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RESEARCH ARTICLE

EVALUATION OF ANTIDIABETIC ACTIVITY OF BILE FROM GALLUS GALLUSDOMESTICUS

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Abstract

Diabetes mellitus is a chronic condition characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The current study aimed at evaluating the antidiabetic activity of bile from *Gallus gallusdomesticus*. The zoochemical analysis revealed the presence of steroids. The *invitro* antioxidant activity of the HMEB and in vitroantiinflammatory activity of HMEB was evaluated. The *invitro*antidiabetic activity was investigated by α-Amylase inhibition assay, and glucose uptake assay. The gene expression study was carried out for GLUT-4 and PPAR-γ was compared with the housekeeping gene β-actin. The animal toxicity study was performed as per OECD test guidelines 423 limit test The OGTT was conducted in normal rats and observed significant blood glucose lowering with HMEB (high dose). Thus hydro-methanolic extract of bile from Gallus gallusdomesticus could be developed as a potential drug candidate for the treatment of diabetes mellitus.

Introduction:-

Diabetes mellitus is a syndrome of multiple etiologies characterized by chronic hyperglycemia with disturbances of carbohydrate, fat, and protein metabolismresulting from defects in insulin secretion, insulin action, or both. This disorder is oftenassociated with long-term complications, involving organs like eyes, kidneys, nerves,heart, and blood vessels.[1] Types of DM include type 1 diabetes, type 2 diabetes, gestational diabetes, and other types of diabetes (Monogenic diabetes, pancreatic diabetes, drug-induced diabetes, etc.).[2]There is an urgent need for strategies to prevent or slow down the emerging epidemic of diabetes and to reduce the associated complications. Chicken bile obtained from the avian species *Gallus gallusdomesticus* have many constituents similar to both pig bile and crucian carp bile.[3]In our country, bile from the avian species *Gallus gallusdomesticus* is not used for edible purposes, it can be developed as a remedy against diabetes mellitus.So, this investigation on chicken bile is done to evaluate its effect on diabetes mellitus.

Materials and Methods:-

Extraction of bile

Freshly removed gallbladders (24Nos.) of chicken were collected from the slaughterhouse near Govt. Medical College Kannur, Pariyaram. 50ml bile sample were extracted with 200ml methanol-water solution (1:1;V/V) in a

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centrifuge tube for 2 hour using an ultrasonic cleaner at room temperature. Centrifuged at 4000 rpm for 20 minutes. Supernatant was collected and solvent evaporated by rotary evaporator.[4]1

FTIR analysis and
FTIR analysis was performed using fresh raw bile juice.[5]

Stability study of bile extract using UV
About 100µg/ml of hydro-methanolic extract of the bile was prepared in methanol and the absorbance of the extract was measured between 300-600nm. The same procedure was repeated with the extract preparation after one month and their difference in absorbance was analysed.[5]

***Invitro*antioxidant activity assays**
DPPH radical scavenging assay
The ascorbic acid stocksolution was prepared in distilled water (1 mg/ ml; w/v). A 60µM solution of DPPH in methanolwas freshly prepared and a 200µl of this solution was mixed with 50µl of the test sample at various concentrations The plates were kept inthe dark for 15 minutes at room temperature and the decrease in absorbance was measured at 515nm. Control was prepared with DPPH solution only, without any extract or ascorbic acid. 95%methanol was used as blank.[6,7]

ABTS radical scavenging assay
The reaction was initiated by the addition of 200µl of diluted ABTS to different concentrations of sample extract and in control 50 µl of methanol was used instead of the sample. Methanol was used as a blank. The absorbance was read at 734 nm and the percentage inhibition was calculated.[8]

Detection of Lipid peroxidation (TBARS Assay)
The following contents mentioned in the below table constitutes the blank and testsamples.[10]
NB: Test indicates samples with cell lysate.

Contents	Blank (ml)	Test (ml)
Cell lysate	--	200 µL
Distilled water	500 µL	300 µL
MDA reagent	1000 µL	1000 µL
Heated in water bath for 15 minutes & cooled		
Centrifuged at 1000 rpm for 10 minutes		
The supernatant was taken and read at 535 nm		

Table No.1:- TBARSassay procedure.

***In vitro*antioxidant activity assays**
COX assay
The cell lysate in Tris-HCl buffer (pH 8) was incubated with glutathione 5 mM/L, and hemoglobin 20 µg/L for 1 minute at 25°C. The reaction was initiated by the addition of arachidonic acid 200 mM/L and terminated after 20 minutes of incubation at 37°C, by the addition of10% trichloroacetic acid in 1 N hydrochloric acid. After the centrifugal separation and the addition of 1% thiobarbiturate, COX activity was determined by reading absorbance at 632 nm.[11]

LOXassay
The reaction mixture (2 mL final volume) contained Tris-HCl buffer (pH 7.4), 50 µL of cell lysate, and sodium linoleate (200 µL; 10mg/ml). The LOX activity was monitored as difference in absorbance at 234 nm, which reflects the formation of 5-hydroxyeicosatetraenoic acid from linoleate.[12]

***In vitro*cytotoxicity activity**
MTT assay
L929 (Mouse fibroblast cell line) was procured from the National Centre for Cell Sciences (NCCS), Pune, India.

[1]HMEB- Hydromethanolic extract of bile from Gallus gallusdomesticus

Assay Procedure

L929 cells (2500 cells/well) were seeded on 96 well plates and allowed to acclimatize to the culture conditions such as 37°Cand 5% CO_2 environment in the incubator for 24 h. The test samples were prepared in DMEM media (100 mg/mL) and filter sterilized using 0.2 µm Millipore syringe filter. The samples were further diluted in DMEM media and added to the wells containing cultured cells at final concentrations of 6.25, 12.5, 25, 50, 100 µg/mL respectively. Untreated wells were kept as control. All the experiments were done in triplicate and average values were taken in order to minimize errors. After treatment with the test samples the plates were further incubated for 24 h. After incubation period, the media from the wells were aspirated and discarded. 100 µL of 0.5 mg/mL MTT solution in PBS was added to the wells. The plates were further incubated for 2 h for the development of formazan crystals. The supernatant was removed and 100 µL DMSO (100%) were added per well.

The absorbance at 570 nm was measured with micro plate reader (MultiskanSkyHigh, Thermoscientific, India). Two wells per plate without cells served as blank. All the experiments were done in triplicates.[12, 13]

***In vitro*antidiabetic activity screening**

α- Amylase Inhibition assay

500mL of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) containing 0.5 mg/mL of α- amylase enzyme and different concentrations (in µg) of the test sample as enzyme inhibitor were pre-incubated at 37°C for 10 minutes. After the pre-incubation, 500 µL of a 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9) was added to each tube and incubated at room temperature for 5 minutes. The reaction was stopped using 1.0 mL of dinitrosalicylic acid (DNSA) reagent. The test tubes were incubated in a boiling water bath for 5 min and then cooled to room temperature. The volume of the reaction mixture was made up to 10mL by adding distilled water, and the absorbance was measured at 540 nm using UV-Visible spectrophotometer. The absorbance was compared with the controls and blank that contained buffer instead of test sample.[14]

α -Glucosidase inhibition assay

400 µL of α-glucosidase (0.067 U/mL) was preincubated with different concentrations of the sample for 30 min. Then 200 µL of 3.0 mM (pNPG) used as substrate dissolved in 0.1M sodium phosphate buffer (pH 6.9) was then added to start the reaction. The reaction mixture was incubated at 37°C for 30 min and stopped by adding 2 mL of 0.1 M Na_2CO_3. The α-glucosidase activity was determined by measuring the yellow-colouredpara- nitro phenol released from pNPG at 400 nm. The results were expressed as percentage of inhibition. Same procedure was done with Acarbose (1mg/ml stock) which was used as standard.[15]

Glucose uptake assay

L6 Cells were cultured on 48 well plates and incubated for 48 hours at 37°C in a CO_2 incubator. When semi-confluent monolayer was formed, the culture was renewed with serum free DMEM containing 0.2% BSA and incubated for 18 hours at 37°C in the CO_2 incubator. After 18 hours, the medium was discarded and cells were washed with PBS (pH 7.4) buffer once and treated with 1000 µg/ml glucose along with test compound (25, 50, 100 µg/ml) for 1 hour. Glucose uptake was calculated as the difference between the initial and final glucose content in the incubated medium. The final glucose concentration was estimated by anthrone method with the aid of a glucose standard graph. The glucose uptake in L6 cells treated with test compounds were compared with that of control cells (untreated). If the treated cells showed improved glucose uptake compared to that of control cells we can assume that the compound have medicinal value.[16]

Gene expression studies

qPCR analysis of the samples for the target genes

The PCR reaction was carried out in 10µl reaction mixture containing 5µl 2X SYBER GREEN qPCR Mix (G bioscience), 1µl of 10µM forward and reverse primer, 1µl of template DNA and 2µl Nuclease free water.

Table No.2:- Components of the PCR reaction mixture.

PCR Components	Stock	Volume to be taken
Nuclease free Water	-	1 µl
Forward Primer	10 µM	1 µl
Reverse primer	10 µM	1 µl
SYBR Green qPCR mix	2X	5 µl
Template C- DNA	-	1 µl

Total Volume		10 µl

Following primers were used for the reaction:
Table No.3:-Primer sequence used for PCR.

Primer	Sequence
Beta Actin Mus F	ACT ATT GGC AAC GAG CGG TT
Beta Actin Mus R	AGG GTG TAA AAC GCA GCT CA
GLUT4F	GGCCATCTTCTTCTGTGGTG
GLUT4R	ACGATGGCCAATTGGTTCAG
pAPGF	AATCAAAGTGGAGCCTGCAT
pAPGR	ACCCTTGCATCCTTCACAAG

The amplification reaction was performed in a thermal cycler (Bio rad) with gradient temperatures using the PCR conditions with the steps of initial denaturation at 95°C for 10 minutes followed by 40 cycles of 95°C for 10 secs, annealing temperature as mentioned below for 40 sec, and 72°C for 40 secs. The data analysed by the software Bio-rad CFX Maestro. The annealing temperatures of all the genes for the current primers were calculated using the Tm of the forward and reverse primers.[17]

Table No.4:- Various steps of PCR along with temperature conditions and time taken.

