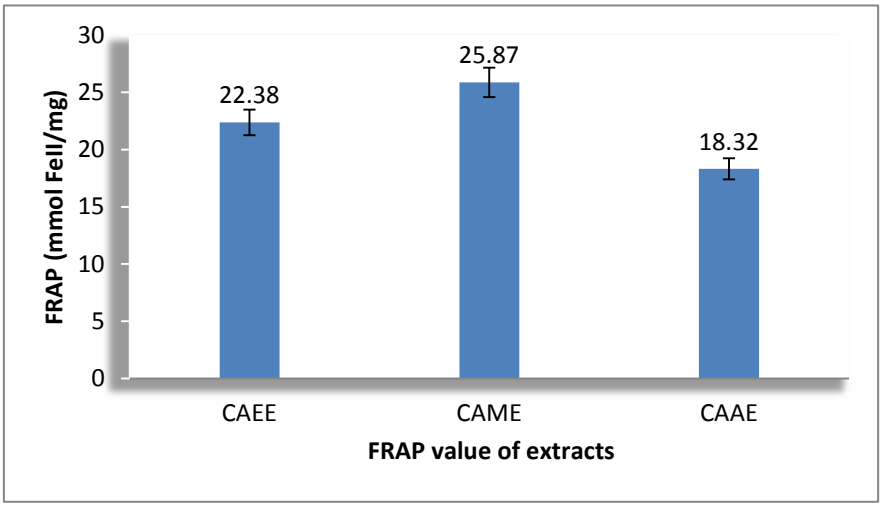


Fig.4.5: Calibration curve of $FeSO_4.7h_2O$ for determination of FRAP value

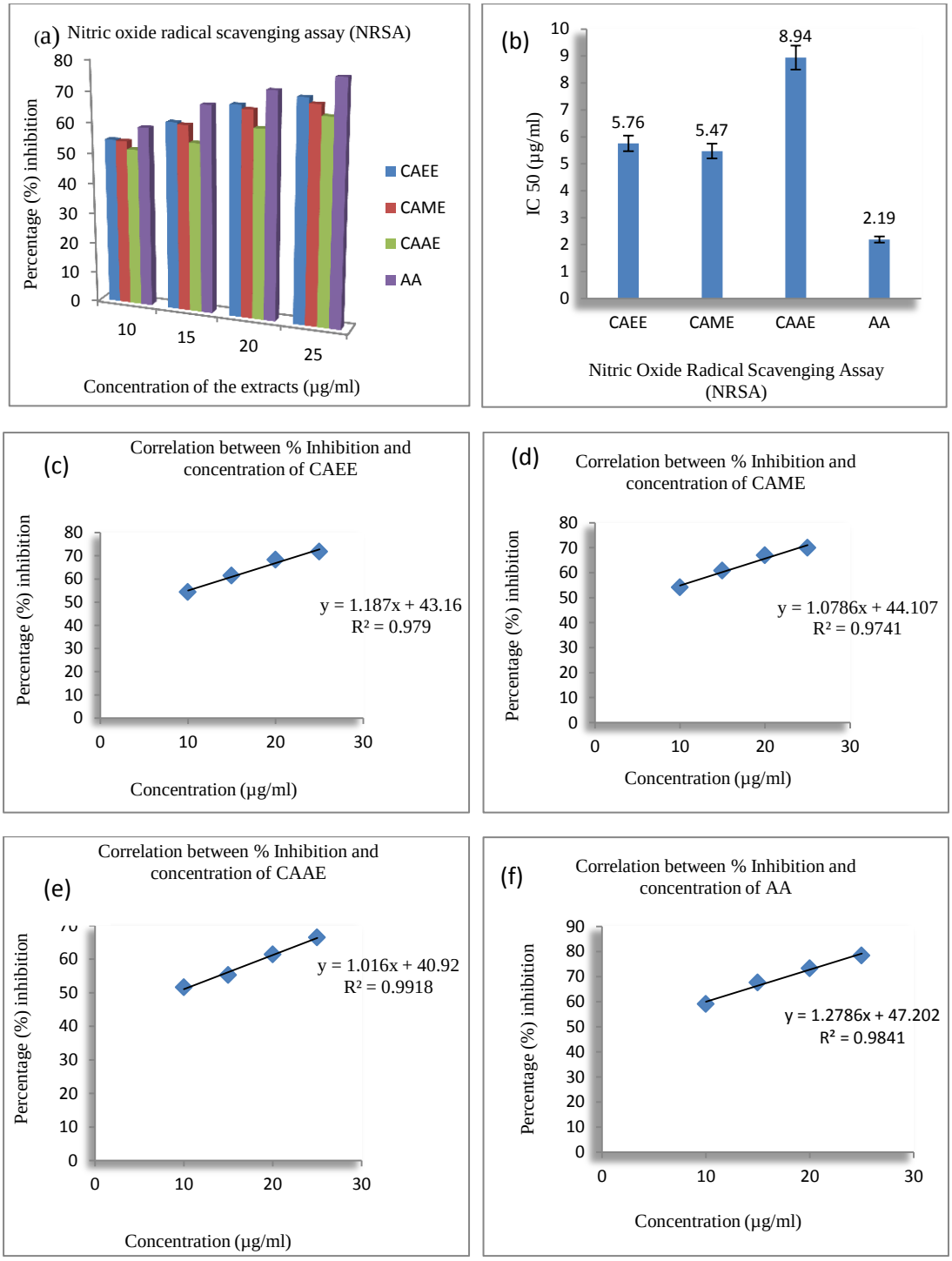


Extracts showed a significnt defference at p<0.05

Fig.4.6: FRAP value of CAEE, CAME and CAAE

4.4.4. Nitric oxide radical scavenging assay (NRSA)

The nitric oxide radical scavenging activity of the extracts is presented in Fig.4.7(a). CAME showed the higher nitric oxide radical scavenging activity then CAEE and CAAE but not so effective as AA. In Fig.4.7(a), the percent inhibition of the extracts, which corresponds to a range of concentration (10, 15, 20 and 25µg/ml) are compared with that of the standard. The percent inhibition of the samples increases linearly with concentration. The $IC_{50,}$ which is defined as the efficient concentration of antioxidant/extract necessary to decrease the initial NO radicals concentration by 50%, was determined from the percent inhibition versus log (extract concentration) curve and is illustrated in Fig.4.7(b).



a, b indicates $p<0.05$ and $p<0.01$ respectively when compared with AA.

Fig.4.7: (a) Graph showing NRSA activity of the extracts; (b) Graph showing IC_{50} for NRSA activity of the extracts; (c), (d), (e) and (f), Percent inhibition of extracts versus log (extract concentration) curve.

The IC_{50} value of CAME and CAEE are 5.47µg/ml and 5.76µg/ml respectively; which is significantly ($p<0.05$) different from IC_{50} of AA. IC_{50} of CAAE (8.94µg/ml) is significantly different from AA (2.19µg/ml) at $p<0.01$. Fig.4.7 (c), (d), (e) and (f)

are the percent inhibition versus log (extract concentration) curve of CAEE, CAME, CAAE and AA respectively.

4.4.5. Correlation between the DPPH activity, FRAP value, Reducing power activity, and NO radical scavenging activity with Total Phenolic Content of CAEE, CAME and CAAE.

The correlation between the antioxidant activity and phenolic content of CAEE, CAME and CAAE was demonstrated by linear regression analysis and is presented in Fig.4.8. The strongest correlation was observed between the DPPH radical scavenging activity and TPC of the extracts (R^2=0.999) [Fig.4.8(a)]. FRAP value and Reducing Power activities of the extracts are also highly correlated with TPC with R^2=0.996 and R^2=0.955 respectively. [Fig.4.8 (b) and (c)]. However NRSA and TPC of the extracts showed the lowest correlation (R^2=0.887) as compared to other assays [Fig.4.8 (d)].

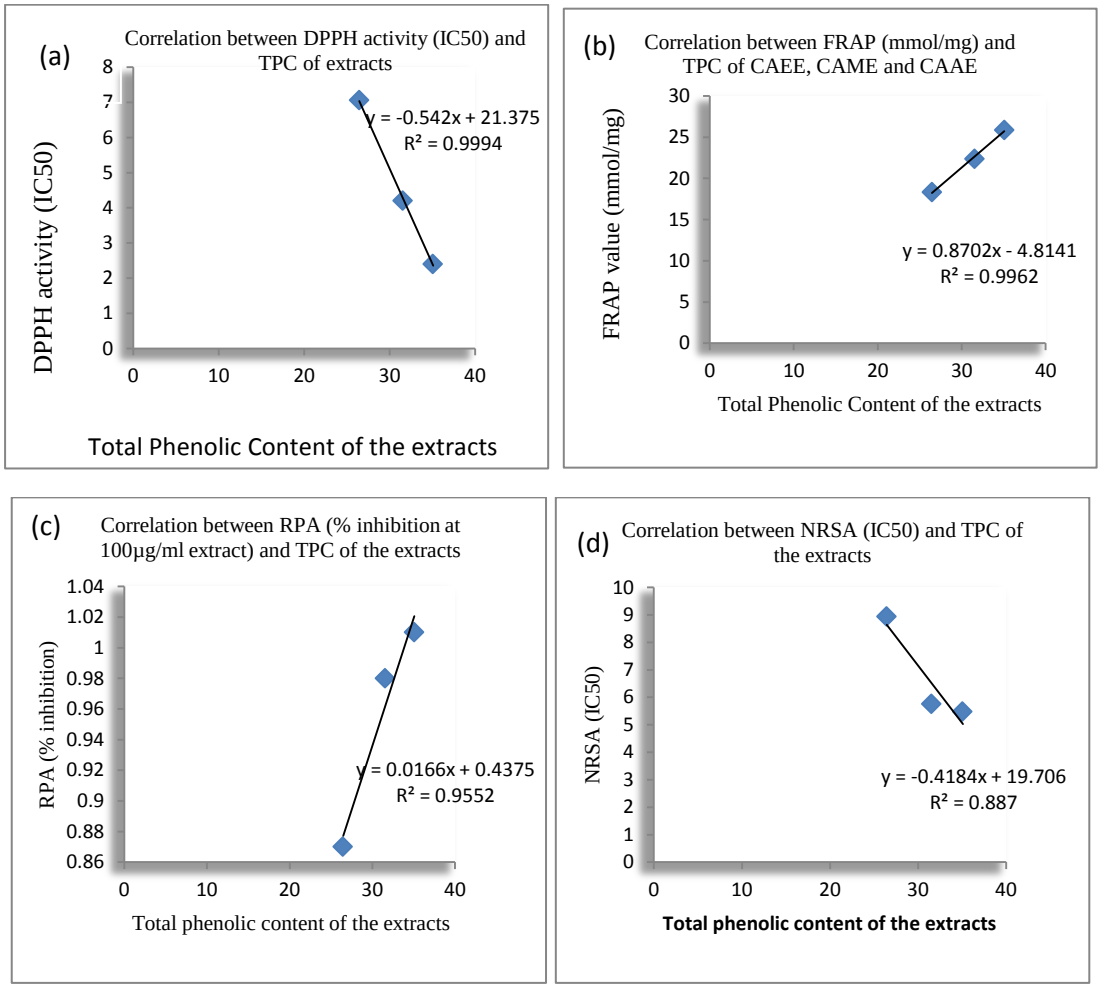


Fig.4.8: Graphs demonstrating correlation between the Total Phenolic Content (TPC) and DPPH activity [Fig.4.8(a)], FRAP value [Fig.4.8(b)], RPA [Fig.4.8(c)] and NRSA [Fig.4.8(d)].

4.5. In vitro antidiabetic activity assessment

The inhibition potential of CAEE, CAME and CAAE on carbohydrate digesting enzymes was investigated using two enzymes: α-amylase and α-glucosidase. Influence of different concentrations of CAEE, CAME, CAAE and positive control acarbose on α-amylase and α-glucosidase activity are presented in Table.4.4 and Table.4.5 respectively. The inhibitory potential is expressed in terms of % inhibition and $IC_{50.}$

4.5.1. Effect of CAEE, CAME and CAAE on α-amylase activity

The effect of CAEE, CAME and CAAE on α-amylase activity is summarized in Table: 4.4.

