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Extraction and Isolation of Marmelosin from *Aegle Marmelos*, Synthesis and Evaluation of Their Derivative as Antidiabetic Agent

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ABSTRACT

Diabetes is one of the major health problems, the world is facing today. It is rising in an alarming rate necessitating alternative treatment methods. The present study has been undertaken to evaluate the anti-diabetic activity of marmelosin extracted from Aegle marmelos. The extraction of plant material in different solvent like petroleum ether, chloroform and ethanol by soxhelation was carried out. Marmelosin was separated out from extraction of Aegle marmelos by Column chromatography methods. Marmelosin derivatives were synthesized using Nitration, Sulphonation and Bromination reaction. The synthesized compounds were evaluated for anti-diabetic activitiy. The effect of marmelosin and its derivatives on hyperglycemia induced with alloxan on repeated administration was studied on male wistar rat. The estimation of blood sugar was carried out by the method GOD-PAP enzymatic method. The results were satisfactory, as significant decrease in the blood glucose levels after repeated administration was observed.

Key Words: Aegle marmelos, Marmelosin, Extraction, Antidiabetic Activity.

INTRODUCTION

Diabetes mellitus is a metabolic disorder initially characterized by a loss of glucose homeostasis with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both.

Without enough insulin, the cells of the body cannot absorb sufficient glucose from the blood; hence blood glucose level increase, which is termed as hyperglycemia. If the glucose level in the blood remains high over a long period of time, this can result in long-term damage to organs, such as the kidneys, liver, eyes, nerves, heart and blood vessels. Complications in some of these organs can lead to death.[1]
Globally, the prevalence of diabetes, without type distinction, was estimated to be 4% in 1995. According to WHO, it is estimated that 3% of the world's population have diabetes and the prevalence is expected to double by the year 2025 to 6.3%.

There will be a 42% increase from 51 to 72 million in the developed countries and 170% increase from 84 to 228 million, in the developing countries. Thus, by the year 2025, over 75% of all people with diabetes will be in the developing countries, as compared to 62% in 1995. The reasons behind this projected increase in prevalence rate are due to urbanization, westernization and their associated lifestyle changes, increase in life expectancy at birth, physical inactivity and obesity and possibly a genetic predisposition. [1,2]

Age, ethnic, regional and racial differences have also been found to play a role for the diabetic incidence in heterogeneous populations within the same area. [3]

In many countries, herbal therapies are among the most popular of all "alternative treatments". *Aegle marmelos* has been used for centuries as an herbal medicine. It is commonly known as Bael, is indigenous to India and is one of the most useful medicinal plants in India. Its stem, bark, root, leaves and fruits have medicinal value. The ancient systems of medicine, including Roman, Ayurveda, Greek, Siddha and Unani have mentioned its therapeutic applications in cardiovascular disorders, diabetes, diarrhoea and dysentery. Other actions like antifungal, antibacterial, antiprotozoal, hypoglycemic, antioxidant, antiviral and cardioprotective effects have been studied using various parts of the plant. Besides its antioxidant properties, *Aegle marmelos* unripe fruit aqueous extract interacts by various other mechanisms in a complex way to elicit its therapeutic effects.

Roots and fruits contain coumarins such as scoparone, scopoletin, umbelliferone, marmesin and skimmin. Fruits in addition, contain xanthotoxol, imperatorin and alloimperatorin and alkaloids like aegeline and marmeline identified as N-2-hydroxy-2-[4 -(3', 3'-dimethyl allyloxy) phenylethyl] cinnamide. β- sitosterol and its glycoside are also present in the fruits. Roots and stem barks contain a coumarin-aegelinol. Roots also contain psoralen, xanthotoxin, 6,7-dimethoxy coumarin, tembamide, mermin and skimmianine.