Profile	Temperature	Time
Lid temperature	98°C	
Initial denaturation	95°C	00:10:00
Denaturation	95°C	00:00:10
Annealing	Beta Actin 58 GLUT4 57 pAPG 56	00:00:40
Extension	72°C	00:00:50
Meltcurve	65°C to 95°C	

Acute toxicity study
Albino Wistar rats weighing between 120- 160g were used for the study. Animals were brought from Biogen Laboratory Animal Facility, Banglore, Karnataka and were maintained in the animal house of College of Pharmaceutical Sciences, Government Medical College Kannur, Kerala. All the animals were acclimatized at least for 7 days to the laboratory conditions before experimentation. The study was conducted after obtaining approval from CPCSEA (CPS-GMC/ IAEC/22/23-01). Study conducted according to OECD guideline 423.

Hydroalcoholic extracts of bile from Gallus gallusdomesticus(2000 mg/kg b.w.) was administered. Animals were observed individually after dosing at least once during the first 30 minutes, periodically during the first 24 hours, with special attention given during the first 4 hours and daily thereafter, for a total of 14 days, except where they need to be removed from the study and humanely killed for animal welfare reasons or are found dead.[18]If toxic signs or lethality is not observed, then $1/5^{th}$ and $1/20^{th}$ of the limit test dose were considered as test doses for the present investigation.

***In vivo*antidiabetic activity**
OGTT
The Oral Glucose Tolerance Test (OGTT) was performed on overnight fasting normal rats. Distilled water, HMEB high dose, HMEB low dose and glibenclamide (2 mg/ Kg) were administered to four groups of rats, respectively. Glucose (2 g/ Kg) was fed 30 min after pretreatment with distilled water, high dose, HMEB low dose and glibenclamide (2 mg/ Kg). Blood glucose levels were measured at -30, 0, 30, 60, 120 and 180 minutes after glucose load to access the effect of extract on blood glucose levels of the glucose loaded animals. The blood glucose was measured using blood glucose strips.[19]

Results:-

FTIR analysis

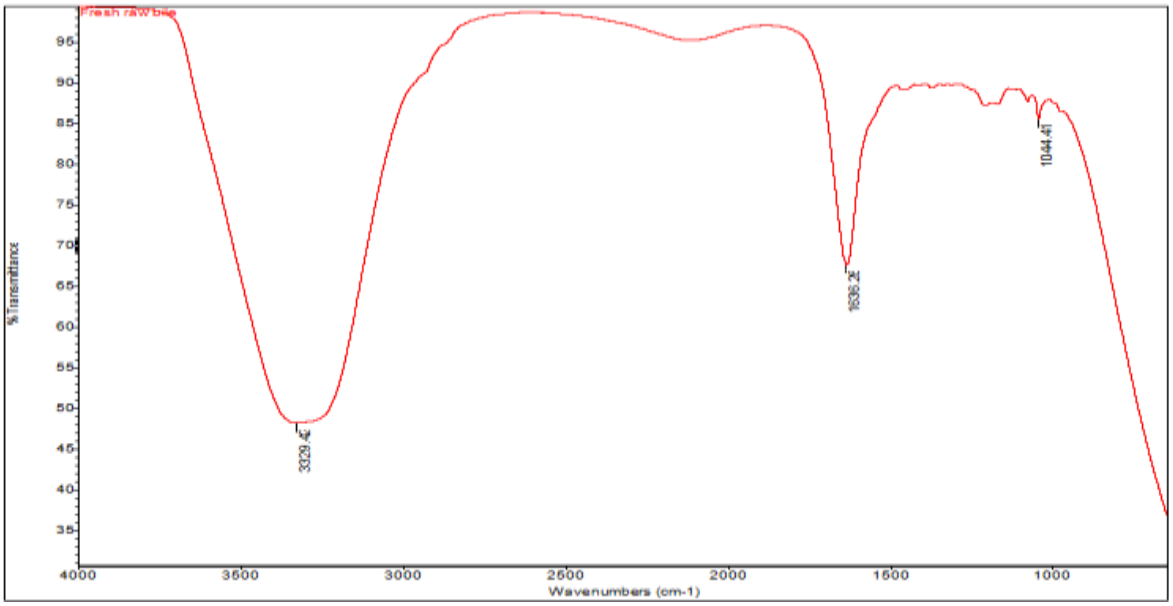


Figure No.1:- IR spectra of fresh raw chicken bile.

Stability study of bile extract using UV

The absorbance of a freshly generated hydro-methanolic extract of bile from *Gallus gallusdomesticus*was measured and then repeated after one month. The lack of a substantial variation in absorbance suggested that the chemical was stable during its one-month storage period.

Figure No.2:- UV absorption spectra of freshly prepared HMEB.

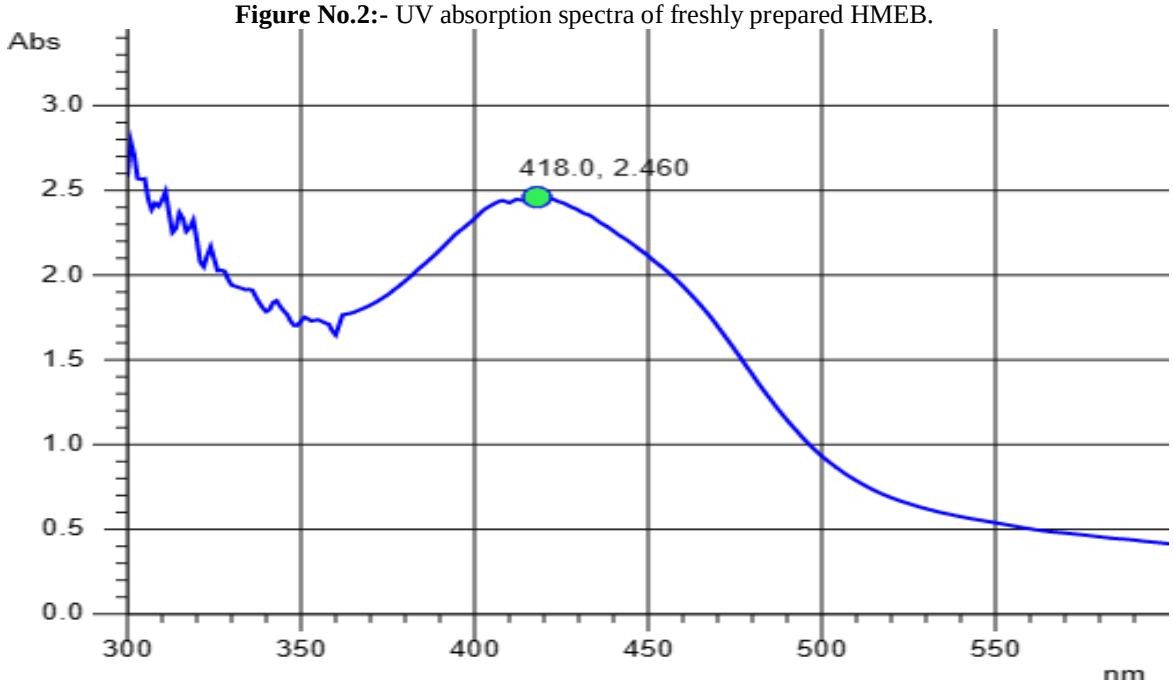
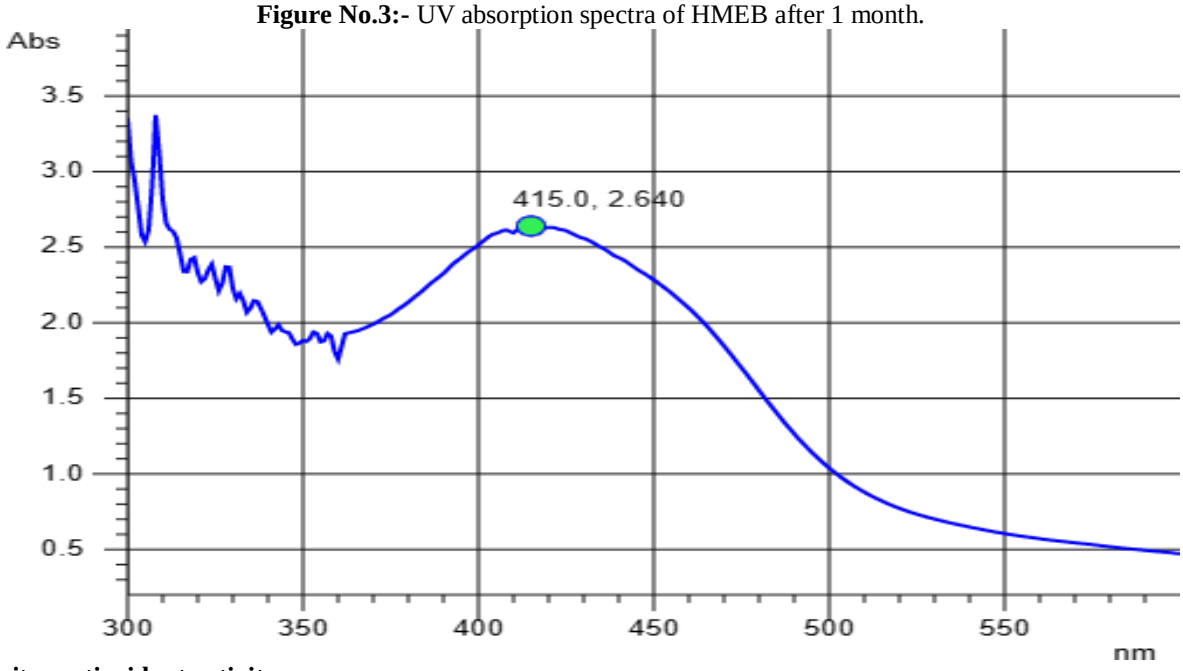


Figure No.3:- UV absorption spectra of HMEB after 1 month.



***In vitro* antioxidant activity assays**

DPPH radical scavenging assay

*In vitro*antioxidant activity of the hydro-methanolic extract of bile from *Gallus gallusdomesticus*was evaluated by DPPH radical scavenging method. The HMEB showed significant antioxidant activity in a dose-dependent manner.The results are shown in Table No.5 and Figure No.4. HMEB showed maximum inhibition of 27.88% while standard (Ascorbic acid) showed a maximum of 89.75% at 200µg/ml.

Table No.5:- In vitro antioxidant activity determination by using DPPH assay.