Table: 4.4. Influence of different concentrations of CAEE, CAME, CAAE and Acarbose on α-amylase activity expressed in terms of %inhibition and $IC_{50.}$

Test sample	Dose (µg/ml)	%Inhibition of α-Amylase	IC_{50} (µg/ml)	Test sample	%Inhibition of α-amylase	IC_{50} (µg/ml)
CAEE	100	43.53±0.81[a]		CAME	45.21±0.54[a]	
	200	46.74±0.79[a]			47.32±0.71[a]	
	300	51.67±0.89	262.62		52.58±0.92[a]	235.58
	400	56.10±0.35[a]			57.01±0.48[a]	
	500	59.86±0.90[a]			61.96±0.21[a]	
CAAE	100	39.29±0.71[b]		A	47.00±0.50	
	200	41.27±0.50[b]			49.41±0.39	
	300	45.70±0.80[a]	409.74		55.50±1.30	189.33
	400	48.40±0.56[b]			58.36±0.35	
	500	54.83±0.80[b]			65.20±1.06	

Each value were represented as mean ± S.D., n=3. [a]p<0.05 CAME, CAEE and CAAE compared with Acarbose of corresponding concentration. [b]p<0.01 CAME, CAEE and CAAE compared with Acarbose of corresponding concentration

At the concentration range of 100, 200, 300, 400 and 500µg/ml the extracts exhibited α-amylase inhibitory activity in a dose dependent manner. Corresponding to the above mentioned concentrations, the percentage (%) inhibition of α-amylase activity exhibited by CAEE is 43.53%, 46.74%, 51.67%, 56.10%, 59.86% respectively; CAME is 45.21%, 47.32%, 52.58, 57.01%, 61.96% respectively; and CAAE is 39.29%, 41.27%, 45.70%, 48.40%, 54.83%, respectively. The inhibitory activity of the extracts are compared with inhibitory activity of standard drug acarbose (A) which is 47.00%, 49.41%, 55.50%, 58.36% and 65.20%. Through linear regression analysis between doses of the test samples and percentage inhibition, linear equation was deduced (presented in Fig.4.9) from which the IC_{50} of each extract was calculated. IC_{50} value of CAEE, CAME, CAAE and acarbose are 262.62µg/ml, 235.58 µg/ml, 409.74 µg/ml and 189.33 µg/ml respectively.

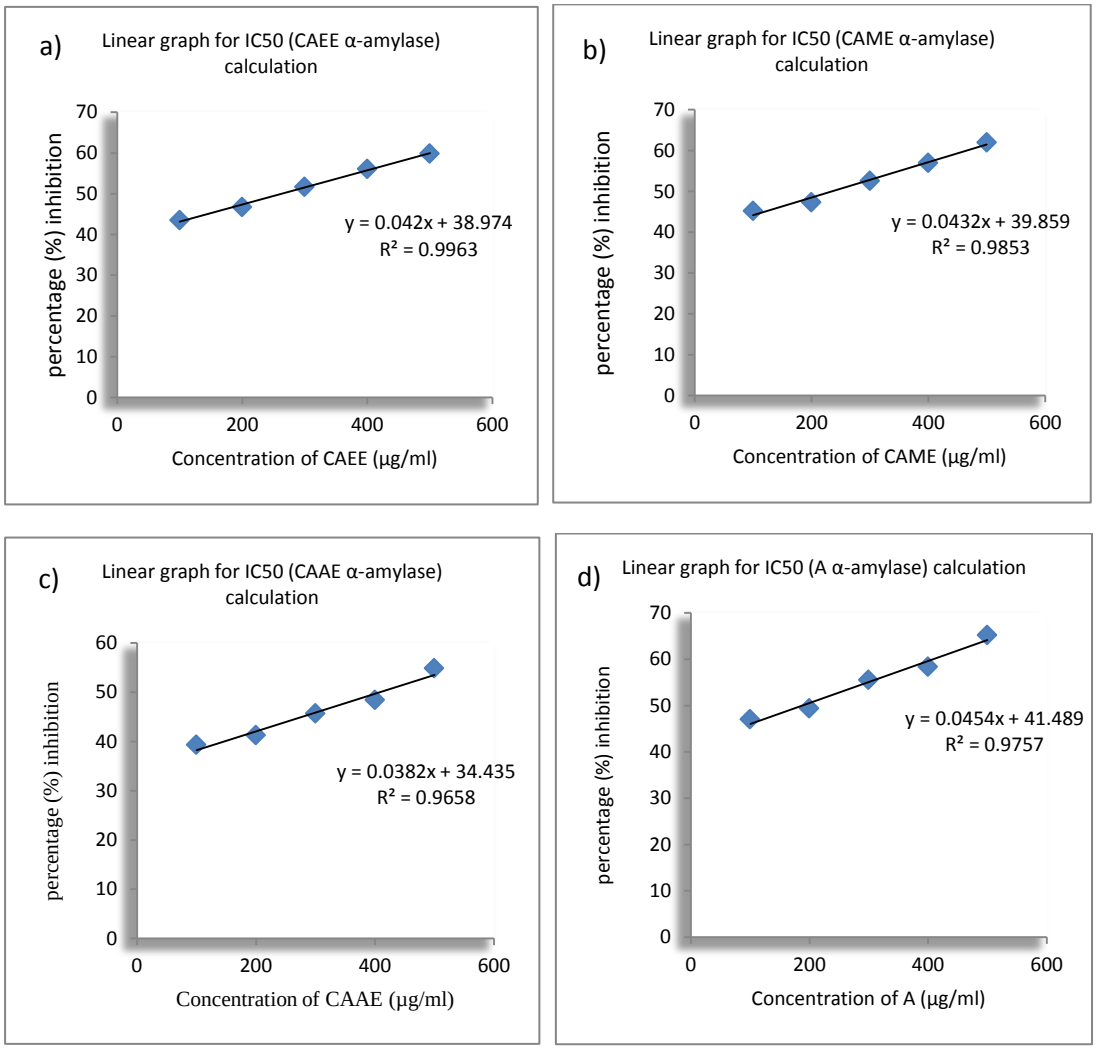


Fig.4.9: Linear regression graph for α-amylase IC_{50} calculation a) CAEE, b) CAME, c) CAAE and d) A.

4.5.2. Effect of CAEE, CAME and CAAE on α-glucosidase activity

The effect of CAEE, CAME and CAAE on α-glucosidase activity is summarized in Table.4.5.

Table.4.5. Influence of different concentrations of CAEE, CAME, CAAE and Acarbose on α-glucosidase activity expressed in terms of %inhibition and IC_{50}.

Test sample	Dose (µg/ml)	%Inhibition of α-glucosidase	IC_{50} (µg/ml)	Test sample	%Inhibition of α-glucosidase	IC_{50} (µg/ml)
CAEE	100	42.74±0.67[a]		CAME	46.38±0.56[b]	
	200	44.37±0.50[b]			49.15±0.27[b]	
	300	48.64±0.41[b]	313.5		54.55±0.33[a]	202.5
	400	53.63±0.36[a]			58.29±0.5[a]	
	500	58.20±0.30[a]			62.14±0.23[a]	
CAAE	100	38.55±0.61[b]		A	49.55±0.68	
	200	39.25±3.56[b]			52.27±0.46	
	300	44.37±0.47[b]	464.29		58.40±0.85	129.09
	400	47.63±0.37[b]			60.43±0.79	
	500	52.06±0.35[b]			67.71±0.96	

Each value were represented as mean ± S.D., n=3. [a]p<0.05 CAME, CAEE and CAAE compared with Acarbose of corresponding concentration. [b]p<0.01 CAME, CAEE and CAAE compared with Acarbose of corresponding concentration

At the concentration range of 100, 200, 300, 400 and 500µg/ml the extracts exhibited α-glucosidase inhibitory activity in a dose dependent manner. In correspond to the above mentioned concentrations of extracts, the percentage (%) inhibition of α-glucosidase activity exhibited by CAEE is 42.74%, 44.37%, 48.64%, 53.63%, 58.20%; CAME is 46.36%, 49.15%, 54.55%, 58.29, 62.14%; and CAAE is 38.55%, 39.25%, 44.37%, 47.63%, 52.06%. The inhibitory activity of the extracts are compared with inhibitory activity of standard drug acarbose (A) which is 49.55%,

52.27%, 58.40%, 60.43% and 67.71%. Through linear regression analysis between doses and percentage inhibition of the extracts, linear equation was deduced (presented in Fig.4.10) from which the IC_{50} of each extract was calculated. IC_{50} value of CAEE, CAME, CAAE and acarbose are 313.5µg/ml, 202.5 µg/ml, 464.29 µg/ml and 129.09 µg/ml respectively.

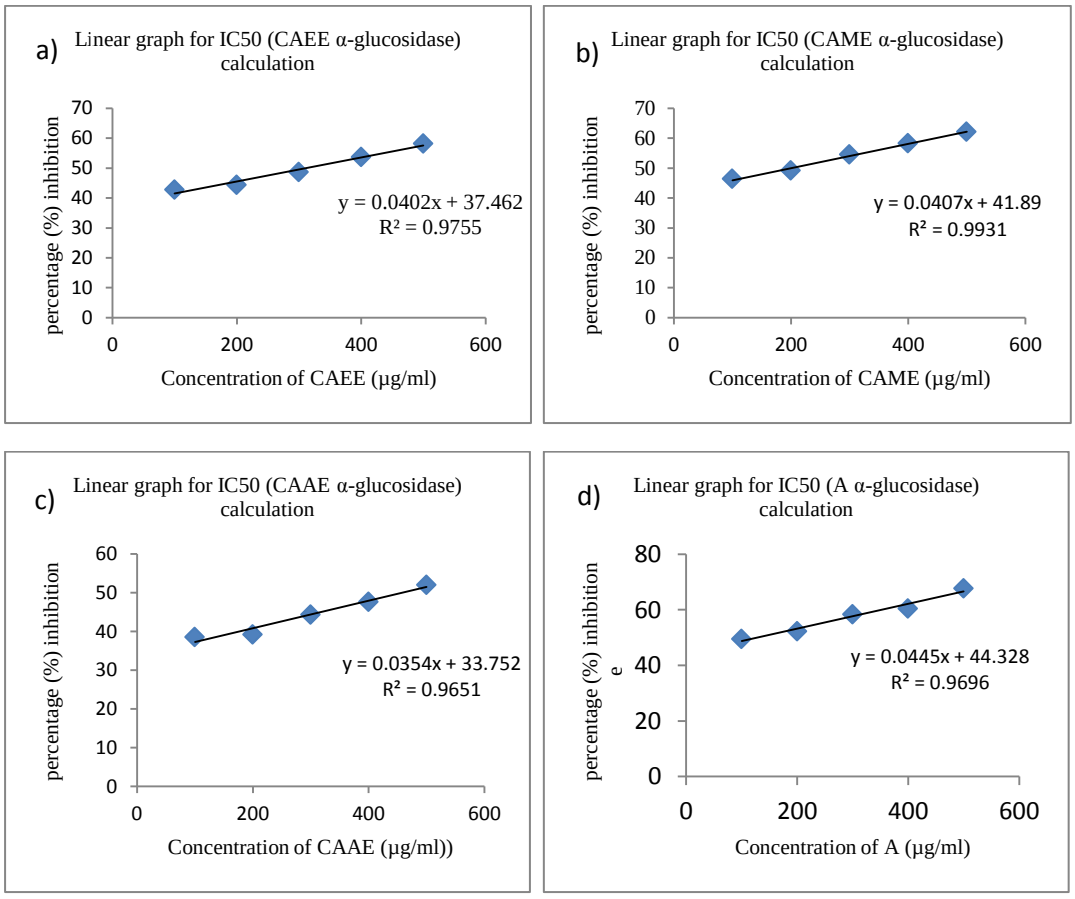


Fig.4.10: Linear regression graph for α-glucosidase IC_{50} calculation a) CAEE, b) CAME, c) CAAE and d) A.

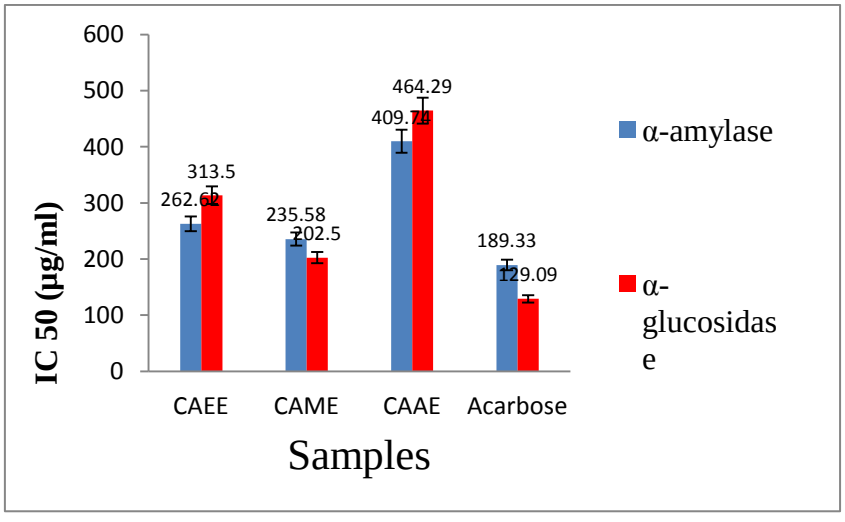


Fig.4.11: Graph of Inhibitory effect of extracts (IC_{50}) on α-amylase and α-glicosidase activity.

4.6. Acute oral toxicity test of the extracts

Extracts of CA did not produce any mortality and sign of lethality throughout the study period of 14 days even when the limit dose was maintained at 3000mg/kg body weight. There was no sign of tremors, convulsions, salivation, diarrhoea, lethargy, sudden or drastic decrease of body weight and coma. And also there were no changes in eyes, respiratory circulation, sleep, etc. Hence, testing the extracts at a higher dose may not be necessary and the extracts were non-toxic. There was no drastic decrease of body weight of the mice. The percentage change in body weight of the mice is presented in Fig.4.12.