Leaves contain the alkaloids O-(3,3-dimethyl allyl)-halfordinol, N-2-ethoxy-2-(4-methoxy phenyl)ethyl cinnamide, N-2-methoxy-2-[4-(3',3'-dimethyl allyloxy) phenylethyl] cinnamide, N- 2-[4-(3',3'-dimethyl allyloxy) phenylethyl] cinnamide, N-2-hydroxy-2-[4-(3',3'-dimethyl allyloxy) phenylethyl] cinnamide, N-4-methoxy steryl cinnamide and N-2-hydroxy-2-(4-hydroxy phenyl) ethyl cinnamide. Mermesinin, rutin and β -sitosterol - β -D-glucoside are also present in the leaves. [4,5]

2. Extraction and Purification

The plant of *Aegle marmelos* was collected from the Jodhpur District of Rajasthan, India Authentifiaction No.-BSI/AZRC/A.19014/SE-1/Estt. The voucher specimen was deposited for the future reference in Jodhpur national university, Jodhpur.

2.1 Preliminary Phytochemical Tests

It comprises of different chemical tests and chemical assays. The purity of crude drugs is ascertained by quantitative estimation of active chemical constituents present in them. The method may be useful in determining single active constituent or the group of related constituents present in the same drug. [6]

2.2. Extraction by Continuous hot percolation (Soxhlet extraction)

The plant material was grinded in pastel mortar and mixer. The powder was passed through mesh No.10 and retain on mesh No.60 was used for extraction. The drug was packed in a paper cylinder made from a filter paper and it was placed in the body of Soxhlet extractor. The solvent was placed in the flask. The apparatus was then fitted as appropriate manner. [7]

2.3. Seperation of Marmelosin from Extract

2.3.1. Thin layer chromatography

The extract was subjected to thin layer chromatography. The techniques formulated by Stahl (1965) were used in TLC method for the optimization of solvent for column chromatography.

2.3.2. Column chromatography

In column chromatography, the mobile phase is a solvent and the stationary phase is a finely divided solid, such as silica gel. Wet method was used for the preparation of column; slurry of the silica gel was prepared for column by ethyl acetate, methanol,and hexane as an eluent in (1:1:1.5) ratio and then carefully poured into the column. The eluent was collected in different test tube with a volume of 2 ml in each test tube. The eluent was dried and marmelosin was separated out. Confirmation of marmelosin (Figure 1) was done by chemical test, melting point and spectroscopy (IR and NMR). [8]

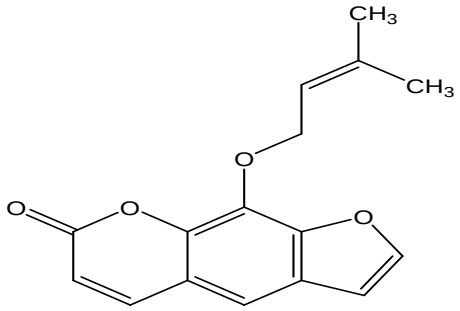

Figure 1: Structure of marmelosin

Characterization: Melting point: 99-100 °C, Rf value: 0.52, λ max (nm): 282, IR (cm^{-1}): 3050.45, 1612.25, 2901.25, 1640.25, 1078.71. ^1H NMR (400 MHz, $CDCl_3$): 6-8.5(1H, s, Ar-H), 3.3-4(1H, s, C-O-C), 1.7(2H, m, -C-C=CH_3), 1.17-1.88(3H, m, -CH)

3. SYNTHESIS OF MARMELOSIN DERIVATIVE

3.1. Synthesis of 9-[(2-methylprop-1-en-1-yl)peroxy]-5-nitro-7H-furo[3,2-*g*]chromen-7-One.

In a 250 ml round bottom flask, 0.35 ml of concentrated nitric acid and 0.4 ml of concentrated sulphuric acid was taken The round bottom flask was immersed in the cold water, then added 0.95 gm of compound and shaken frequently so that the temperature does not rise above 55°C. After that fitted a reflux condenser and heated in water bath at 60°C for 40-45 minutes with vigorous shaking. Poured the reaction mixture in a beaker containing 500 ml of cold water, stirred well and filtered off the residual liquid, washed the residual liquid with water, discarded the aqueous layer, dried the residue and recrystallized with ethanol. (Figure 2)