Sl. No	Groups	Concentration (µg/ml)	Absorbance (Mean±SD)	%Inhibition	IC_{50} (µg/ml)
1	Control	-	0.8229±0.0003	-	
2	Standard-Ascorbic acid	12.5	0.5515±0.0002	32.98	28.19
		25	0.4587±0.0002	44.26	
		50	0.1466±0.0001	82.19	
		100	0.0926±0.0003	88.75	
		200	0.0844±0.0005	89.75	
3	HMEB	12.5	0.7126±0.0003	16.78	-
		25	0.6908±0.0001	19.32	
		50	0.6565±0.0002	23.33	
		100	0.6319±0.0003	26.21	
		200	0.6175±0.0002	27.88	

Values are mean±SD (N=3)

ABTS radical scavenging assay

*In vitro*antioxidant activity of the hydro-methanolic extract of bile from *Gallus gallusdomesticus*was evaluated by ABTS radical scavenging assay.The results are shown in Table No.6 and Figure No.5. HMEB showed maximum inhibition of 20.58% while standard (Ascorbic acid) showed a maximum of 94.76% at 200µg/ml.

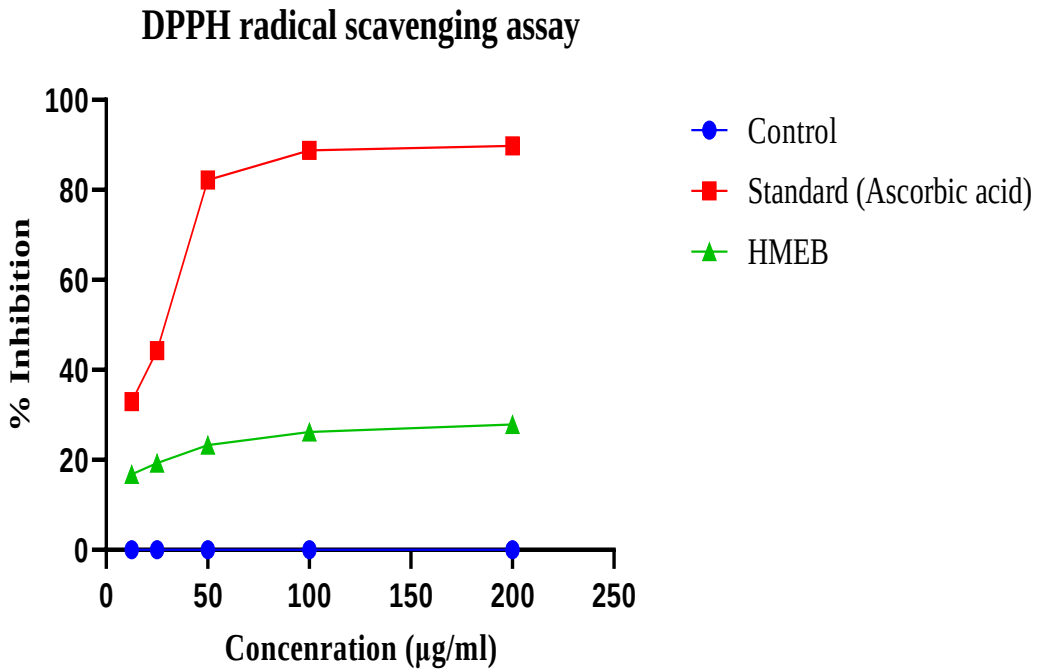


Figure No.4:- DPPH radical scavenging activity.

Table No.6:- *In vitro* antioxidant activity determination by using ABTS assay.

Sl. No	Groups	Concentration (µg/ml)	Absorbance (Mean±SD)	%Inhibition	IC_{50} (µg/ml)
1	Control	-	0.7837±0.0004	-	-
2	Standard-Ascorbic acid	12.5	0.4464±0.0004	43.04	19.69
		25	0.3416±0.0003	56.42	
		50	0.1824±0.0005	76.73	
		100	0.0746±0.0002	90.48	
		200	0.0414±0.0003	94.72	
3	HMEB	12.5	0.6938±0.0001	11.50	-
		25	0.6783±0.0003	13.49	
		50	0.6571±0.0003	16.18	
		100	0.6326±0.0001	19.32	
		200	0.6227±0.0002	20.58	

Values are mean±SD (N=3)

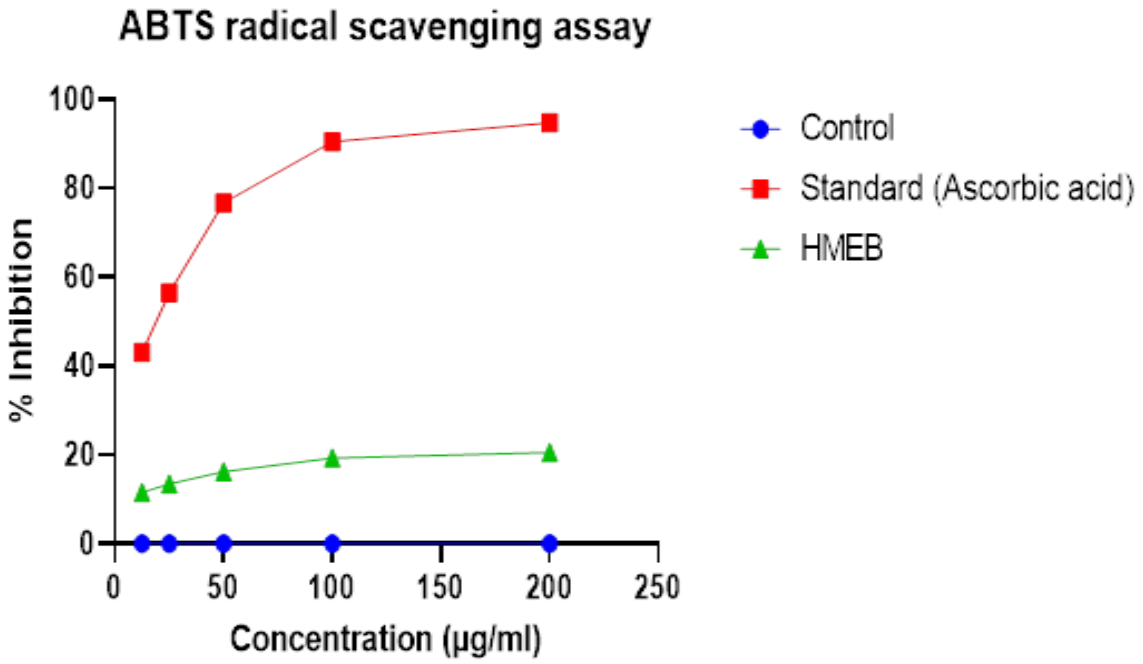


Figure No.5:- ABTS radical scavenging activity.

Detection of Lipid peroxidation (TBARS assay)

The HMEB was found to cause no significant enhancement in lipid peroxidation in L929 cell lines.This indicates that the HMEB does not cause the generation of free radicals as well as associated cell membrane damage in normal cells.

Concentration (µg/ml	Triplicate 1	Triplicate 2	Triplicate 3	Average	Concentration of TBARS (nano moles/ml)
Control	0.082	0.068	0.088	0.079	6.78
6.25	0.083	0.070	0.09	0.081	6.92
12.5	0.084	0.071	0.091	0.082	7
25	0.086	0.075	0.091	0.084	7.17
50	0.087	0.077	0.092	0.085	7.29
100	0.088	0.079	0.093	0.086	7.40

Table No.7:- Detection Lipid peroxidation (TBRAS)

***Invitro*antiinflammatory activity assays**

COX assay

*In vitro*anti-inflammatory activity of the hydro-methanolic extract of bile from *Gallus gallusdomesticus*was evaluated by COX assay.The results are shown in the Table No.8 and Figure No.6.HMEB showed maximum inhibition of 40.94% while standard (Diclofenac) showed a maximum of 55.71% at 100µg/ml.

Table No.8:- *In vitro*anti inflammatory activity by COX assay.

Sl.No	Groups	Concentration (µg/ml)	Absorbance (Mean±SD)	% Inhibition	IC_{50} (mg/ml)
1.	Control	-	0.7360±0.0091	-	-
2.	Standard-Diclofenac	6.25	0.7310±0.0083	13.25	82.91
		12.5	0.6340±0.1504	21	
		25	0.5760±0.0090	29.15	
		50	0.5180±0.0135	37.76	

		100	0.4550±0.0117	55.71	
3.	HMEB	6.25	0.6810±0.0070	7.42	-
		12.5	0.6440±0.0090	12.40	
		25	0.6050±0.0050	17.70	
		50	0.5400±0.0070	26.53	
		100	0.4340±0.0075	40.94	

Values are mean±SD (N=3)

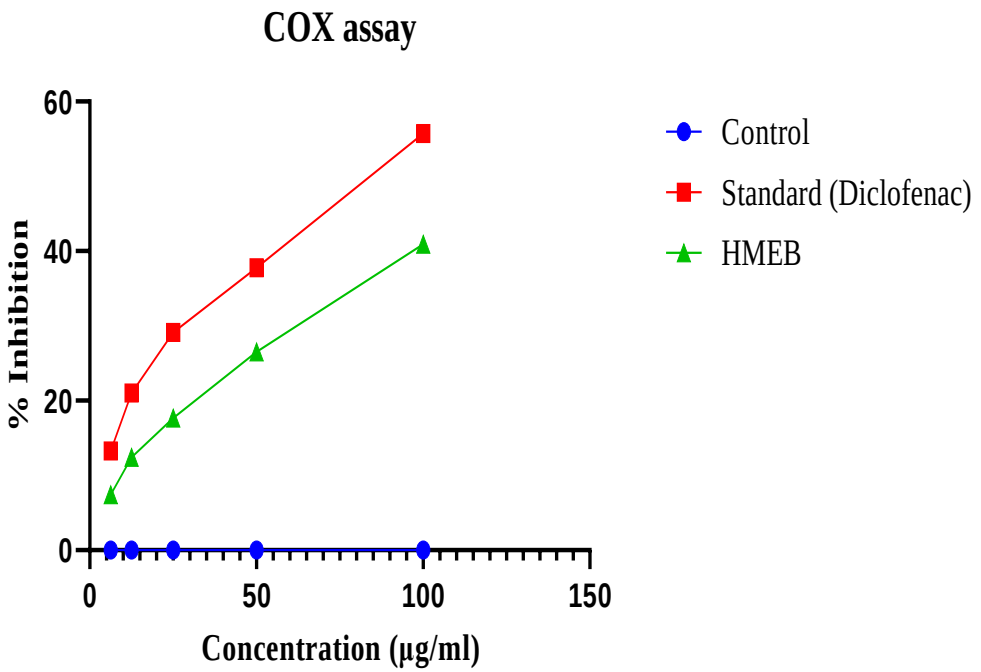


Figure No. 6:- *In vitro*antiinflammatory activity by COX assay.