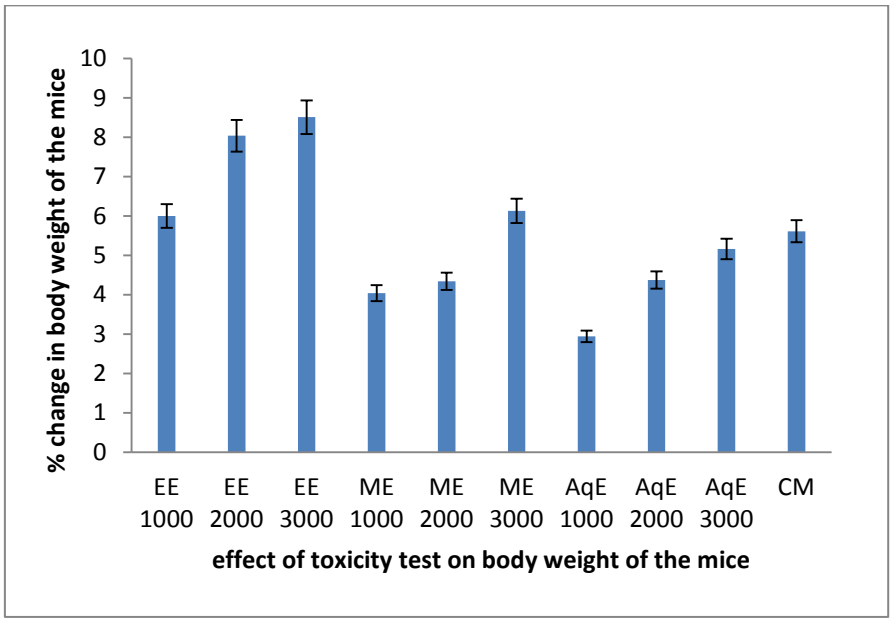


Fig.4.12: Effect of acute oral toxicity test of extracts on body weight of mice.

4.7. Oral Glucose Tolerance Test (OGTT)

Oral Glucose tolerance test of the extracts was performed in two groups, in normal group and STZ induced diabetic group.

4.7.1. Effect of CAEE, CAME and CAAE on OGTT in normal mice

The effect of CAEE, CAME and CAAE on OGTT in normal mice group was assessed at different time intervals and is depicted in Table.4.6. The blood glucose level at 0min, 30min, 90min and 120min were compared with the initial baseline blood glucose level of their respective groups. The BGL after the glucose load reached a peak at 30min and decreased subsequently over time, in all the groups of mice. The percentage change in BGL at 30min, 60min, 90min and 120min, from initial/baseline BGL (0min) are – 91.42%, 60.87%, 46.72% and 40.85% respectively in NC mice;

84.85%, 53.94%, 42.11% and 21.90% respectively in GTNM; 85.33%, 55.51%, 47.44% and 39.17% respectively in EETNM; 87.56%69.02%, 49.53% and 27.20% respectively in METNM; 88.66%, 71.89%, 50.34% and 43.28% respectively in AqETNM.

Table.4.6: Effect of extracts on oral glucose tolerance test in normal group mice.

Groups	BGL mg/dL at various time intervals in OGTT (normal group)				
	0min	30min	60min	90min	120min
NC	80.56±1.62	154.21±1.23	129.6±2	118.2±1.7	113.47±2.3
GTNM	86.2±1.9	159.34±2.01	132.7±1.8	122.5±2.1	105.08±2.4
EETNM	84.31±1.72	156.25±1.77	131.11±1.8	124.31±2.7	117.33±2.07
METNM	86.41±2.02	162.07±2.14	146.05±2.6	129.21±2.5	109.91±2.8
AqETNM	82.9±1.53	156.4±2.53	142.5±2.46	124.63±2.74	117.95±3.2

NC, normal control; GTNM, glibenclamide treated normal mice; EETNM, ethanol extract treated normal mice; METNM, methanol extract treated normal mice; AqETNM, Aqueous extract treated normal mice.

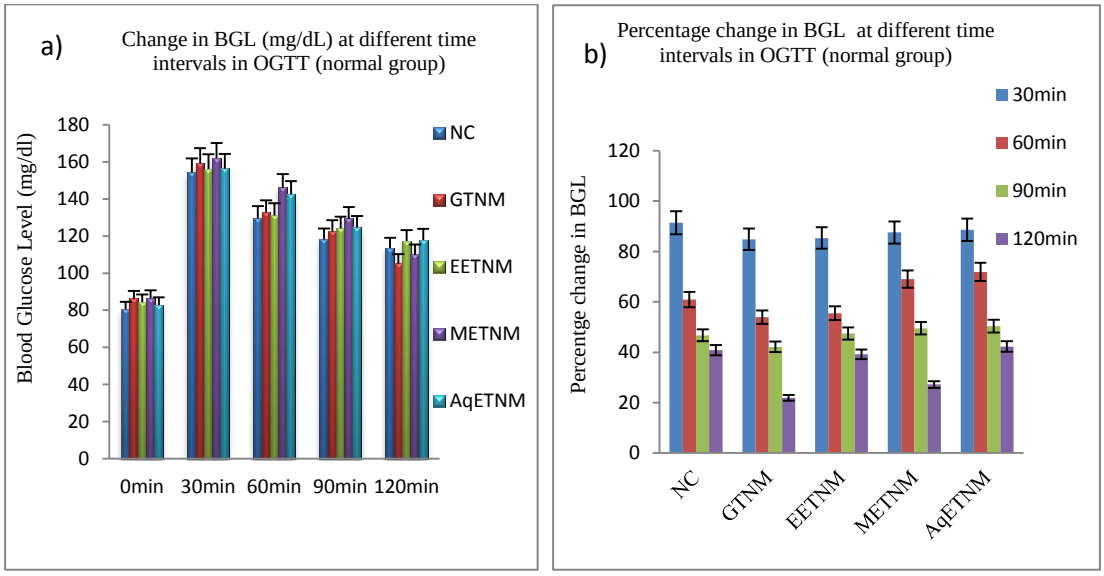


Fig.4.13: Effect of extracts on OGTT in normal group mice. a) Change in BGL (mg/dL) at different time intervals, b) Percentage change in BGL at different time intervals.

4.7.2. Effect of CAEE, CAME and CAAE on OGTT in STZ induced diabetic mice

The effect of CAEE, CAME and CAAE on OGTT in STZ induced diabetic mice was measured at different time intervals and is depicted in Table.4.7.

Table.4.7: Effect of extracts on oral glucose tolerance test in STZ induced diabetic mice group.

Groups	BGL mg/dL at various time intervals in OGTT (Diabetic group)				
	0min	30min	60min	90min	120min
DC	204.81±2.5	341.02±2.7	322.44±2.87	311.46±3.66	299.79±3.25
GTDM	225.06±1.81	346.27±1.99	253.75±2.6	218.43±3.1	186.93±3.7
EETDM	214.37±2.2	328.01±2.54	284.17±3.3	249.43±2.98	227.05±3.1
METDM	217.53±2.7	330.82±3.1	276.48±3.8	232.78±3.77	211.84±3.33
AqETDM	221.46±1.3	341.56±2.43	289.35±3.87	245.32±4.1	220.73±4.05

NC, normal control; GTNM, glibenclamide treated normal mice; EETNM, ethanol extract treated normal mice; METNM, methanol extract treated normal mice; AqETNM, Aqueous extract treated normal mice.

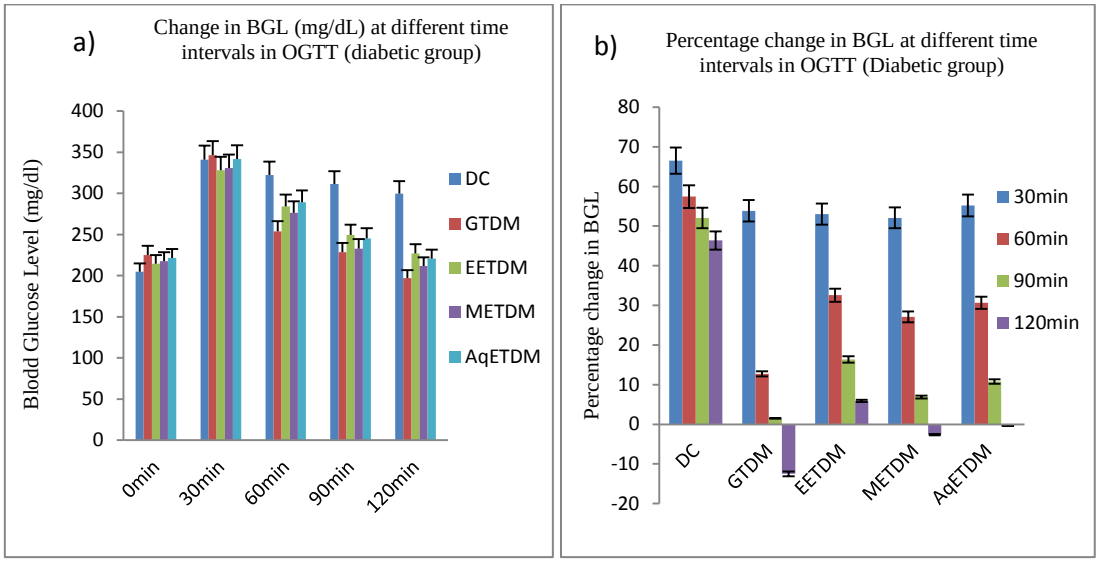


Fig.4.14: Effect of extracts on OGTT in diabetic group mice. a) Change in BGL (mg/dL) at different time intervals, b) Percentage change in BGL at different time intervals.

The blood glucose level at 30min, 60min, 90min and 120min were compared with the initial baseline (0min) blood glucose level of their respective groups. The BGL after the glucose load reached a peak at 30min and decreased subsequently over time, in all the groups of mice. The percentage change in BGL at 30min, 30min, 90min and 120min, from initial/baseline BGL (0min) are – 66.51%, 57.43%, 52.07% and 46.38% respectively in DC mice; 53.51%, 12.75%, 1.50% and -12.50% respectively in

GTDM; 53.01%, 32.56%, 16.36% and 5.92% respectively in EETDM; 52.08%, 27.10%, 6.87% and -2.62% respectively in METDM; 55.21%, 30.65%, 10.77% and -0.33 respectively in AqETDM.

4.8. In-vivo antidiabetic activity assessment of the extracts

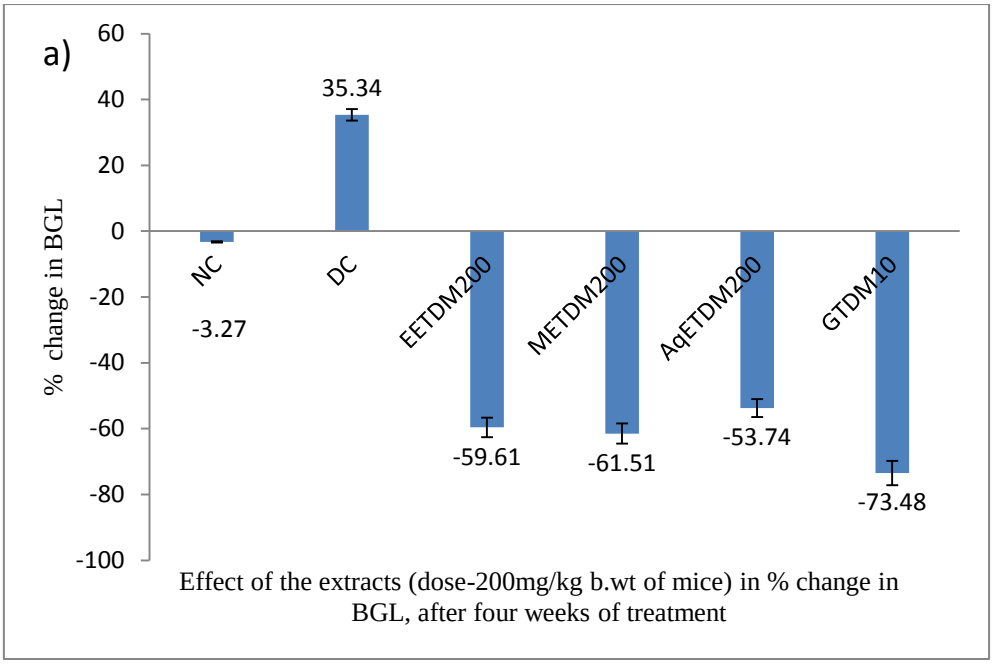
4.8.1. In vivo antidiabetic effect of extracts on fasting BGL in STZ induced diabetic mice

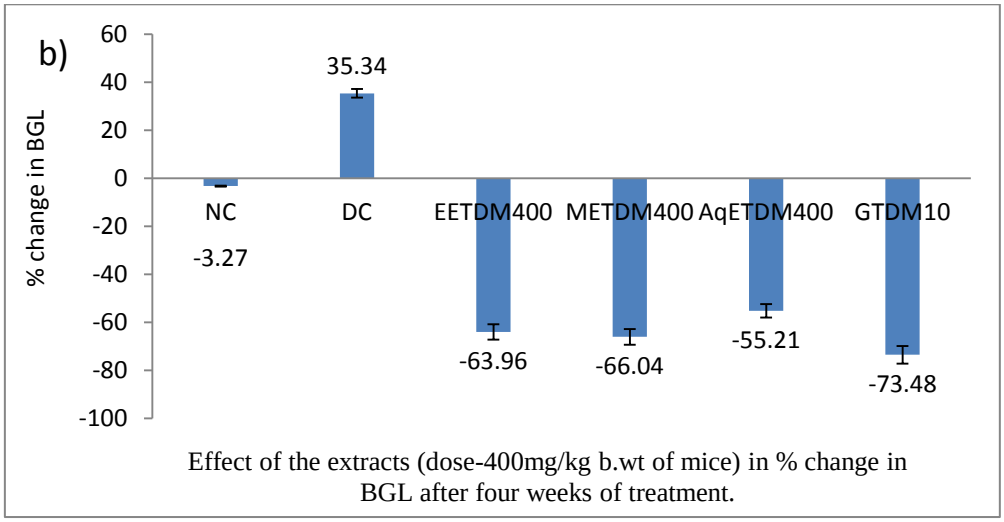
The effect of the extracts on fasting BGL in STZ induced diabeteic mice during treatment is presented in Table.4.8.