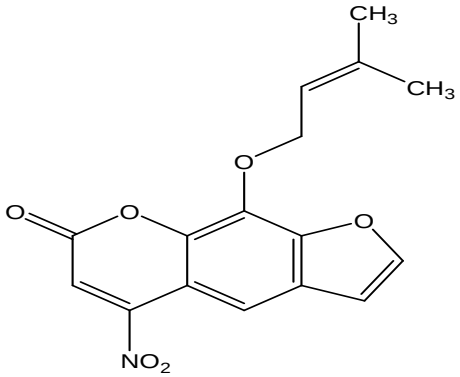

Figure 2: 9-[(2-methylprop-1-en-1-yl)peroxy]-5-nitro-7H-furo[3,2-*g*]chromen-7-One.

Characterization: Melting point: 192-194 °C, Yield: 88.34 %, Rf value: 0.54, λ max (nm): 277.2, IR (cm^{-1}): 3050.25, 1612.09, 2900.15, 1640.25, 1079.73, 1542.69. ^1H NMR (400 MHz, $CDCl_3$): 6-8.5(1H, s, Ar-H), 3.3-4(1H, s, C-O-C), 1.17-1.88(3H, m, -CH), 0.9(3H, m, -CH_3).

3.2 Synthesis of 9-[(2-methylprop-1-en-1-yl)peroxy]-7-oxo-7H-furo[3,2-*g*]chromene-5-sulfonic acid.

0.07 ml of concentrated sulphuric acid and 1 gm of compound was taken in a round bottom flask, then 0.35 ml of cold water and 0.8 gm of sodium bi carbonate was added to this solution with constant stirring. Now added 0.065 gm of sodium chloride to it and heated until it dissolved, filtered the solution while hot and cool the filtrate in an ice bath with frequent stirring when the sodium chloride separated out and filtered the product washed with 0.05 ml of filtered solution of saturated sodium chloride, dried in oven at 110°C and recrystallized with ethanol. (Figure 3)

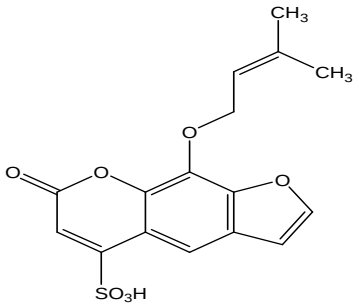

Figure 3: 9-[(2-methylprop-1-en-1-yl)peroxy]-7-oxo-7H-furo[3,2-*g*]chromene-5- sulfonic acid

Characterization: Melting point: 202-204 °C, Yield: 81.40 %, Rf value: 0.47, λ max (nm): 281, IR (cm^{-1}): 3050.25, 1612.09, 2900.15, 1640.25, 1079.73, 1169.72. ^{1}H NMR (400 MHz, $CDCl_3$): 6-8.5(1H, s, Ar-H), 3.3-4(1H, s, C-O-C), 1.7(2H, m, -C-C=CH_3).

3.3. Synthesis of 5-bromo-9-[(2-methylprop-1-en-1-yl)peroxy]-7H-furo[3,2-*g*]chromen-7-one.

In a 250 ml round bottom flask, taken 0.5 mol of pyridine and 1 gm of compound. The round bottom flask was fitted with reflux condenser and placed in cold water. Poured 0.24 ml bromine, heated on water bath at 25-30°C for 1 hour with occasionally shaking heated further at 65-70°C for 45 minutes. Now added 10% aqueous sodium hydroxide solution shaken it vigorously, filtered off the residual liquid, wash the residual liquid with water again and again so that the alkali is completely removed, dried the residue and recrystallized with ethanol. (Figure 4)