LOX assay

*Invitro*antiinflammatory activity of the hydro-methanolic extract of bile from *Gallus gallusdomesticus*was evaluated by LOX assay.The results are shown in Table No.9 and Figure No.7HMEB showed maximum inhibition of 35.54% while standard (Diclofenac) showed a maximum of 62.37% at 100µg/ml.

Table No.9:- In vitro anti-inflammatory activity by LOX assay.

Sl.No	Groups	Concentration (µg/ml)	Absorbance (Mean ± SD)	% Inhibition	IC_{50} (µg/ml)
1.	Control	-	0.6390±0.0091	-	-
2.	Standard-Diclofenac	6.25	0.5990±0.0102	6.305	45.98
		12.5	0.5590±0.0104	12.558	
		25	0.4640±0.0170	27.410	
		50	0.3050±0.0070	52.214	
		100	0.2400±0.0115	62.376	
3.	HMEB	6.25	0.6040±0.0070	4.43	-
		12.5	0.5870±0.0060	7.12	
		25	0.5290±0.0070	16.19	
		50	0.4610±0.0140	27.05	
		100	0.4070±0.0106	35.54	

Values are mean±SD (N=3)

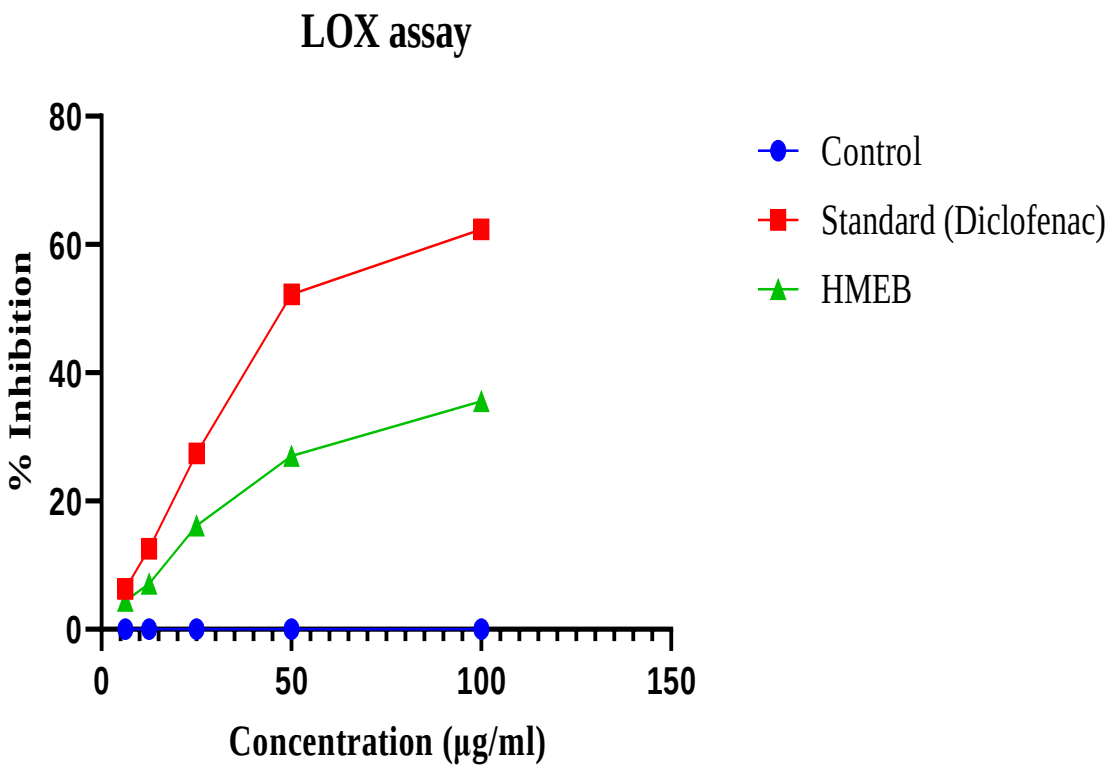


Figure No.7: In vitro anti-inflammatory activity by LOX assay.

MTT assay

*Invitro*cytotoxicity of the hydro-methanolic extract of bile from *Gallus gallusdomesticus*was evaluated by MTT assay. The HMEB was found to cause no significant reduction in cell viability. This indicates that the sample is not cytotoxic to the normal cells.The results are shown in Table No.10 and Figure No.8. HMEB showed a percentage cell viability of 97.41% at 100µg/ml.

Table No.10:- In vitrocytotoxic activity determination by using MTT assay.

Concentration (µg/ml)	Triplicate reading 1 OD	Triplicate reading 2 OD	Triplicate reading 3 OD	Triplicate reading average	Percentage viability
Control	0.687	0.678	0.683	0.683±0.005	-
6.25	0.679	0.685	0.682	0.682±0.003	99.90
12.5	0.674	0.677	0.683	0.683±0.005	99.32
25	0.671	0.673	0.679	0.679±0.004	98.78
50	0.665	0.669	0.676	0.676±0.006	98.14
100	0.658	0.666	0.671	0.671±0.007	97.41

Values are mean±SD (N=3)

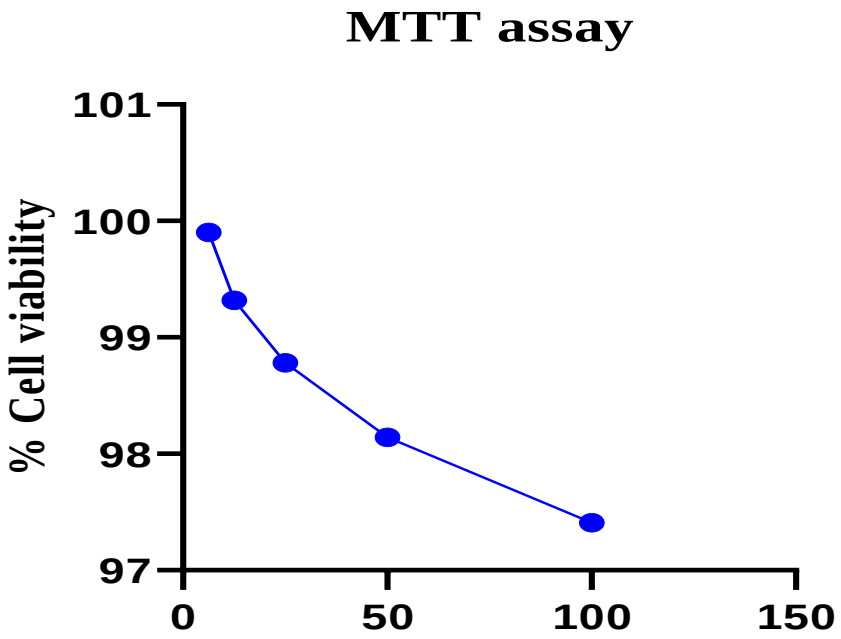


Figure No.8:- In vitrocytotoxic activity determination by using MTT assay.

***Invitro*antidiabetic activity screening methods**

α-Amylase inhibition assay

*Invitro*antidiabetic activity of the hydro-methanolic extract of bile from *Gallus gallusdomesticus*was evaluated by α-amylase inhibition assay. The results are shown in Table No.11 and Figure No.9.HMEB showed maximum inhibition of 75.80 % while standard (Acarbose) showed a maximum of 89.57% at 200µg/ml. The IC_{50} value of the standard-Acarbose was found to be 49.52 µg/ml and that of HMEB was found to be 32.39 µg/ml.

Table No.11:- In vitroantidiabetic activity determination by using α-Amylase inhibition assay.

Sl.No	Groups	Concentration (µg/ml)	Absorbance (Mean ± SD)	% Inhibition	IC_{50} (µg/ml)
1.	Control	-	0.0310±0.0010	-	-
Of		12.5	0.9000±0.0100	8.43	
	Standard-	25	0.8500±0.0100	13.70	
2.	Acarbose	50	0.6400±0.0152	35.83	49.52
		100	0.3400±0.0030	67.84	
		200	0.1300±0.0017	89.57	
		12.5	1.0810±0.0002	11.64	
		25	0.7680±0.0032	37.86	
3.	HMEB	50	0.6970±0.0001	43.80	32.39
		100	0.4920±0.0003	60.97	
		200	0.3150±0.0002	75.80s	

Values are mean±SD (N=3)

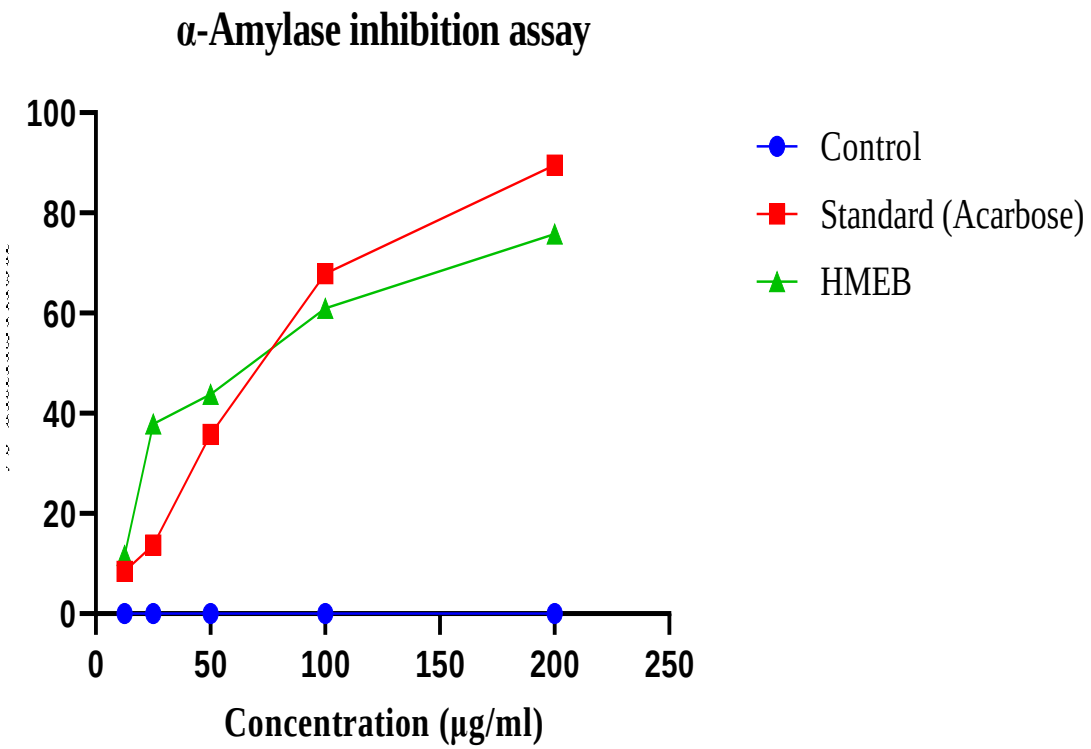


Figure No.9:- *In vitro*antidiabetic activity determination by using α-Amylase inhibition assay.