Table.4.8: *In vivo* antidiabetic effect of extracts on fasting BGL in STZ induced diabetic mice after 28 days of treatment.

Groups	Dose (mg/kg)	Blood glucose (mg/dL)				
		0 day	day 7	Day 14	Day 21	Day 28
NC	-	62.01±6.90	65.42±7.19	63.72±5.65	64±9.78	63.28±8.91
DC	-	58.31±7.97	230.56±10.18***	256.02±11.12***	291.8±11.13***	312.04±7.87***
CAEE	200	59.39±5.00	255.44±9.22	193.35±8.49^{a}	148.06±9.30^{a}	103.16±8.05^{a}
	400	60.16±5.18	251.64±8.46	180.81±10.18^{a,ee}	136.46±6.30^{a,ee}	90.68±6.76^{a,e}
CAME	200	61.58±6.43	244.51±5.86	170.38±6.57^{b,eee}	129.5±6.92^{b,ee}	94.11±7.34^{b,e}
	400	58.02±5.03	271.95±6.48	172.91±5.93^{b}	118.64±6.47^{b}	92.35±7.33^{b}
CAAE	200	56.71±6.64	238.71±7.13	202.91±8.26^{c}	141.89±7.97^{c}	110.41±6.96^{c}
	400	59.72±4.66	237.33±5.98	199.28±7.03^{c}	139.10±8.88^{c}	106.29±6.95^{c}
GTDM	10	61.26±7.92	261.04±9.67	163.71±9.57^{d}	99.51±7.83^{d}	69.23±7.33^{d}

Each value was expressed as mean ±SD (n=6). ***p<0.001, DC compared with control; ^{a}p<0.001, CAEE 200, 400 mg/kg dose compared with DC; ^{b}p<0.001 CAME 200, 400 mg/kg dose compared with DC; ^{c}p<0.001 CAME 200, 400 mg/kg dose compared with DC ^{d}p<0.001, glibenclamide 10 mg/kg dose compared with DC; ^{e}p<0.001, ^{ee}p<0.01, ^{eee}p<0.05 glibenclamide 10mg/kg dose compared with CAEE400 mg/kg dose.





NC, normal control; DC, diabetic control; EETDM, Ethanol Extract Treated Diabetic Mice; Methanol Extract Treated Diabetic Mice; AqETDM, Aqueous Extract Treated Diabetic Mice; GTDM, Glibenclamide Treated Diabetic Mice.

Fig: 4.15. Percentage (%) change in BGL in NC, DC, EETDM, METDM, AqETDM and GTDM. a). Effect of the extracts (dose-200mg/kg b.wt of mice) in % change in BGL, after four weeks of treatment, b). Effect of the extracts (dose-400mg/kg b.wt of mice) in % change in BGL after four weeks of treatment.

Administration of STZ in mice significantly ($P<0.001$) increased the blood glucose level compared to normal control mice (Table.4.8). The effect of oral administration of 200 and 400 mg/kg dose of CAME, CAEE and CAAE on blood glucose levels in STZ-diabetic mice are showed in Table.4.8. Percentage (%) change in BGL of each group was calculated from initial BGL (day 7, before extract/Glibenclamide treatment) and final BGL (day 28, after treatment). A significant ($P< 0.001$) reduction of blood glucose levels was observed in CAME [for 200 mg/kg dose, 61.51%

reduction (% change in BGL is – 61.51%)]; for 400 mg/kg dose, 66.04% reduction [% change in BGL is – 66.04%)], CAEE [for 200 mg/kg dose, 59.62% reduction ((% change in BGL is – 59.62%); for 400 mg/kg dose, 63.96% reduction (% change in BGL is – 63.96%), CAAE [for 200mg/kg dose, 53.74% reduction (% change in BGL is - 53.74%); for 400 mg/kg dose, 55.21% reduction (% change in BGL is – 55.21%)] and standard drug glibenclamide [10mg/kg dose, 73.48% reduction (% change in BGL is – 73.48%)] treated mice, where effectiveness depends on dose and duration of treatment. The maximum reduction was noticed on 28th day.

4.8.2. Lipid profile and total protein changes in serum showing the effect of extracts

As shown in Table.4.9, the increased serum levels of TG and TC were significantly suppressed, while the decreased serum HDL levels were significantly improved after treatment with CAME, CAEE, CAAE and glibenclamide at $^{a}P<0.001$, $^{b}p<0.001$ and $^{c}p<0.001$ respectively when compared with diabetic control.

Table.4.9: Lipid profile and total protein changes in serum showing the effect of extracts.

Groups	Dose (mg/kg)	TC	TG	HDL	Total protein
NC	-	74.05±3.87	87.95±2.90	64.92±3.53	8.36±4.47
DC	-	128.78±2.28	137.48±3.96	40.05±2.10	5.07±2.30
EETDM	200	82.47±2.45^{a}	94.93±4.40^{a}	53.74±3.54^{a}	7.45±5.36^{a}
	400	79.21±4.01^{a}	91.65±3.17^{a}	55.30±4.93^{a}	7.72±4.40^{a}
METDM	200	78.37±3.76^{a}	95.42±1.68^{a}	55.02±3.33^{a}	7.55±2.11^{a}
	400	76.85±2.04^{a}	89.77±4.31^{a}	56.48±4.04^{a}	8.01±3.28^{a}
AqETDM	200	89.02±4.65^{b}	99.72±3.49^{b}	49.00±1.65^{b}	6.40±3.46^{b}
	400	85.89±4.04^{b}	98.24±1.64^{b}	51.75±2.64^{b}	6.88±3.29^{b}
GTDM	10	75.55±4.11^{c}	88.05±2.27^{c}	57.48±4.44^{c}	8.05±4.35^{c}

All data are expressed as mean ± SD (n=6). Each value is expressed as mean±SD. $^{a}p<0.001$, CAEE 200 and 400 mg/kg dose compared with DC. $^{b}p<0.001$, CAAE 200 and 400 mg/kg dose compared with DC. $^{c}p<0.001$, glibenclamide 10mg/kg dose compared with DC. TC, Total Cholesterol. TG, Tri-Glycerides. HDL, High Density Lipoprotein. NC, Normal Control; DC, Dibetic Control; METDM, methanol extract treated diabetic mice; EETDM, Ethanol Extract Treated Diabetic Mice; METDM, Methanol Extract Treated Diabetic Mice; AqETDM, Aqueous Extract Treated Diabetic Mice; GTDM, Glibenclamide Treated Diabetic Mice.

In diabetic mice treated with CAEE 200mg/kg TC level was decrease by 35.96%, TG level was decrease by 30.95%, HDL was elevated by 34.18%, total protein was increased by 46.94% when compared with the diabetic control mice. In mice treated with CAEE400mg/kg TC level was decrease by 38.49%, TG level was decrease by 33.34%, HDL was elevated by 38.08%, total protein was increased by 52.27% when compared with the diabetic control mice. In mice treated with CAME 200mg/kg TC level was decrease by 39.14%, TG level was decrease by 30.59%, HDL was elevated by 34.21%, total protein was increased by 48.92% when compared with the diabetic control mice. In mice treated with CAME 400mg/kg TC level was decrease by 40.33%, TG level was decrease by 33.34%, HDL was elevated by 37.38%, total protein was increased by 52.29% when compared with the diabetic control mice. In mice treated with CAAE 200mg/kg TC level was decrease by 30.87%, TG level was decrease by 27.47%, HDL was elevated by 22.35%, total protein was increased by26.23% when compared with the diabetic control mice. In mice treated with CAAE 400mg/kg TC level was decrease by 33.31%, TG level was decrease by 28.54%, HDL was elevated by 29.21%, total protein was increased by 35.7% when compared with the diabetic control mice. In mice treated with glibenclamide TC level was decrease by 1.33%, TG level was decrease by 35.94%, HDL was elevated by 43.52%, total protein was increased by 58.78% when compared with the diabetic control mice.

4.8.3. Changes in SGOT and SGPT on serum and Hepatic glycogen in the liver tissue homogenate of normal and diabetic mice after 28 days of treatment with CAME, CAEE and CAAE

Table.4.10. illustrated the effect of *Cassia alata* on SGOT, SGPT, GST and hepatic glycogen. The activities of SGOT and SGPT increases significantly ($p<0.001$) in diabetic mice as compared to normal control. Treatment with both the doses (200, 400 mg/kg) of CAME, CAEE, CAAE and glibenclamide (10 mg.kg dose) in diabetic mice decreases the SGOT and SGPT activities significantly ($p<0.001$) as compared to diabetic control mice. The level of SGOT was decreased by 34.45%, 39.28%, 23.01% and 44.62%, in CAEE (200mg/kg dose), CAME (200mg/kg dose), CAAE (200mg/kg dose) and glibenclamide treated mice respectively when compared with diabetic control. The level of SGPT was decreased by 35.00%, 36.33%, 19.40% and 38.93%, in CAEE (200mg/kg dose), CAME (200mg/kg dose), CAAE (200mg/kg dose) and glibenclamide treated mice respectively when compared with diabetic control. The

level of hepatic glycogen was increased by 101.35%, 124.19%, 94.59% and 136.04% in CAEE (200mg/kg dose), CAME (200mg/kg dose), CAAE (200mg/kg dose) and glibenclamide treated mice respectively, when compared with diabetic control.

Table: 4.10. Changes in SGOT and SGPT on serum and Hepatic glycogen in the liver tissue homogenate of normal and diabetic mice after 28 days of treatment with CAEE and CAAE.

Groups	Dose (mg/kg)	SGOT (U/dL)	SGPT (U/dL)	HG (mg/g of wet tissue)
NC	-	37.63±2.68	52.98±2.01	40.64±2.72
DC	-	70.85±3.17***	87.99±3.12***	14.934±1.56***
EETDM	200	46.44±2.81[a]	57.19±3.19[a]	30.07±1.43[a]
	400	44.68±2.49[a]	55.60±3.72[a]	33.67±2.07[a]
METDM	200	43.02±3.24[a]	56.02±2.43[a]	33.48±1.92[a]
	400	40.14±1.89[a]	54.00±2.7[a]	34.13±3.54[a]
AqETDM	200	54.55±3.97[b]	70.92±4.49[b]	29.06±1.67[b]
	400	50.35±2.75[b]	66.11±4.28[b]	31.29±2.15[b]
GTDM	10	39.24±2.6[c]	53.74±3.49[c]	35.25±1.70[c]

Each value is expressed as mean±SD. ***p<0.001, DC compared with NC; [a]p<0.001, CAME, CAEE 200 and 400 mg/kg dose compared with DC; [b]p<0.001, CAAE 200 and 400 mg/kg dose compared with DC; [c]p<0.001, glibenclamide 10mg/kg dose compared with DC. SGOT, Serum Glutamic Oxaloacetic Transaminase. SGPT, Serum Glutamic Pyruvic Transaminase.
NC, Normal Control; DC, Dibetic Control; METDM, methanol extract treated diabetic mice; EETDM, Ethanol Extract Treated Diabetic Mice; METDM, Methanol Extract Treated Diabetic Mice; AqETDM, Aqueous Extract Treated Diabetic Mice; GTDM, Glibenclamide Treated Diabetic Mice.

The level of SGOT was decreased by 36.94%, 43.35% and 28.93%, in CAEE (400mg/kg dose), CAME (400mg/kg dose) and CAAE (400mg/kg dose) treated mice respectively, when compared with diabetic control. The level of SGPT was decreased by 36.81%, 38.63% and 24.87%, in CAEE (400mg/kg dose), CAME (400mg/kg dose) and CAAE (400mg/kg dose) treated mice respectively, when compared with diabetic control. The level of hepatic glycogen was increased by 125.46%, 128.54% and 109.52%, in CAEE (400mg/kg dose), CAME (400mg/kg dose) and CAAE (400mg/kg dose) treated mice respectively, when compared with diabetic control. Hepatic glycogen content in liver decreases significantly ($p<0.001$) in diabetic mice compared to normal control. Treatment of diabetic mice with CAME, CAEE, CAAE and glibenclamide increases the glycogen content significantly, $^{a}p<0.001$, $^{b}p<0.001$, $^{c}p<0.001$ and $^{d}p<0.001$ respectively, as compared to diabetic control.