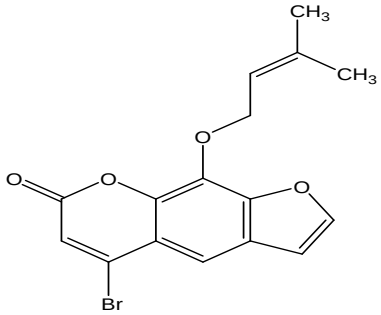

Figure 4: 5-bromo-9-[(2-methylprop-1-en-1-yl)peroxy]-7H-furo[3,2-*g*]chromen- 7-one

Characterization: Melting point: 198-200 °C, Yield: 87.24 %, Rf value: 0.57, λ max (nm): 280, IR (cm^{-1}): 3050.25, 1612.09, 2900.15, 1640.25, 1079.73, 1169.72, 550.02. ^{1}H NMR (400 MHz, $CDCl_3$): 6-8.5(1H, s, Ar-H), 3.3-4(1H, s, C-O-C), 1.7(2H, m, -C-C=CH_3).

4. Anti-Diabetic Activity of Synthesized Compounds on Male Wistar Rats

Antidiabetic studies were carried out using male and female wistar albino rats (150-300 g). The animal were grouped and housed in clean polyacrylic cages (38x23x10 cm) with not more than five animals per cage and mentioned under standard laboratory conditions (temperature 25±2°C) and light cycle (12 h light and 12 h dark). They were fed with standard pellet diet and water *ad. labitum*. All procedures described were reviewed and approved by the Institutional Animal Ethical Committee.

Alloxan monohydrate was purchased from Chemdyes Corporation, Ahmadabad. All other chemicals used for this study were of analytical grade. The solutions were prepared by dissolving synthesized derivative in distilled water. Diabetes was induced in the rats by administering 110 mg/Kg of alloxan intraperitonially into the 24 hr fasted rats. After 72 hr, the blood samples were collected and analyzed for blood glucose level. Albino rats which showed more than 180-200 mg/dl blood glucose levels were considered as diabetic. These animals were used for further studies.

Healthy albino rats were randomly distributed into 4 groups of 5 animals in each group. All the animals were fasted for 18 hr before sampling. The groups were divided as following:

Group (I):- *Normal control*
Group (II):- *Diabetic control*
Group (III):- *Diabetic rats, treated aqueous Compound A (50 mg/Kg).*
Group (IV):- *Diabetic rats, treated with Compound B (50 mg/Kg).*
Group (V):- *Diabetic rats, treated with Compound C (50 mg/Kg).*
Group (VI):- *Diabetic rats, treated with Compound D (50 mg/Kg).*
Group (VII):- *Diabetic rats, treated with Standard drug (Gliclazide) (10 mg/Kg).*

4.1. Blood sampling and biochemical analysis

The compound and derivative were dissolved in distilled water and given orally by oral feeding needle. Blood sample were collected at day 0, 3, 7 and 14 for all 4 groups by retro orbital plexus of rats with the help of micro capillary tubes. The blood samples were collected in micro centrifuge tubes (Eppendorf tubes) and centrifuge for 10 minutes at 10,000 rpm. Blood glucose level was estimated by GOD - PAP method by using Elitech glucose SL kit (Logotech pvt. ltd., Delhi) with the help of autoanalyser (Rapid, star 21) at filter range 500 - 550 nm. The blood glucose level was expressed as mg/dl of blood.

4.2. Antidiabetic Activity of Marmelosin and Their Derivatives

As describe in the section 4.8, Antidiabetic activity of synthesized compounds on male wistar rats were determined. The result were complied in Table No. 1-7 and Figure 5.