α- Glucosidase inhibition assay

*In vitro*antidiabetic activity of the hydro-methanolic extract of bile from *Gallus gallusdomesticus*was evaluated by α- glucosidase inhibition assay.The results are shown in Table No.12 and Figure No.10.HMEB showed maximum inhibition of 26.98% while standard (Acarbose) showed a maximum of 97.64% at 200µg/ml.

Table No.12:- In vitroantidiabetic activity determination by using α-Glucosidase inhibition assay.

Sl.No	Groups	Concentration (µg/ml)	Absorbance (Mean ± SD)	% Inhibition	IC_{50} (µg/ml)
1.	Control	-	0.0260±0.0050	-	-
2.	Standard-Acarbose	6.25	0.5670±0.0001	20.20	16.72
		12.5	0.4420±0.0003	38.64	
		25	0.2080±0.0108	73.15	
		50	0.0500±0.0020	96.46	
		100	0.0420±0.0003	97.64	
3.	HMEB	6.25	1.5870±0.0100	13.49	-
		12.5	1.5160±0.0023	17.40	
		25	1.4670±0.0010	20.10	
		50	1.4050±0.0001	23.51	
		100	1.3420±0.0020	26.98	

Values are mean±SD (N=3)

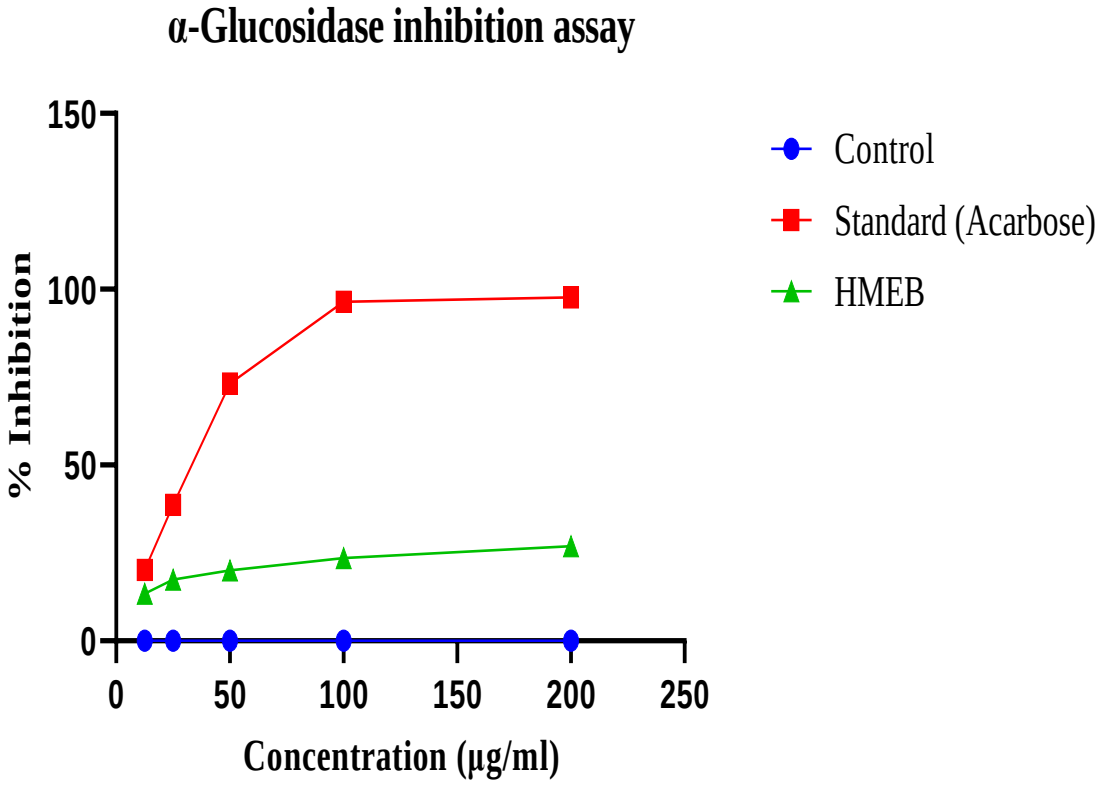


Figure No.10:- *In vitro*antidiabetic activity determination by using α-Glucosidase inhibition assay.

Glucose uptake assay

*In vitro*antidiabetic activity of the hydro-methanolic extract of bile from *Gallus gallusdomesticus*was evaluated by glucose uptake assay.The results are shown in Table No.22 and Figure No.32.HMEB showed maximum uptake of 67.20% while standard (Insulin) showed a maximum of 90.30% at 100µg/ml.

Table No.13:- *In vitro*antidiabetic activity determination by using Glucose uptake assay.

Sl. No	Groups	Concentration (µg/ml)	Absorbance (Mean±SD)	% Glucose uptake
1	Control	-	0.6420±0.0127	36.20
2	Standard-Insulin	25	0.3973±0.0138	60
		50	0.2933±0.0126	70.60
		100	0.0976±0.0101	90.30
3	HMEB	25	0.5400±0.0055	46
		50	0.4460±0.0062	55.40
		100	0.3280±0.0100	67.20

Values are mean±SD (N=3)

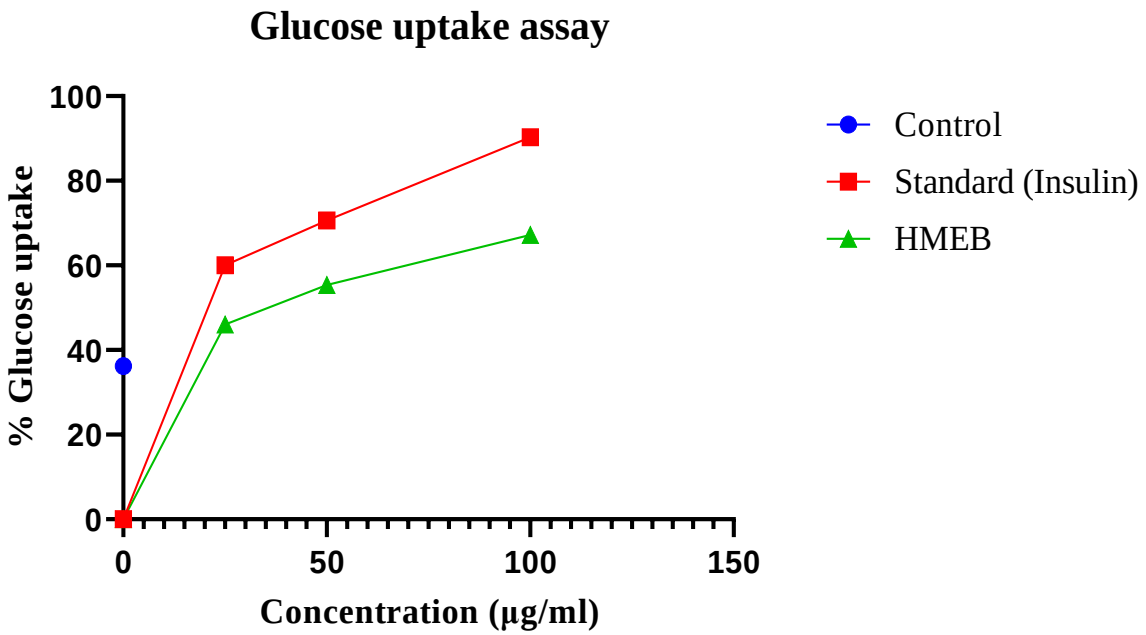


Figure No.11:- *In vitro*antidiabetic activity determination by using Glucose uptake assay.

Gene expression studies

RNA quantification

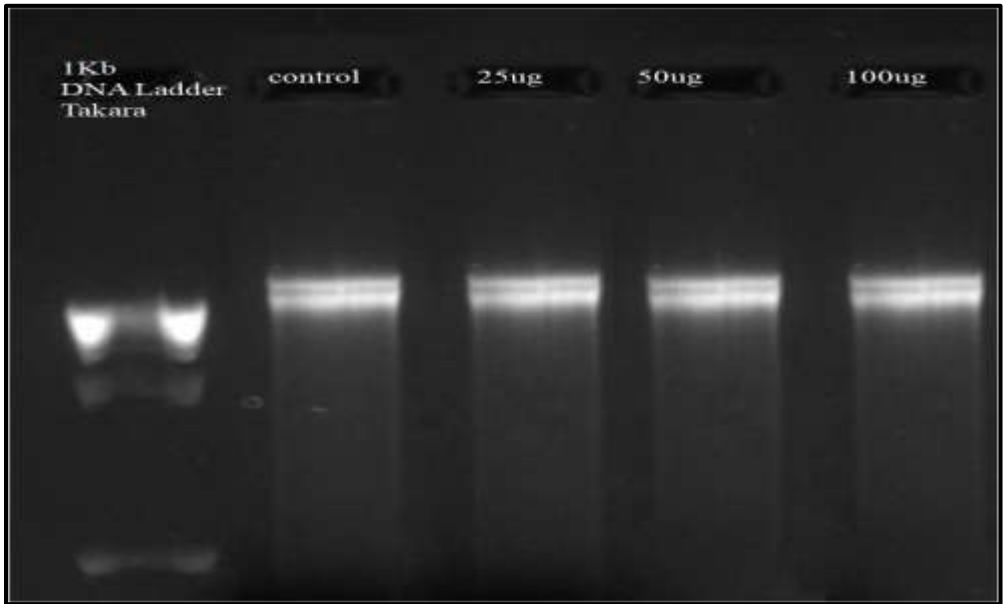


Figure No.12:- RNA as seen on 2 % agarose gel.

Table No.14:- Concentrations of obtained total RNA.

Sample	Concentration (uDrop plate reader)	A260/280	A260/230
Control	100.4 ug/ml	2.14	2.33
25 ug	104 ug/ml	2	2.64
50 ug	76 ug/ml	2.1	2
100 ug	102 ug/ml	2.01	2.8

The absorbance ratio at 260 and 280 nm is used to determine DNA purity. The 260/280 ratio for RNA should ideally be near to 2.0, while the 260/230 ratio should be close to 2. The RNA ratio obtained was within the range, indicating that the RNA isolated was of high quality.