4.8.4. Histological studies

Haematoxylin and eosin stained pancreatic sections of normal mice showed Langerhans Islets (LI) with normal cellular structure which are distinctively surrounded by the pancreatic acini (PA). The islets appeared lightly stained than the surrounding acinar cells (Fig: 4.16b). Langerhans Islets were almost completely destroyed leaving empty space in diabetic mice (Fig. 4.16a). In glibenclamide, CAME, CAEE and CAAE treated mice the Langerhans Islets maintained regular structure almost similar to the normal (Fig.4.16c, d, e and f).

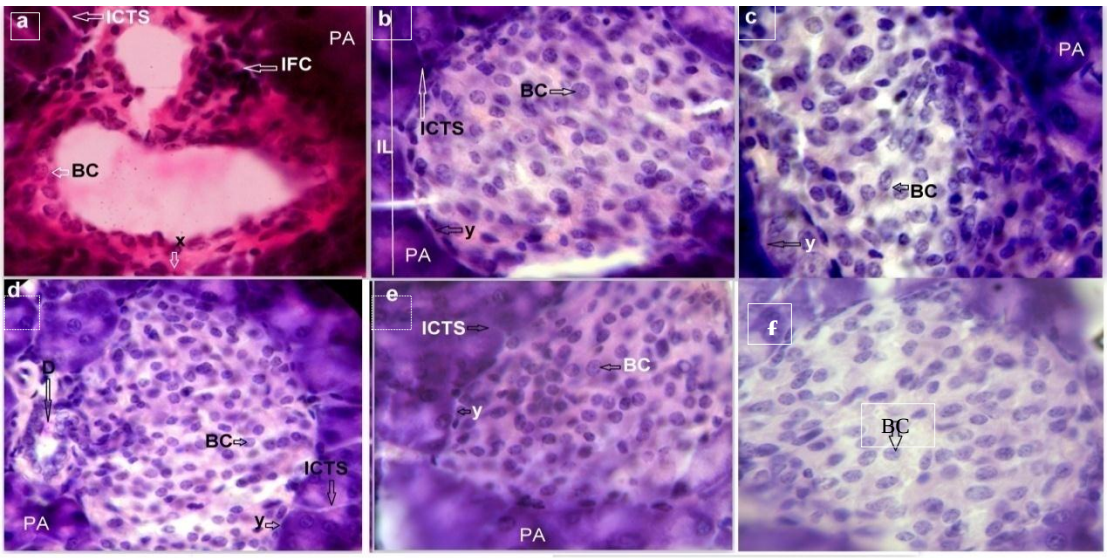


Fig..4.16: Micrograph of mice pancreatic tissue of [a]. Diabetic mice, [b]. Control mice showing normal lobular architecture, [c]. Glibenclamide treated mice revealed restoration of the normal architecture, [d], [e] and [f]. CAME, CAEE and CAAE treated mice respectively, showing normal cellular structure of the IL. Duct (D). Islets of Langerhans (IL), pancreatic acini (PA), Beta cells (BC).

Examined liver sections of normal control mice showed regular architecture of cellular structure. Hepatocytes are located in rows that radiate out from the central vein (CV). Microscopic blood channels called liver sinusoids (S) are seen in between the hepatocyte rows. Kupffer cells are seen in the linings of the liver sinusoids (Fig.4.17b). In liver sections of diabetic mice, infiltrations of inflammatory cells through hepatic tissue are seen indicating injury of the liver tissue. Normal radiating forms of the hepatocytes are seemed to be distorted (Fig.4.17a). Examined sections of treated mice showed the CA's ability to reduce cell necrosis and helped in retaining the regular form of cellular arrangement around the central vein (Fig.4.17c, d, e and f).

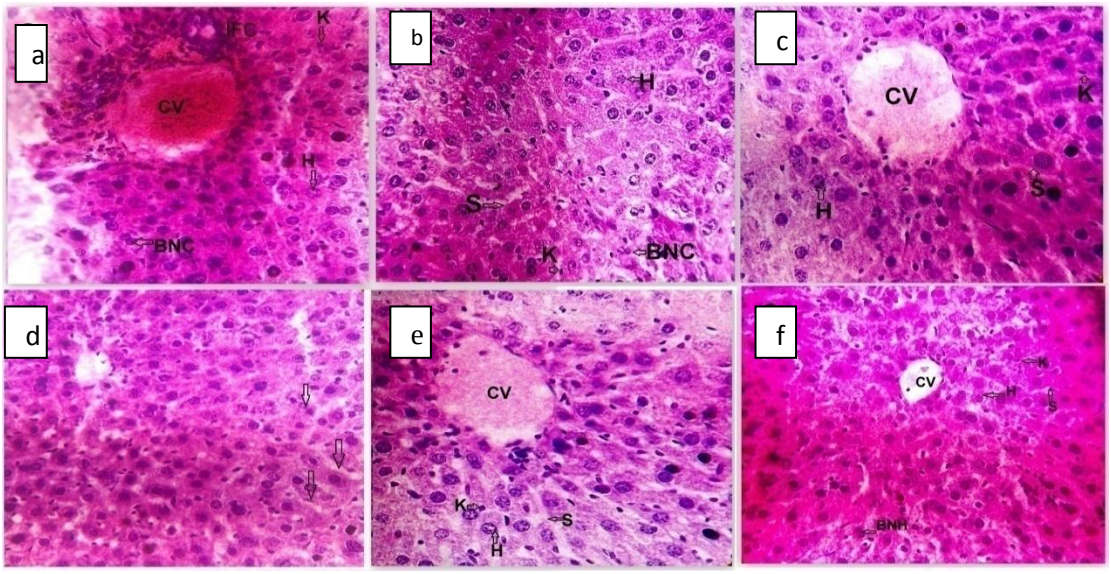


Fig.4.17: Micrograph of liver sections of [a]. Diabetic mice showing necrosis of liver, [b]. Control mice showing normal radiating cords of hepatocytes, [c][d][e] and [f]. Glibenclamide, CAME, CAEE and CAAE treated mice respectively revealing normal architecture of cells. Central vein (CV), hepatocytes (H), binucleated hepatocytes (BNH), sinusoids (S), kupffer cells (K), infiltrating inflammatory cells (IFC).

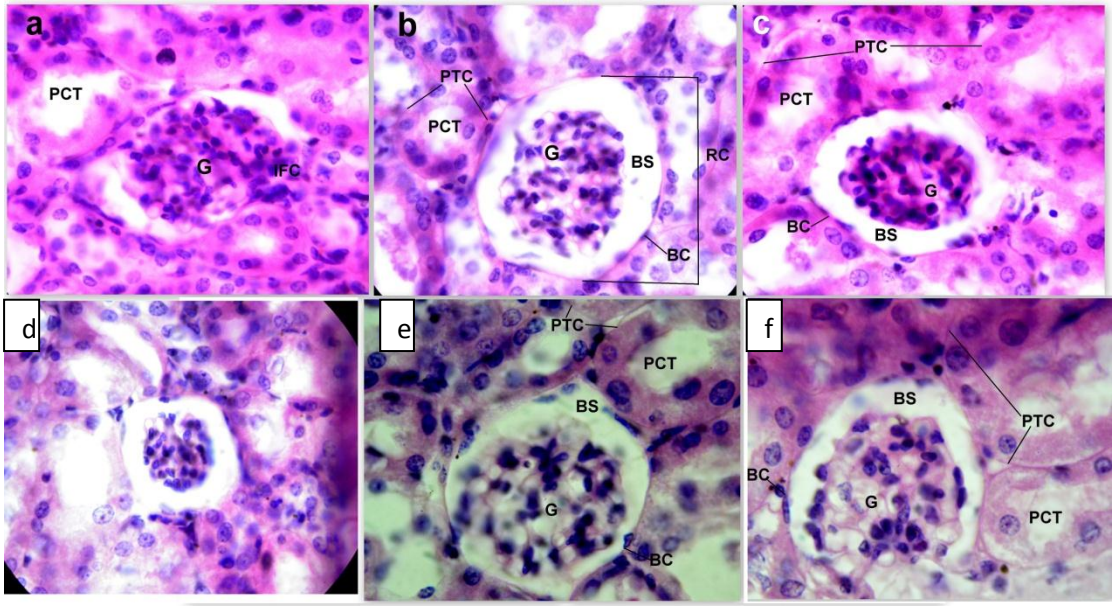


Fig.4.18: Micrograph of kidney sections of [a]. Dianetic mice, [b]. Normal mice, [c]. Glibenclamide treated mice, [d]. CAME treated mice, [e]. CAEE treated mice, [f] CAAE treated mice. Renal corpuscles (RC), Glomerulus (G), proximal convoluted tubules (PCT), proximal tubular capillaries (PTC), Glomerulus G), infiltration of inflammatory cells (IFC), Bowman's capsule (BC), Bowman's space (BS).

Histological studies of the kidney of normal mice revealed regular glomerulus surrounded by the Bowman's capsule, proximal convoluted tubules without any inflammatory changes and proximal tubular capillaries are distinct (Fig.4.18b).

Kidneys of untreated diabetic mice showed narrow Bowman's space due to infiltration of the inflammatory cells (Fig.4.18a). However the treated mice showed fewer infiltrations of inflammatory cells (Fig.4.18 c, d, e and f).

4.9. Effects of CAME on secretory function of pancreatic MIN6 β-cells

The effects of CAME on pancreatic insulin secretion in mouse insulinoma MIN6 β-cell line is presented in Fig.4.19. CAME increased insulin release from MIN6 β-cells in a dose dependent manner over the concentration gradient (0.01-25mg/ml). At 0.01, 0.05, 0.1, 0.5 1, 5, 10 and 25 mg/ml, the measured insulin secretion enhanced by CAME were 0.095, 0.195, 0.16, 0.29, 0.8, 1.2, 1.35 and 1.61 ng/50.000 cells/20min; and measured insulin secretion enhanced by gliclazide were 0.063, 0.18, 0.11, 0.24, 0.86, 1.12, 1.24 and 1.35 ng/50.000 cells/20min respectively; measured insulin secretion enhanced by control was 0.72 ng/50.000 cells/20min. The minimum effective concentration of CAME was 1mg/ml (11.11% increased when compared with negative control). At concentrations 1, 5, 10 and 25 mg/ml, the effect of CAME was 1.11, 1.67, 1.88 and 2.25 fold increase respectively and the effect of gliclazide was 1.19, 1.56, 1.72 and 1.88 fold increase respectively, when compared with control.

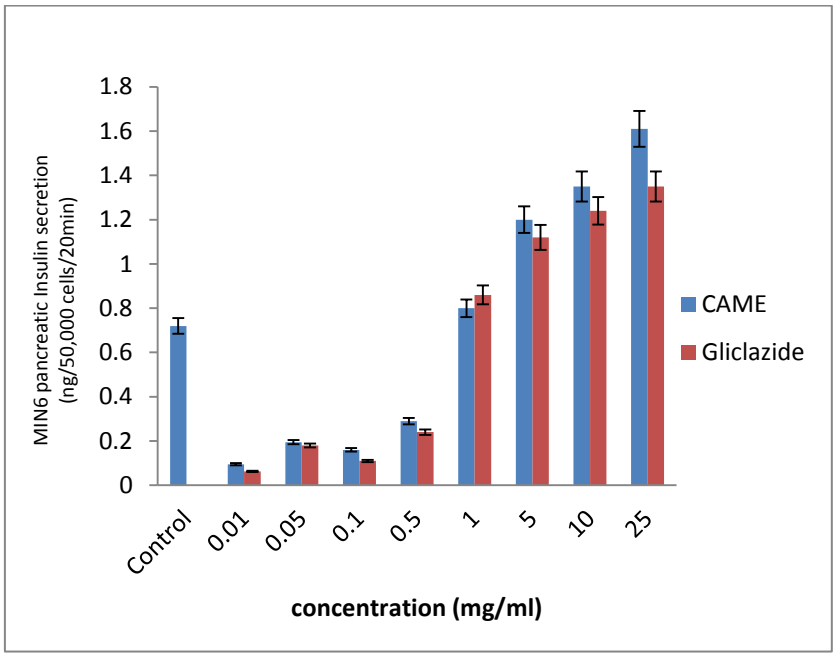


Fig.4.19: Effects of CAME on secretory function of pancreatic MIN6 β-cells.

The Ca^{2+} dependency of the insulinotropic of CAME is presented in Fig.4.20. The marked insulinotropic activity of CAME in Ca^{2+} free KRB medium was slightly increased (1.07 to 1.25 fold) when compared with corresponding concentrations of CAME activity in 2.5mM Ca^{2+} KRB medium (Fig.4.20A). Similarly, the effective

insulinotropic potential of gliclazide in Ca^{2+} free KRB medium was increased (2.23 to 5.71 fold) when compared with corresponding concentrations of gliclazide activity in 2.5mM Ca^{2+} KRB medium (Fig.4.20B).