Table:1 Group (I) Normal control

S.No.	Animal mark	Body wt. (gm)	Blood glucose levels (mg/dl)			
			Day 0	Day 3	Day 7	Day 14
1	Head & Back	178	112.2	114.3	119.2	120
2	Tail	164	96.4	101.2	106	102
3	Head &Tail	156	120.1	118	116	118
4	Head	182	106.5	109.1	113	114
5	Unmark	194	109.2	106	104	105
Mean			**108.88**	**111.64**	**109.72**	**111.8**
Standard deviation (SD)			**8.63**	**6.48**	**5.93**	**7.94**
Standard error mean (SEM)			**3.86**	**2.89**	**2.65**	**3.55**

TABLE 2: Group (II) Diabetic control

S. No.	Animal mark	Body wt. (gm)	Blood glucose levels (mg/dl)			
			Day 0	Day 3	Day 7	Day 14
1	Head & Back	170	252	241	248	251
2	Tail	195	243	242	240	242
3	Head &Tail	184	248	244	246	245
4	Head	160	270	265	260	280
5	Unmark	159	278	273	268	278
Mean			**108.88**	**111.64**	**258.2**	**259.2**
Standard deviation (SD)			**8.63**	**6.48**	**15.03**	**18.37**
Standard error mean (SEM)			**3.86**	**2.89**	**6.72**	**8.21**

Table 3: Group (III) Effect of Compound A on blood glucose level after daily administration for 14 days

S.No.	Animal mark	Body wt. (gm)	Blood glucose levels (mg/dl)			
			Day 0	Day 3	Day 7	Day 14
1	Back	245	223	211	181	139
2	Tail	235	230	214	176	132
3	Unmark	213	250	226	187	133
4	Head &Tail	208	291	255	193	129
5	Back & Tail	194	235	208	171	124
Mean			**245.8**	**222.8**	**181.6**	**131.4**
Standard deviation (SD)			**27.14**	**19.25**	**8.70**	**5.50**
Standard error mean (SEM)			**12.13**	**8.61**	**3.89**	**2.46**

Table 4: Group (IV) Effect of Compound B on blood glucose level after daily administration for 14 days

S.No.	Animal mark	Body wt. (gm)	Blood glucose levels (mg/dl)			
			Day 0	Day 3	Day 7	Day 14
1	Back	221	238	219	171	133
2	Tail	200	229	213	168	135
3	Unmark	245	231	211	165	128
4	Head &Tail	208	271	234	189	138
5	Back & Tail	194	239	207	173	126
Mean			**241.6**	**216.8**	**173.2**	**132**
Standard deviation (SD)			**16.99**	**10.54**	**9.33**	**4.94**
Standard error mean (SEM)			**7.60**	**4.71**	**4.17**	**2.21**

TABLE 5: Group (V) Effect of Compound C on blood glucose level after daily administration for 14 days

S.No.	Animal mark	Body wt. (gm)	Blood glucose levels (mg/dl)			
			Day 0	Day 3	Day 7	Day 14
1	Back	153	234	225	203	148
2	Tail	183	219	230	222	153
3	Unmark	240	229	201	193	137
4	Head & Tail	152	235	190	188	132
5	Back & Tail	165	226	215	143	131
Mean			**228.6**	**212.2**	**189.8**	**140.2**
Standard deviation (SD)			**6.50**	**16.63**	**29.21**	**9.83**
Standard error mean (SEM)			**2.90**	**7.43**	**13.06**	**3.74**

TABLE 6: Group (VI) Effect of Compound D on blood glucose level after daily administration for 14 days

S.No.	Animal mark	Body wt. (gm)	Blood glucose levels (mg/dl)			
			Day 0	Day 3	Day 7	Day 14
1	Back	182	246	231	218	149
2	Tail	225	222	Death	Death	Death
3	Unmark	247	257	250	231	152
4	Head &Tail	190	228	225	213	138
5	Back & Tail	225	236	222	202	135
Mean			**237.8**	**232**	**216**	**143.5**
Standard deviation (SD)			**14.00**	**12.56**	**12.02**	**8.26**
Standard error mean (SEM)			**6.26**	**6.28**	**6.01**	**4.13**