Gene expression analysis

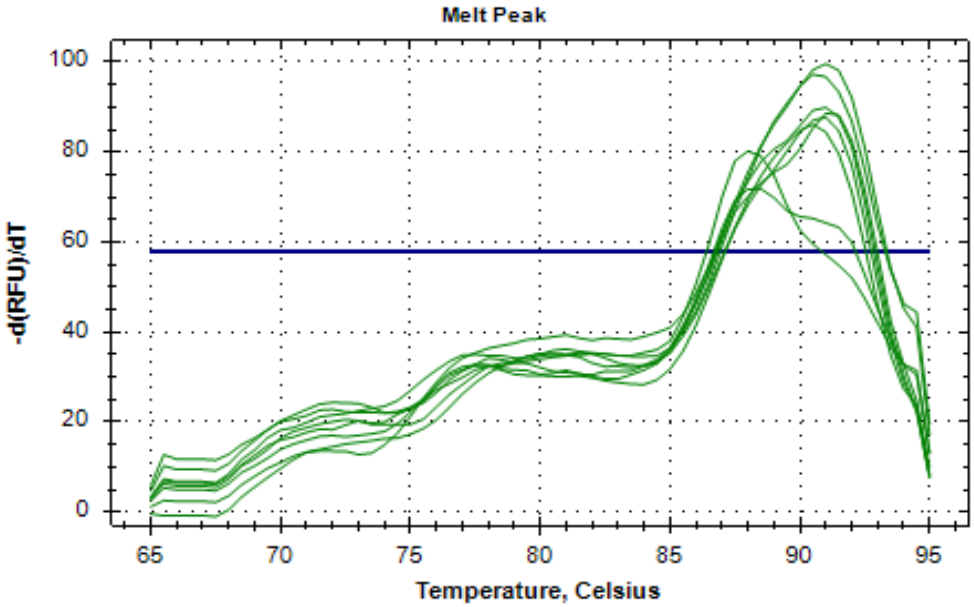


Figure No.13:- Melt peak of β–actin (Raw data of two replicates per sample).

A melting curve records the change in fluorescence observed when double stranded DNA (dsDNA) incorporated dye molecules dissociates or "melts" in to single stranded DNA (ssDNA) as the temperature of reaction is raised. A typical denaturation (melt) melt curve is performed after qPCR cycling, which will typically give rise to a single distinct peak in the plot of the negative derivative of fluorescence versus temperature. In the study majority of the melt curve showed a single distinct peak which indicated that amplified double stranded DNA products are a single discrete species. The presence of multiple peaks in the melt curve typically indicates the presence of contaminating off-target amplification products.

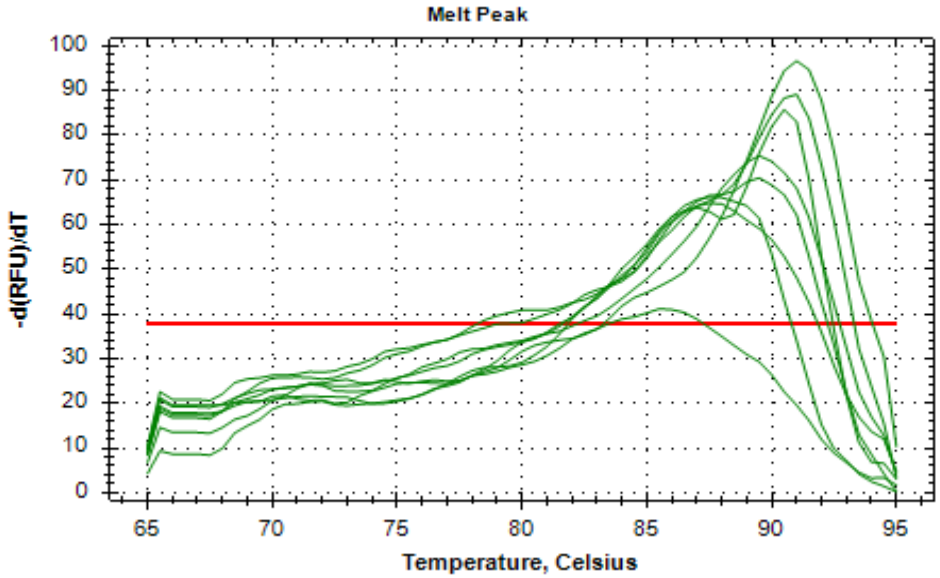


Figure No. 14:- Melt peak of GLUT-4

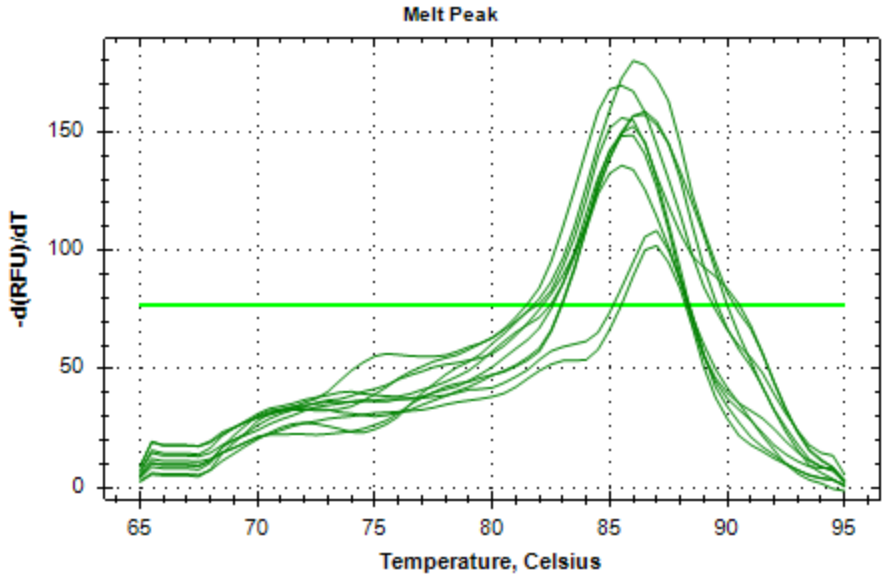


Figure No.15:- Melt peak of pAPG.

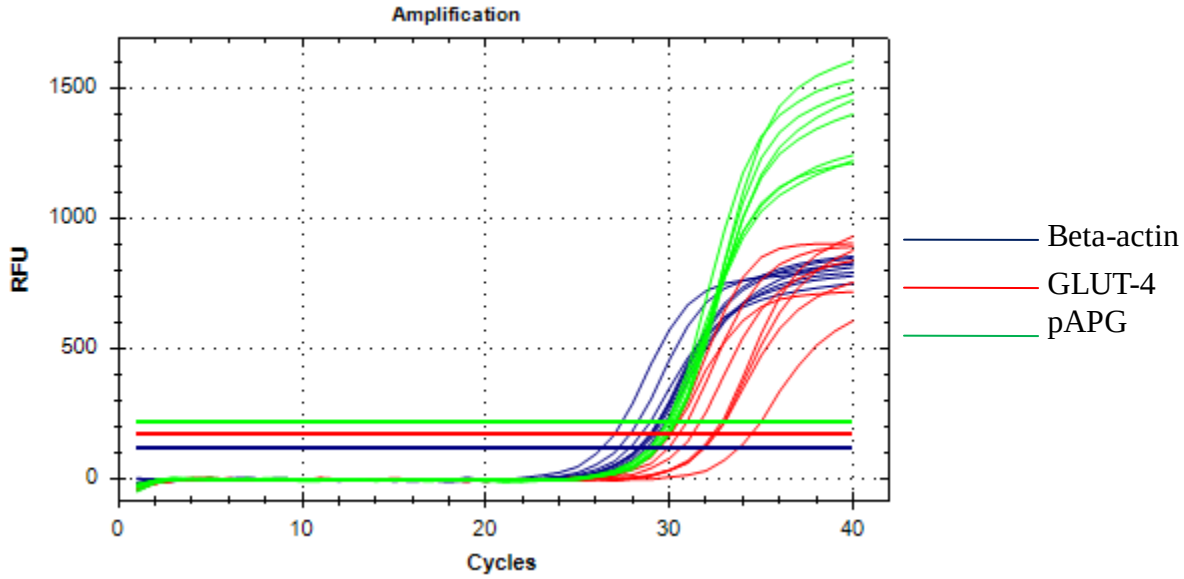


Figure No.16:-Log Scale Amplification curve of samples.

The Cq value is the PCR cycle number at which the samples' reaction curve intersects the threshold line. The higher the Cq value the less the mRNA detected is present because it requires more cycles of amplification to detect the fluorescence.

MeanCq values for β-actin housekeeping gene were 28.49, 26.33, 28.33, and 28.30 for control, 100 µg, 50 µg, and 25 µg respectively, while it was 32.50, 30.04, 32.36, 30.49 for control, 100 µg, 50 µg, and 25 µg respectively for GLUT-4. The average Cq values for pAPG were 29.32, 29.88, 30.35, and 30.15 for control, 100 µg, 50 µg, and 25 µg respectively. In the present study, GLUT-4 and pAPG gene expression of treated groups were compared with control (untreated) after normalization using β-actin gene. The values obtained from the average Cq values of GLUT-4 gene after normalization were found to be 1.0000, 0.5660, and 1.38043 for 100 µg, 50 µg and 25 µg treated groups respectively. The values obtained from the average Cq values of pAPG gene after normalization were found to be 0.9281, 2.2612, and 2.03471 for 100 µg, 50 µg and 25 µg treated groups respectively.

Table No.15:-Ct value β-actin, GLUT 4, pAPG.

Target	Sample	Cq	Cq Mean
GLUT 4	Control	32.50	32.50
GLUT 4	Treated 25	30.49	30.49
GLUT 4	Treated 50	32.36	32.36
GLUT 4	Treated 100	30.04	30.04
β-actin	Control	28.49	28.49
β-actin	Treated 25	28.30	28.30
β-actin	Treated 50	28.33	28.33
β-actin	Treated 100	26.33	26.33
pAPG	Control	29.32	29.32
pAPG	Treated 25	30.15	30.15
pAPG	Treated 50	30.35	30.35
pAPG	Treated 100	29.88	29.88

Normalized expression ΔΔCq

Target	**Sample**	**Expression**	**Expression SEM**	**Mean Cq**	**Cq SEM**
β- actin	Control			28.61	0.1262
β- actin	Treated 100			26.79	0.4550
β- actin	Treated 50			28.07	0.2300
β- actin	Treated 25			28.35	0.0159
GLUT-4	Control	0.33049	0.2246	33.47	0.9723
GLUT-4	Treated 100	1.00000	0.3154	30.05	0.0071
GLUT-4	Treated 50	0.56604	0.4252	30.87	0.3802
GLUT-4	Treated 25	1.38043	0.0294	32.43	0.0733
pAPG	Control	3.54209	0.3388	29.98	0.0558
pAPG	Treated 100	0.92811	0.3200	30.08	0.2009
pAPG	Treated 50	2.26128	0.3438	30.23	0.0808
pAPG	Treated 25	2.03471	0.02827	30.36	0.0083

Table No.16:-Normalized expression relative to control.