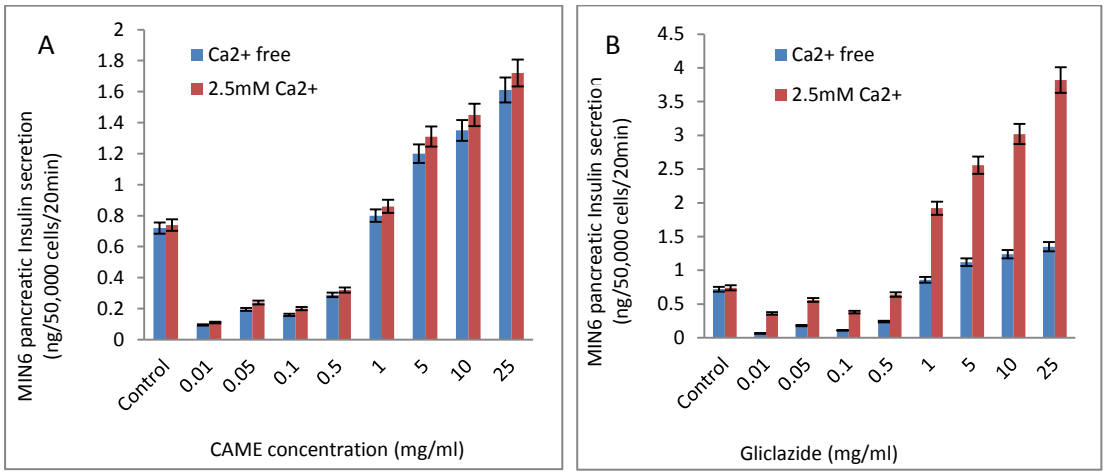


Fig.4.20: The Ca^{2+} dependency of the insulinotropic of A) CAME and B) gliclazide

4.10. GC-MS profile study of CAME

The chromatogram of CAME by GC-MS is shown in Fig.4.20: CAME with the most potent antidiabetic property among the three extracts, when subjected to GC-MS showed 16 peaks revealing 16 compounds (Fig: 4.20). On comparison of the mass spectra of the constituents with the NIST library, the sixteen phytocompounds were characterized and identified and is listed in Table.4.11, along with respective Retention Time, molecular formula and percentage concentration. The mass spectra of all the phytochemicals identified in CAME were presented in Fig.4.21.

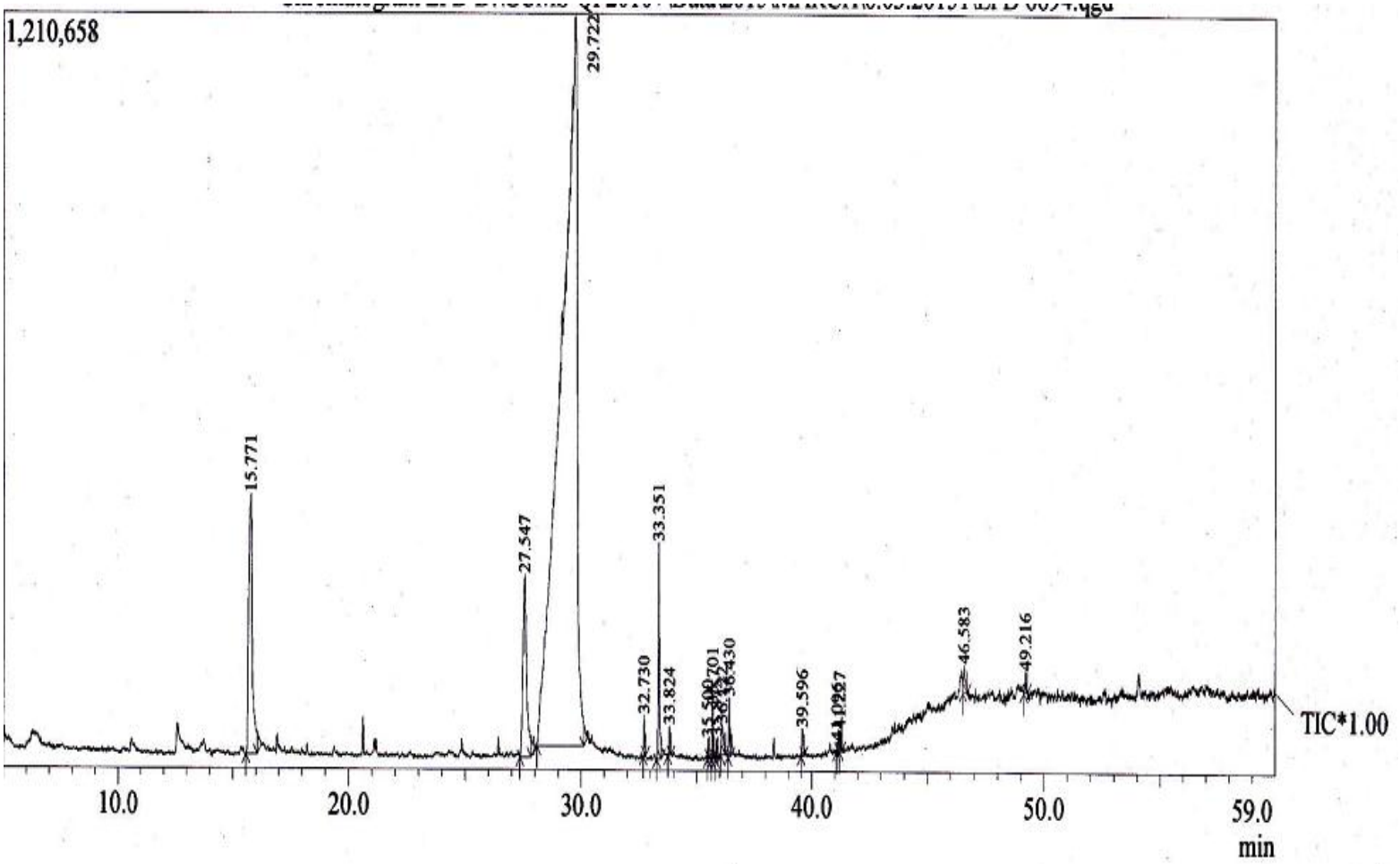


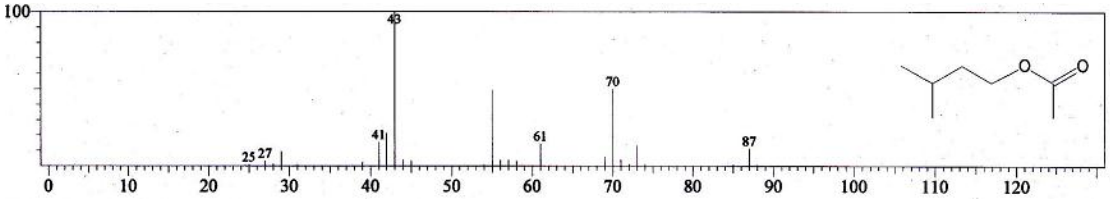
Fig.4.21: Chromatogram of CAME by GC-MS

Table: 4.11. Phytochemicals identified in CAME by GC-MS.

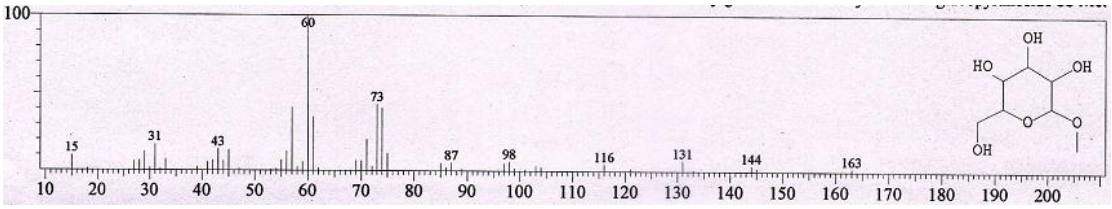
No.	RT	Name of the compound with common name	Molecular formula	Peak area %
1	15.771	l-Butanol, 3-methyl acetate. (Isoamyl acetate)	$C_7H_{14}O_2$	6.66
2	27.547	Methyl β-D-Glucopyranoside	$C_7H_{14}O_6$	4.64
3	29.722	3-o-methyl-d-glucose. (Methylglucose; D-Glucose, 3-O-methyl-; 3-methyl-glucose)	$C_7H_{14}O_6$	84.36
4	32.730	Tridecanoic acid, methyl ester. (Methyl tridecanoate; Methyl ester of tridecanoic acid; Methyl n-tridecanoate; Methyl tridecanoate ester)	$C_{14}H_{28}O_2$	0.22
5	33.351	n-Hexadecanoic acid. (Palmitic acid; Palmitinic acid; Cetylic acid; Glycon P-45; Hexadecanoic acid; 1-Pentadecanecarboxylic acid)	$C_{16}H_{32}O_2$	1.64
6	33.824	Decanoic acid, ethyl ester. (Capric acid, ethyl ester; Ethyl caprate; Ethyl caprinate; Ethyl decanoate)	$C_{12}H_{24}O_2$	0.17
7	35.500	9-Octadecenoic acid (Z)-, methyl ester. (Oleic acid, methyl ester; Methyl cis-9-octadecenoate; Methyl oleate)	$C_{19}H_{36}O_2$	0.09
8	35.701	Phytol. (2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]- ; trans-Phytol)	$C_{20}H_{40}O$	0.30
9	35.875	Docosanoic acid, methyl ester. (Behenic acid, methyl ester; Methyl behenate; Methyl	$C_{23}H_{46}O_2$	0.09

		docosanoate)		
10	36.122	Cyclohexanone, 2,2-dimethyl-5-(3-methyloxiranyl)-, [2.alpha.]-(.+-.)-	$C_{11}H_{18}O_2$	0.44
11	36.430	Octadecanoic acid. (Stearic acid; n-Octadecanoic acid; Glycon DP)	$C_{18}H_{36}O_2$	0.45
12	39.596	2-diacetylamino-3-(1h-indol-3-yl)-propionic acid, methyl ester	$C_{16}H_{19}N_2O_4$	0.24
13	41.096	Cyclohexanone 4-(1 1-dimethylpropyl)-	$C_{11}H_{20}O$	0.04
14	41.227	hexadecanoic acid 2-hydroxy-1-(hydroxymethyl)ethyl ester (Glycerol β-palmitate; 2-Palmitoylglycerol; Palmitic acid β-monoglyceride)	$C_{19}H_{38}O_4$	0.16
15	46.583	Pseduosarsasapogenin-5,20dien	$C_{27}H_{42}O_3$	0.34
16	49.216	Ethyl iso-allocholate	$C_{26}H_{44}O_5$	0.17

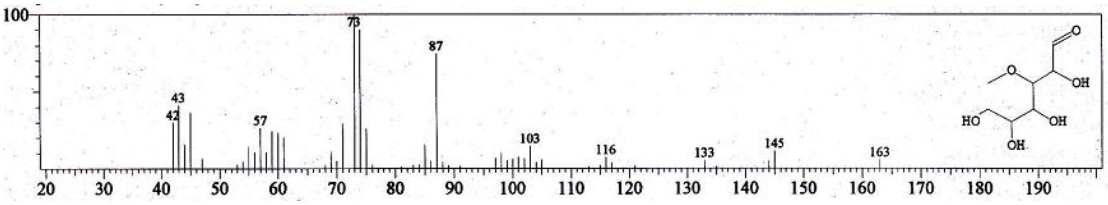
RT, Retention Time.



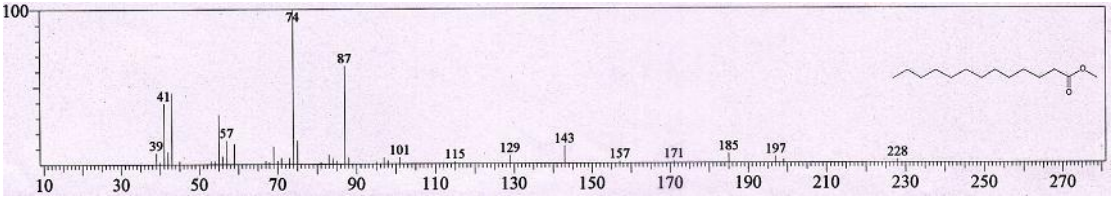
a). l-Butanol, 3-methyl acetate.



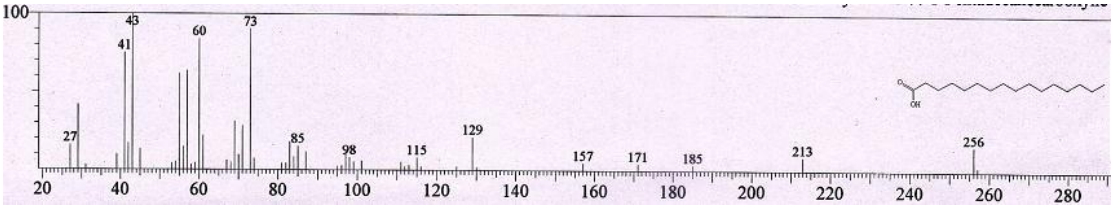
b). Methyl β-D-Glucopyranoside



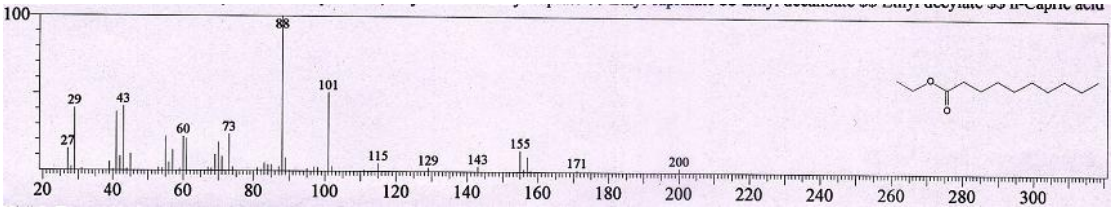
c). 3-o-methyl-d-glucose



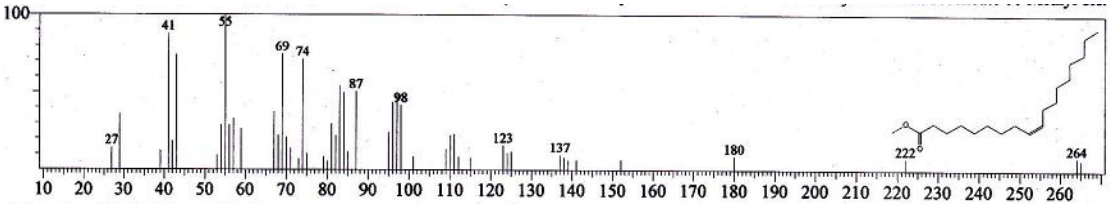
d). Tridecanoic acid, methyl ester



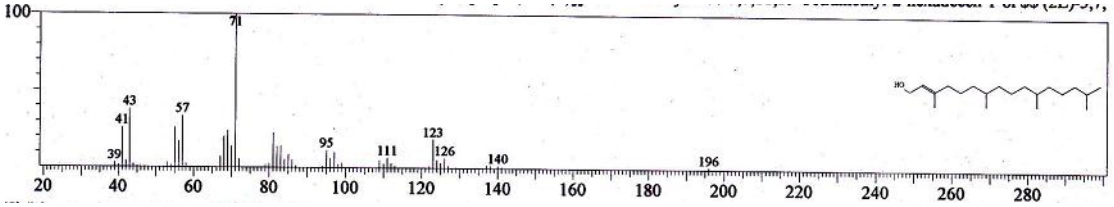
e). n-Hexadecanoic acid.



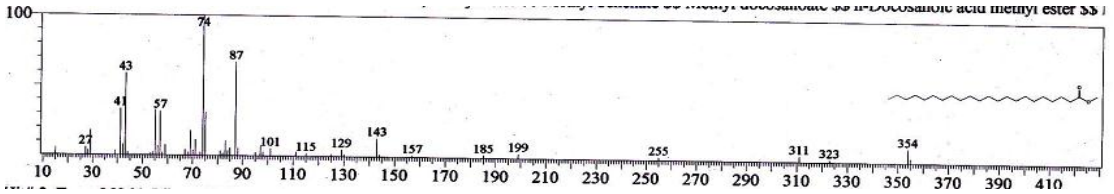
f). Decanoic acid, ethyl ester



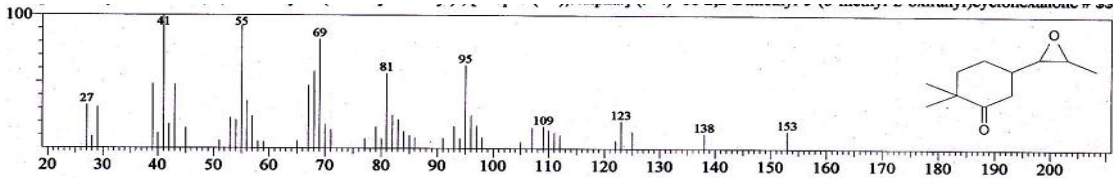
g). 9-Octadecenoic acid (Z)-, methyl ester



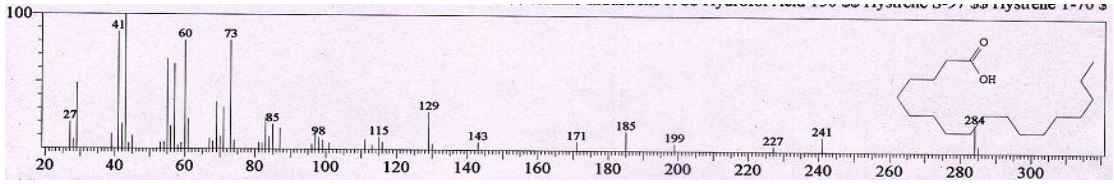
h). Phytol



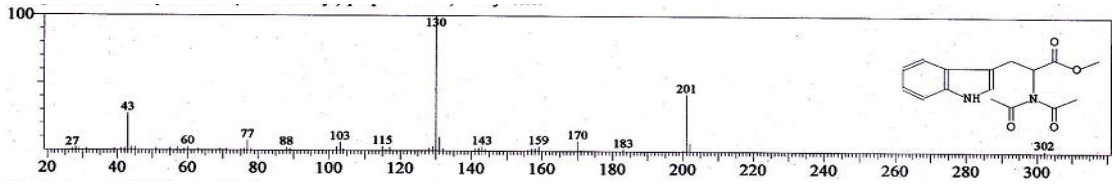
i). Docosanoic acid, methyl ester



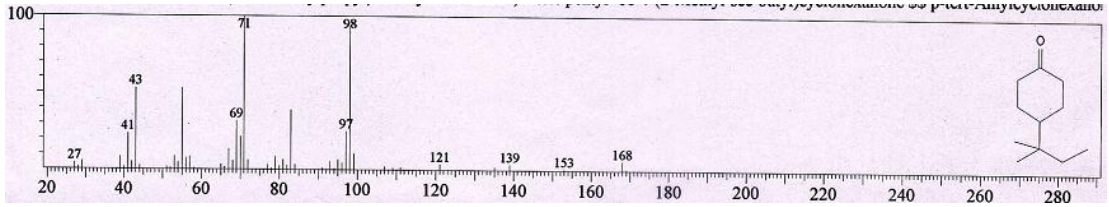
j). Cyclohexanone, 2,2-dimethyl-5-(3-methyloxiranyl)-, [2.alpha.]-(.+-.)-



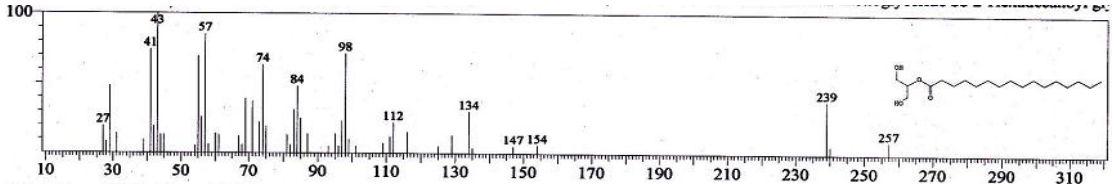
k). Stearic acid



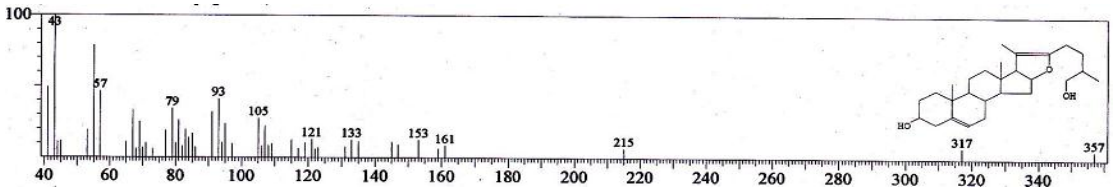
l). 2-diacetylamino-3-(1h-indol-3-yl)-propionic acid, methyl ester



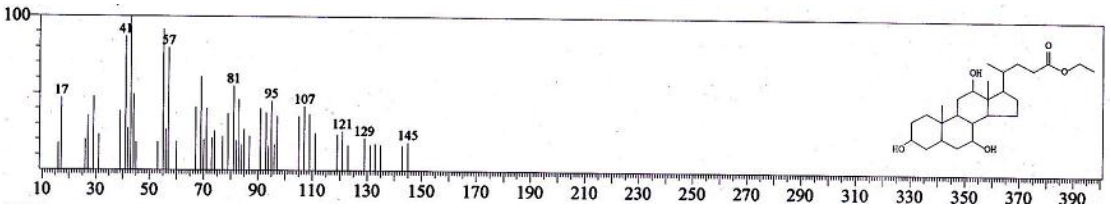
m). Cyclohexanone 4-(1 1-dimethylpropyl)-



n). hexadecanoic acid 2-hydroxy-1-(hydroxymethyl)ethyl ester



o). Pseduosarsasapogenin-5,20dien



p). Ethyl iso-allocholate

Fig.4.22: The compounds identified by matching the spectra with those found in the NIST 05 library.

4.11. Molecular characterization of the plant

Genomic DNA extraction was carried out with a modified CTAB method and it produced significant result. Distinct bands of genomic DNA was observed on 0.8% agarose gel when observed in a Gel Documentation system (BIO-RAD). The image is given below (Fig.4.23).

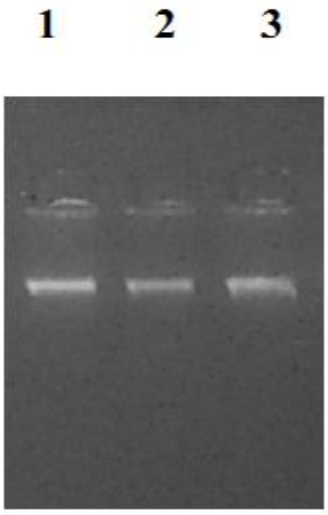


Fig.4.23: Electrophoresis of total DNA extracted from the three samples. Lane 1: F1, Lane 2: F2 and Lane 3: F3.

The extracted genomic DNAs were amplified with three RAPD primers. The amplification patterns revealed a high level of polymorphism. All primers produced

multiple DNA products ranging in size from 0.1 to 1 kb. The RAPD profiling image is shown in Fig 4.24.

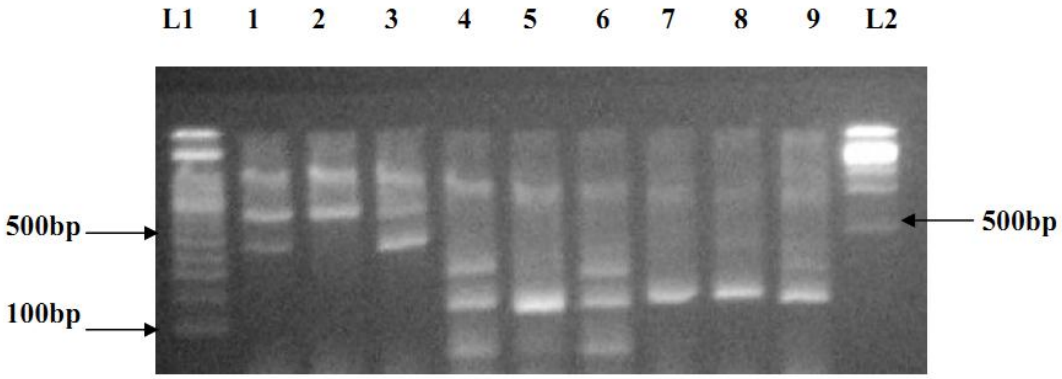


Fig.4.24: RAPD profile of the three samples. Lane 1-3: F1, F2 and F3 with primer 1, Lane 4-6: F1, F2 and F3 with primer 2, Lane 7-9: F1, F2 and F3 with primer 3.

The dendrogram derived from RAPD profiles (Fig 4.25) shows that the collected plant samples, F1 and F3 are most closely related with a similarity level of 0.12. However the plant sample F2 showed clear divergence and showed no similarity with the other two sample plants and appeared separately in the phylogenetic tree. The RAPD profiling data and the phylogenetic tree is in accordance with the phenotypic data. The sample F1 and F3 belong to same species *Cassia alata* Linn. collected from two different places and F2 belongs to a different species *Cassia tora* Linn.