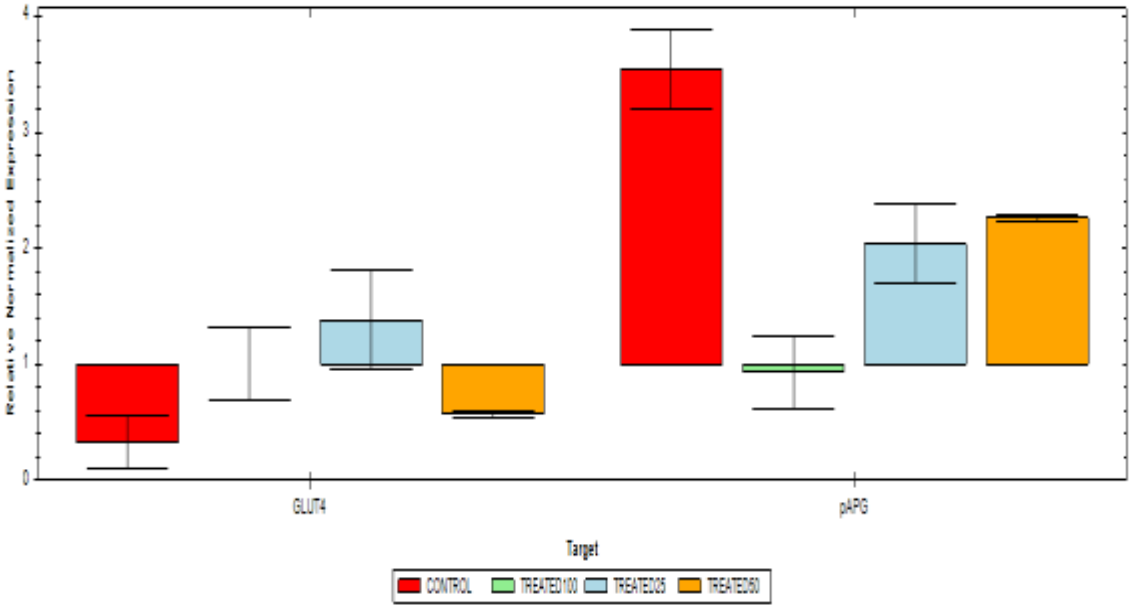


Figure No.17:- Normalized expression relative to control.

In qPCR, normalization is done effectively to correct the differences between the compared sample. Achieving reliable results is only possible after the application of an appropriate normalization method. The above expression data after normalization clearly showed the increased expression of GLUT-4 compared to control on treatment with HMEB whereas the expression of pAPGwas found to be decreased compared to control on treatment with HMEB. The increased expression of the GLUT-4 may due to the fact that GLUT -4 might be the sole responsible gene participating in the glucose absorption when treated with HMEB. It is found that the maximum expression of GLUT-4 gene is found at the concentration of 25 µg/ml and this could be the optimum concentration of GLUT-4 getting expressed in maximum. The increased expression of GLUT-4 increases glucose uptake.[32] The treatment of HMEB in L929 cells thereby upregulates the expression of GLUT-4 which in turn promotes glucose uptake. Increased glucose uptake lowers blood glucose level. [31, 56]

Animal toxicity study

Results of the acute toxicity profile of hydro methanolic extract of bile

A limit test at one dose level of 2000 mg/kg body weight may be carried out with six animals.(Limit test OECD 423). No mortality and morbidity were noticed during the entire study period of HMEB-treated animals. Similarly, there was no significant change in CNS, ANS, and CVS related behavioral activity and the sensory responses of the drug-treated group.

Clinical signs	Animal 1	Animal 2	Animal 3		Animal 4	Animal 5	Animal 6
Lacrimation	Absent	Absent	Absent		Absent	Absent	Absent
Salivation	Absent	Absent	Absent		Absent	Absent	Absent
Animal appearance	Normal	Normal	Normal		Normal	Normal	Normal
Convulsion	Absent	Absent	Absent		Absent	Absent	Absent
Skin colour	Normal	Normal	Normal		Normal	Normal	Normal
Diarrhoea	Absent	Absent	Absent		Absent	Absent	Absent
Touch response	Normal	Normal	Normal		Normal	Normal	Normal
Mortality	Nil	Nil	Nil		Nil	Nil	Nil
Behaviour	Normal	Normal	Normal		Normal	Normal	Normal

Table No.17:- Effect of HMEB on clinical signs of rats in acute toxicity study .

Effect of HMEB on body weights of female rats in an acute toxicity study

As per the OECD guidelines, the acute toxicity limit test study of HMEB was carried out at the dose level of 2000mg/kg.b.w. The test drug HMEB was administered at the maximum of 2000 mg/kg b.w did not reveal any abnormal clinical signs in any of the animals. All the rats survived and no treatment-related mortality occurred during 14 days. No weight loss was observed in any of the groups treated with HMEB.

Table No.18:-Effect of HMEB on the body weight of female rats in the acute toxicity study.

Treatment groups	Bodyweight (g)		
	1st day	7th day	14th day
Animal 1	132	159	166
Animal 2	149	164	173
Animal 3	158	175	186
Animal 4	127	151	166
Animal 5	133	164	179
Animal 6	109	122	134

Oral Glucose Tolerance Test (OGTT)

The results were analysed and AUC during OGTT were calculated using Graph Pad Prism 9.0 software. The reduction of calculated AUC was observed as shown in Figure No.40. The HMEB (high dose) showed significant blood glucose lowering compared to control at 60 minutes.The HMEB (low dose)also lowered blood glucose in normal rats, but found less significant.

Table No.19:- Blood glucose level of treatment groups.

Treatment groups		**Blood glucose (mg/dl)**					
		-30 min	**0 Min**	**30 min**	**60 min**	**120 min**	**180 min**
Control	Rat 1	128	127	169	149	132	129
	Rat 2	127	125	164	142	130	126
	Rat 3	126	126	162	153	128	127
	Rat 4	129	127	160	147	131	126
	Rat 5	126	125	159	149	129	124
	Rat 6	130	128	163	145	134	130
Standard (Glibenclamide)	Rat 1	101	100	105	65	64	62
	Rat 2	94	64	113	98	93	82
	Rat 3	98	87	123	89	85	84
	Rat 4	108	105	131	112	101	99
	Rat 5	95	90	143	113	90	86
	Rat 6	123	120	148	127	109	102
HMEB (low dose 100 mg/Kg)	Rat 1	120	114	152	125	108	102
	Rat 2	122	118	143	124	112	106
	Rat 3	118	113	149	125	104	94
	Rat 4	121	115	135	121	107	100
	Rat 5	119	118	147	123	106	104
	Rat 6	123	120	148	127	109	102
HMEB (high dose 400 mg/Kg)	Rat 1	94	92	121	103	95	90
	Rat 2	115	108	126	122	113	111
	Rat 3	118	96	127	100	74	70
	Rat 4	91	85	118	99	85	82
	Rat 5	87	86	122	112	102	98
	Rat 6	91	90	114	131	125	119

Table No.18:- Mean blood glucose level of treatment groups.

Treatment groups	Blood glucose (mg/dl) at (min)					
	-30	0	30	60	120	180
Control	127.70 ±0.6667	126.30 ±0.4944	162.80 ±1.4470	147.50 ±1.5440	130.70 ±0.8819	127.00 ±0.8944
Standard (Glibenclamide)	103.2 ±4.468	94.33 ±7.740	127.2 ±6.853	100.7 ±8.924	90.33 ±6.302	85.83 ±5.833
HMEB (low dose)	120.50 ±0.7638	116.30 ±1.116	145.70 ±2.445	124.20 ±0.8333	107.70 ±1.116	101.30 ±1.687
HMEB (high dose)	99.33 ±5.518	92.83 ±3.449	121.3 ±1.994	111.2 ±5.326	99.00 ±7.567	95.00 ±7.439

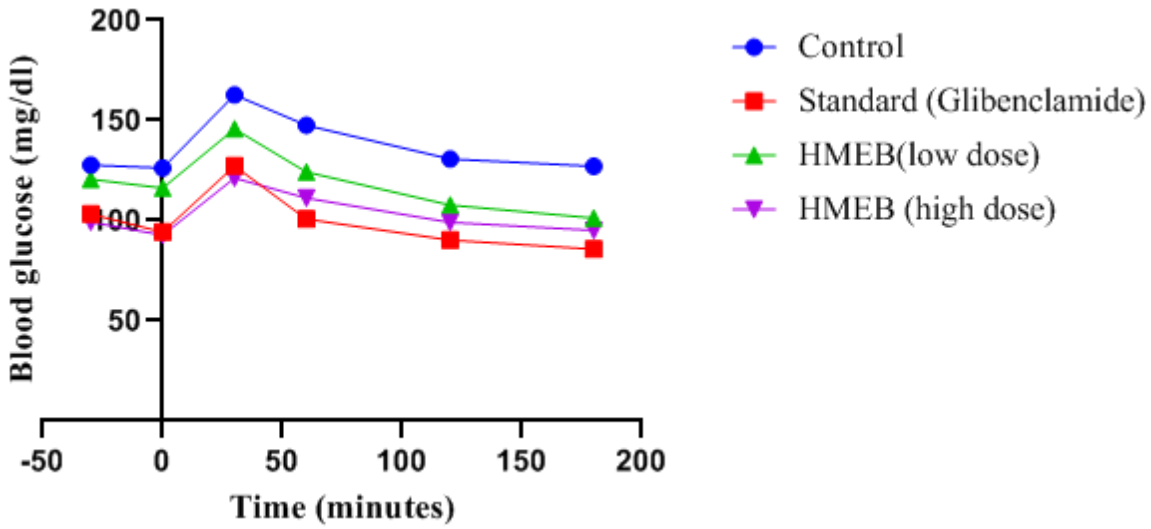


Figure No.18:- Glucose tolerance curve.

Animals	Blood glucose (mg/dl) at 60 minutes			
	Control	**Standard (Glibenclamide)**	**HMEB (low dose)**	**HMEB (high dose)**
1	149	65	125	103
2	142	98	124	122
3	153	89	125	100
4	147	112	121	99
5	149	113	123	112
6	145	127	127	131
Mean ± SEM	147.50 ±1.5440	100.70 ±8.924 ****	124.20 ±0.8333 **	111.20 ±5.326 ****

Discussion:-

The use of bile acid chelates is currently the sole approved therapeutic option for the treatment of diabetes related to bile acids. Bile from domesticated animal species (such as cow, chicken and pig) has been utilized as a substitute for bear bile in Traditional Chinese Medicine practices. The antidiabetic potential of a methanolic extract of chicken bile was explored in this work. The IR spectra of HMEB obtained by FTIR analysis were compared to reference values in the literature which proved the authenticity of the bile collected from *Gallus gallusdomesticus* and the same was utilized for making HMEB.[4] The UV absorbance of freshly prepared hydro-methanolic extract of bile from *Gallus gallusdomesticus* was taken and it was allowed to stand for one month and the measurement was repeated. No significant difference in absorbance indicated the stability of the compound during its one-month storage period.

*In vitro*antioxidant activity of the hydro-methanolic extract of bile from *Gallus gallusdomesticus*was evaluated by DPPH radical scavenging method. HMEB showed a maximum inhibition of 27.88 % while standard (Ascorbic acid) showed a maximum of 89.75% at 200µg/ml.In vitro antioxidant activity of the hydro-methanolic extract of bile from *Gallus gallusdomesticus*was evaluated by ABTS radical scavenging assay. HMEB showed maximum inhibition of 20.58% while standard (Ascorbic acid) showed a maximum of 94.76% at 200µg/ml.*In vitro*antioxidant activity of the hydro-methanolic extract of bile from *Gallus gallusdomesticus*was evaluated by nitric oxide radical scavenging assay. TheHMEB showed maximum inhibition of 31.30 % while standard (Ascorbic acid) showed a maximum of 73.27% at 100µg/ml. The HMEB was found to cause no significant enhancement in lipid peroxidation in L929 cell lines.This indicated that the HMEB does not cause the generation of free radicals as well as associated cell membrane damage in normal cells.

*In vitro*antiinflammatory activity of the hydro-methanolic extract of bile from *Gallus gallusdomesticus*was evaluated by COX assay.HMEB showed maximum inhibition of 40.94% while standard (Diclofenac) showed a maximum of 55.71% at 100µg/ml.*In vitro*antiinflammatory activity of the hydro-methanolic extract of bile from *Gallus gallusdomesticus*was evaluated by LOX assay.HMEB showed maximum inhibition of 35.54% while standard (Diclofenac) showed a maximum of 62.37% at 100µg/ml.

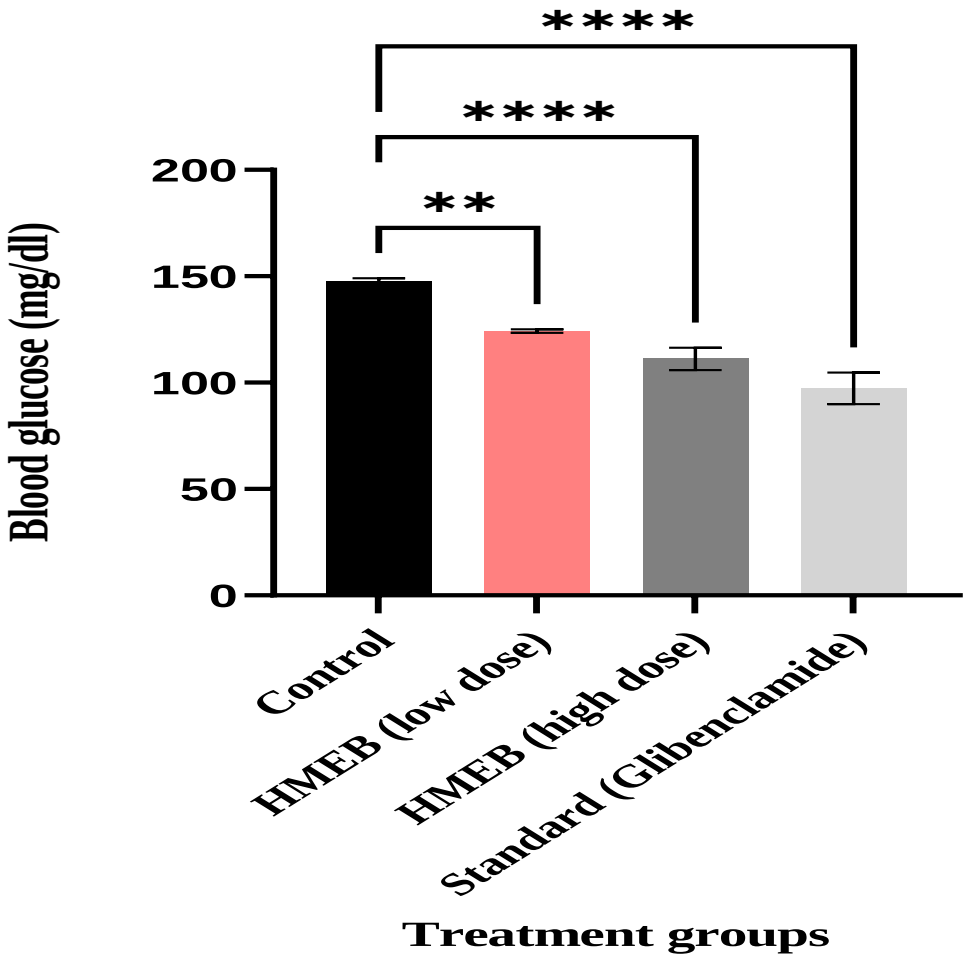


Figure No.19:- Blood glucose level (mg/dl) at 60 minutes of various treatment groups.

*In vitro*cytotoxicity of the hydro-methanolic extract of bile from *Gallus gallusdomesticus*was evaluated by MTT assay. The HMEB was found to cause no significant reduction in cell viability. This indicates that the sample is not cytotoxic to the normal cells.Sample showed percentage cell viability of 97.41% at 100µg/ml.

The α- amylase inhibition assay was used to assess the antidiabetic effect of a hydro-methanolic extract of bile from *Gallus gallusdomesticusin vitro*. At 200 g/ml, HMEB showed a maximum inhibition of 75.80%, while standard (Acarbose) showed a maximum inhibition of 89.57%. The glucose absorption assay was used to assess the antidiabetic effect of a hydro-methanolic extract of bile from *Gallus gallusdomesticus in vitro*. At 100 g/ml, HMEB had a maximum uptake of 67.20%, while insulin had a maximum uptake of 90.30%. The glucose absorption assay was used to determine the antidiabetic effect of a hydro-methanolic extract of bile from *Gallus gallusdomesticus in vitro*. At 100 g/ml, HMEB had a maximum uptake of 67.20%, while insulin had a maximum uptake of 90.30%.

RNA quantification results showed that RNA isolated was of good quality. MeanCq values for β-actin housekeeping gene were 28.49, 26.33, 28.33, and 28.30 for control, 100 µg, 50 µg, and 25 µg respectively, while it was 32.50, 30.04, 32.36, 30.49 for control, 100 µg, 50 µg, and 25 µg respectively for GLUT-4. The average Cq values for

pAPG were 29.32, 29.88, 30.35, and 30.15 for control, 100 µg, 50 µg, and 25 µg respectively. In the present study, GLUT-4 and pAPG gene expression of treated groups were compared with control (untreated) after normalization using β-actin gene. The values obtained from the average Cq values of GLUT-4 gene after normalization were found to be 1.0000, 0.5660, and 0.1.38043 for 100 µg, 50 µg and 25 µg treated groups respectively. The values obtained from the average Cq values of pAPG gene after normalization were found to be 0.9281, 2.2612, and 2.03471 for 100 µg, 50 µg and 25 µg treated groups respectively. The above expression data after normalization clearly showed the increased expression of GLUT-4 compared to control on treatment with HMEB whereas the expression of pAPGwas found to be decreased compared to control on treatment with HMEB. The increased expression of the GLUT-4 may due to the fact that GLUT -4 might be the sole responsible gene participating in the glucose absorption when treated with HMEB. It is found that the maximum expression of GLUT-4 gene is found at the concentration of 25 µg/ml and this could be the optimum concentration of GLUT-4 getting expressed in maximum. The increased expression of GLUT-4 increases glucose uptake.[32]The treatment of HMEB in L929 cells may upregulates the expression of GLUT-4 which in turn promotes glucose uptake. Increased glucose uptake lowers blood glucose level. [31, 56]

Animal toxicity study revealed that the HMEB is safer for oral administration. The clinical signs of the animals were found to be normal and abnormalities were absent. Mean body weight of the animals on Day 1, Day 7, Day 14 shows increment in their body weight.

OGTT was conducted in normal rats. TheHMEB (high dose 400 mg/Kg)and HMEB (low dose100 mg/Kg) shows significant blood glucose lowering compared to control.TheHMEB (low dose100 mg/Kg)also lowered blood glucose level in normal rats, but was found to be less significant.

Conclusion:-

The pharmacological investigation of bile from Gallus gallusdomesticus have been explored in this thesis.Preliminaryzoochemical analysis revealed the presence of steroids.The results showed that hydro-methanolic extract of bile possessed invitro antioxidant activity, antiinflammatory activity, and antidiabeticactivity.The HMEB does not cause significant lipid peroxidation in normal cells and found to be non-toxic to normal cells *in vitro*.

In vitro cytotoxicity of the hydro-methanolic extract of bile from *Gallus gallusdomesticus*was evaluated by MTT assay. The sample caused no significant reduction in cell viability, indicated that sample is not cytotoxic to normal cells.Acute toxicity studies were conducted in rats using OECD guidelines 423 and it was found that the maximum dose of 2000mg/kg was safe. $1/5^{th}$ and $1/20^{th}$doses of 2000 mg/Kg of HMEB were selected for conducting OGTT.

The antidiabetic efficacy of a hydro-methanolic extract of bile from *Gallus gallusdomesticus* was dose dependent. *In vitro*, the sample inhibited α-amylase and α-glucosidase enzymes and showed increased glucose absorption in L6 cells in glucose uptake assay. The HMEB has shownantihyperglycemic effects in vivo. The findings lay the groundwork for the HMEB to be developed as a potential antidiabetic agent. However, more research is needed to investigate its biomolecular targets and antidiabetic effectiveness in humans.

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