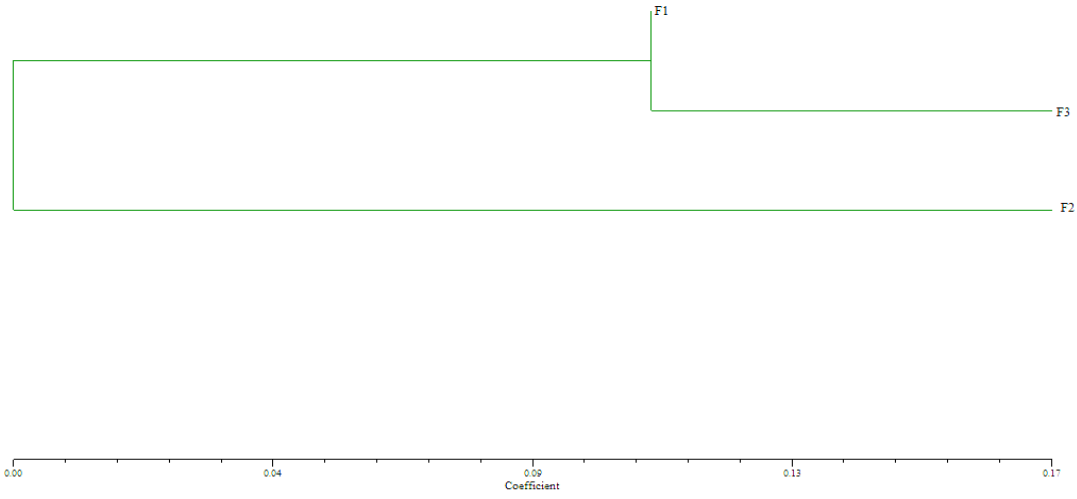


Fig.4.25. The dendrogram derived from RAPD profiles.

The RAPD patterns of the three samples produce by the three primers are presented in Fig.4.26, Fig.4.27, and Fig.4.28.

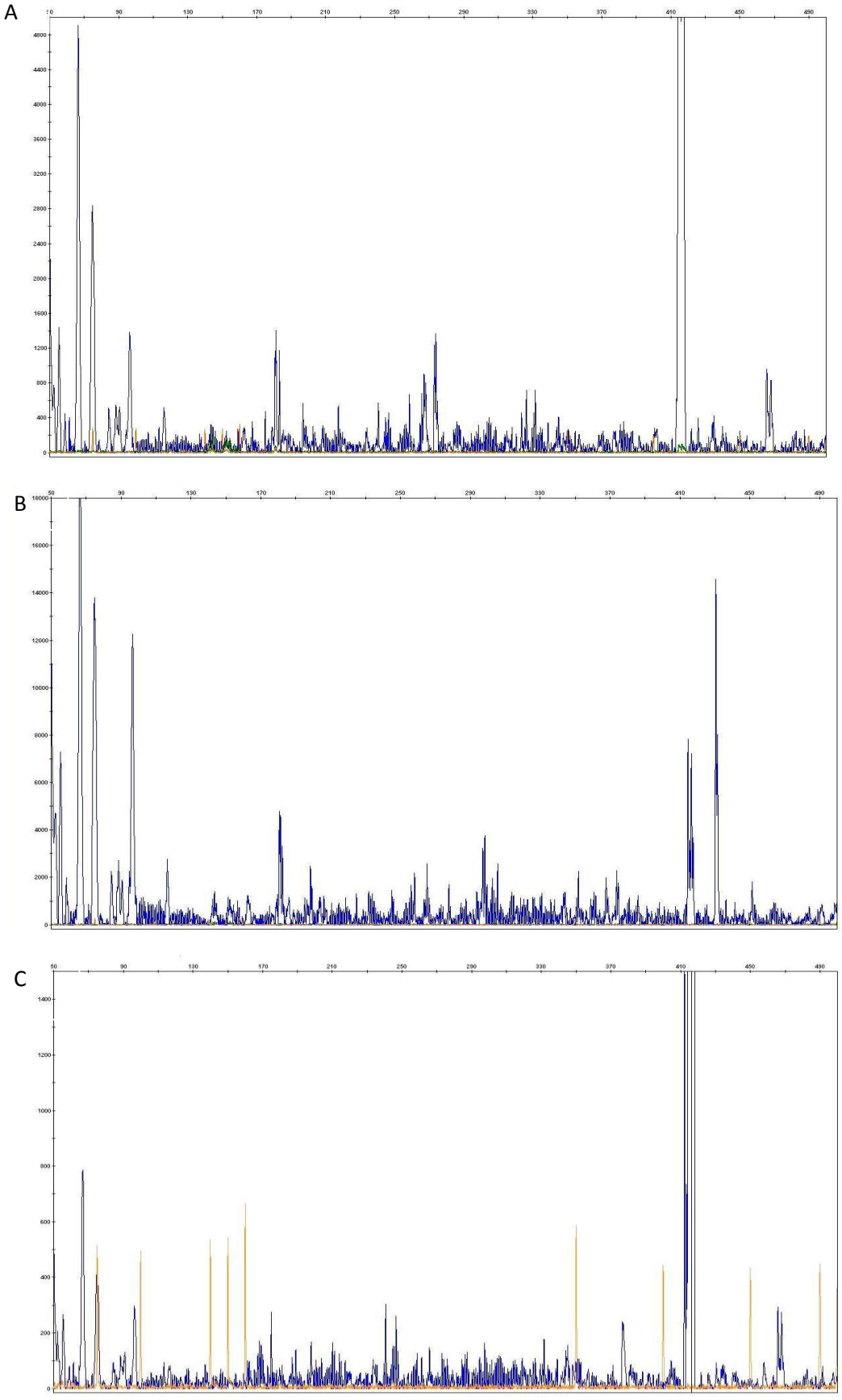


Fig.4.26: RAPD patterns of F1 (A), F2 (B) and F3 (C) using primer 1

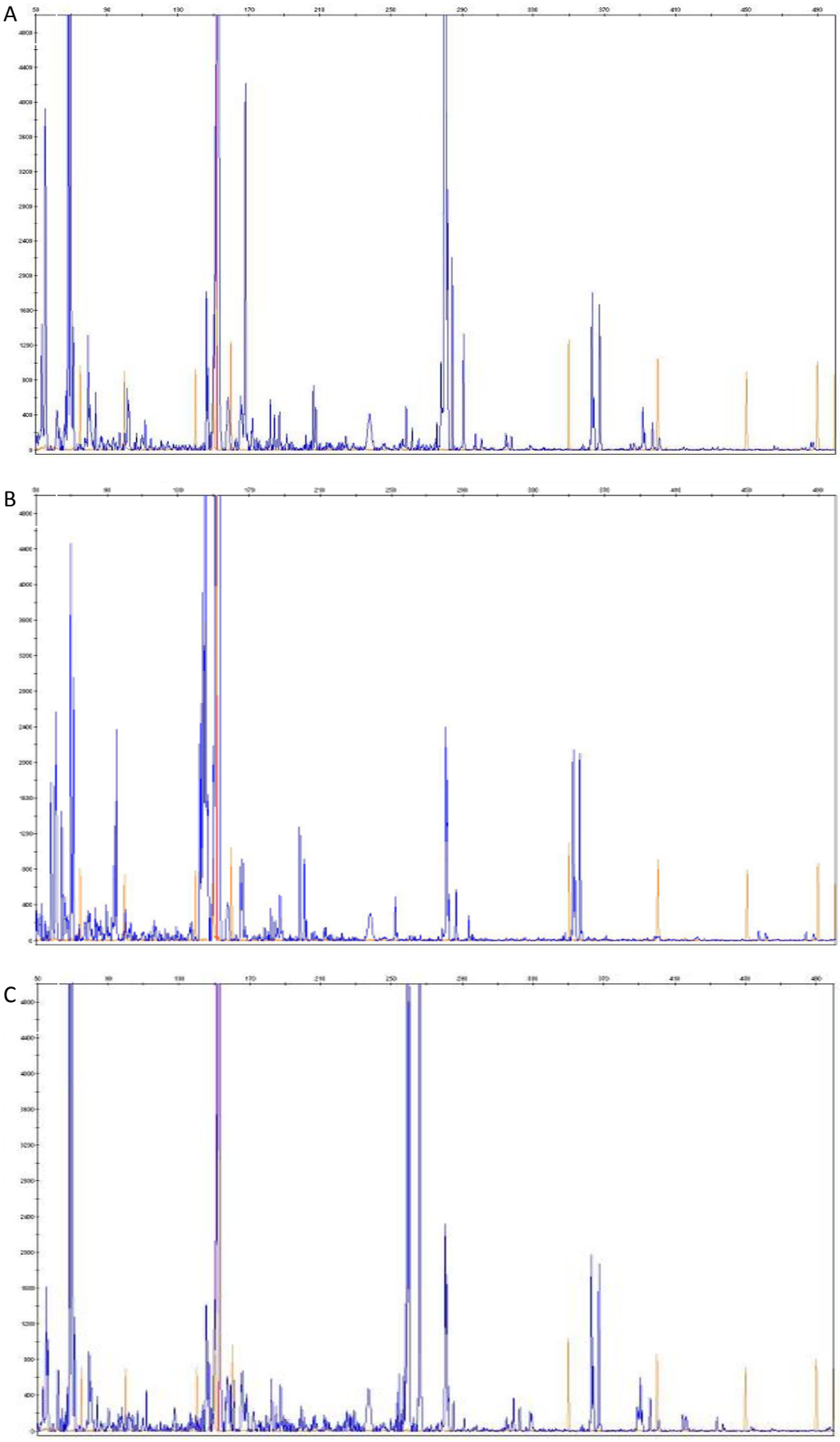


Fig.4.27: RAPD patterns of F1 (A), F2 (B) and F3 (C) using primer 2

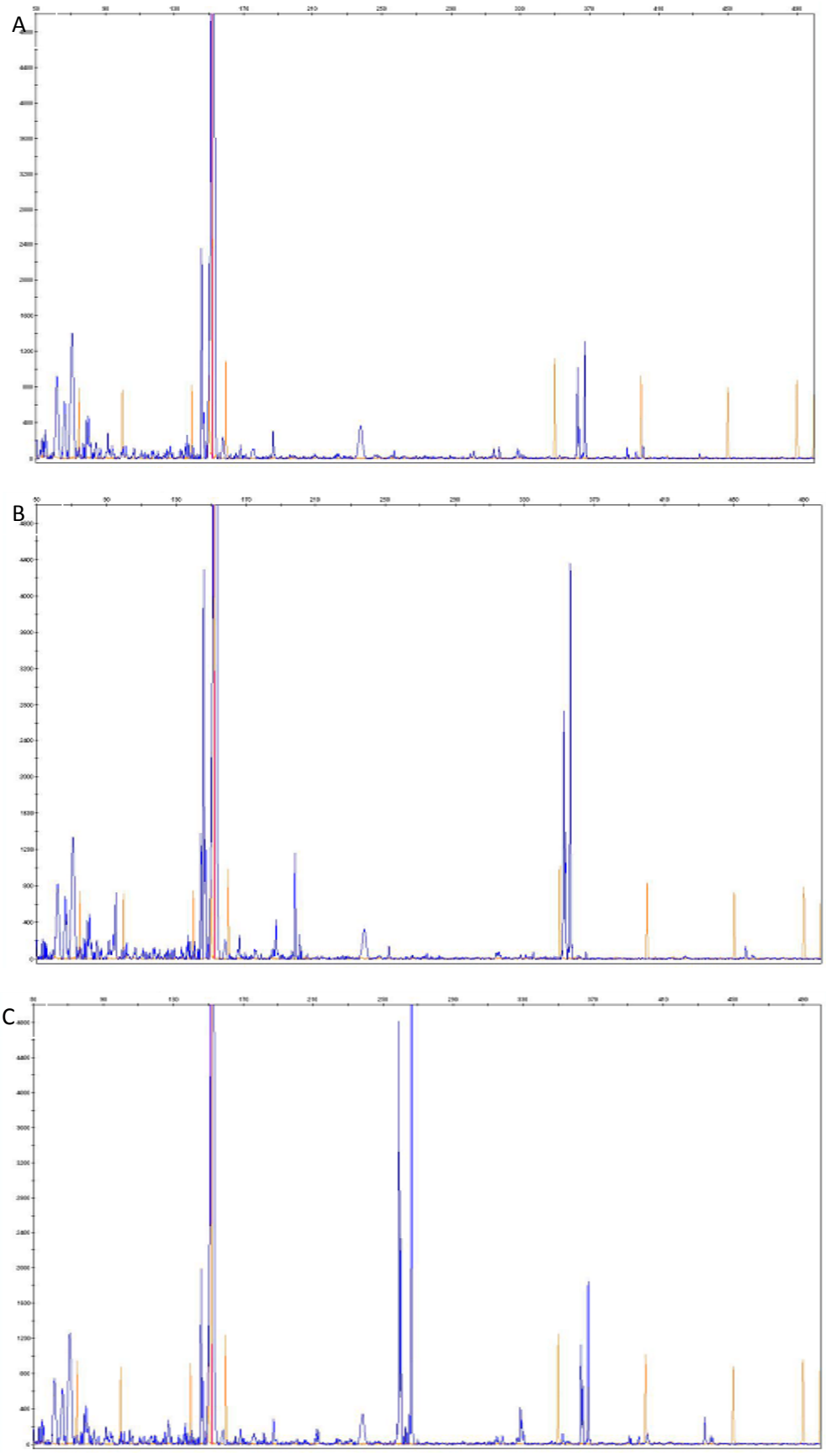


Fig.4.28: RAPD patterns of F1 (A), F2 (B) and F3 (C) using primer 3