TABLE 7: Group (VII) Effect of standard drug (Gliclazide) on blood glucose level after daily administration for 14 days

S.No.	Animal mark	Body wt. (gm)	Blood glucose levels (mg/dl)			
			Day 0	Day3	Day 7	Day 14
1	Back	235	240	180	122	102
2	Tail	220	251	191	127	101
3	Unmark	242	228	177	123	100
4	Head &Tail	195	287	223	135	105
5	Back & Tail	204	246	190	128	103
Mean			**250.4**	**196.67**	**128.67**	**102.67**
Standard deviation (SD)			**22.18**	**18.26**	**5.14**	**1.92**
Standard error mean (SEM)			**9.92**	**8.16**	**2.30**	**0.86**

TABLE 8: One way analysis of variance (ANOVA) compare between day 0 with day 3, 7 and day 14

Group		Day		Day 3	Day 7	Day 14
I	**Normal control**	0	P-value	0.867401	0.583419	0.593285
			F	0.029723	0.326501	0.30936
			F crit.	5.317655	5.317655	5.317655
II	**Diabetic control**	0	P-value	0.598042	0.510706	0.927295
			F	0.301315	0.473803	0.008867
			F crit.	5.317655	5.317655	5.317655
III	**Compound A**	0	P-value	0.160815	0.001006	0.00000153
			F	2.388478	25.36394	85.31525
			F crit.	5.317655	5.317655	5.317655
IV	**Compound B**	0	P-value	0.024195	0.00000483	0.00000716
			F	7.688	62.21489	191.7038
			F crit.	5.317655	5.317655	5.317655
V	**Compound C**	0	P-value	0.074138	0.01994	0.00000883
			F	4.215674	8.400893	328.4192
			F crit.	5.317655	5.317655	5.317655
VI	**Compound D**	0	P-value	0.539635	0.043286	0.00000703
			F	0.415705	6.065492	139.7531
			F crit.	5.591448	5.591448	5.591448
V	**standard drug (gliclazide)**	0	P-value	0.001929	0.00000199	0.00000410
			F	20.50387	146.7575	221.4036
			F crit.	5.317655	5.317655	5.317655

P>0.05 is considered as non-significant

TABLE 9: Effect of marmelosin and its derivatives on blood glucose level after daily administration for 14 days

Groups and Treatment (n=5)		Blood glucose levels mg/dl			
		Day 0	**Day 3**	**Day 7**	**Day 14**
I	Normal control	108.88±3.86	109.72±2.65	111.64±2.89	111.8±3.55
II	Diabetic control	258.2±6.72	253±6.67	252.4±5.07	259.2±8.21
I	Compound A	245.8±12.13	222.8±8.61 (9.4%)$^{\#}$	181.6±3.89** (26%) $^{\#}$	131.4±2.46*** (46.8%) $^{\#}$
II	Compound B	241.6±7.60	216.8±4.71* (10.3%) $^{\#}$	173.2±4.17*** (28.4%) $^{\#}$	132±2.21*** (45.4%) $^{\#}$
III	Compound C	228.6±2.90	212.2±7.43 (7.2%) $^{\#}$	189.8±13.0* (16.9%) $^{\#}$	140.2±3.74*** (38.6%) $^{\#}$
IV	Compound D	237.8±6.26	232±6.28 (2.4%) $^{\#}$	216±6.01* (9.17%) $^{\#}$	143.5±4.13*** (39.66%) $^{\#}$
V	Standard drug (gliclazide)	250.4±9.92	196.6±8.16** (21.46%) $^{\#}$	128.6±2.30*** (48.62%) $^{\#}$	102.6±0.82*** (59%) $^{\#}$

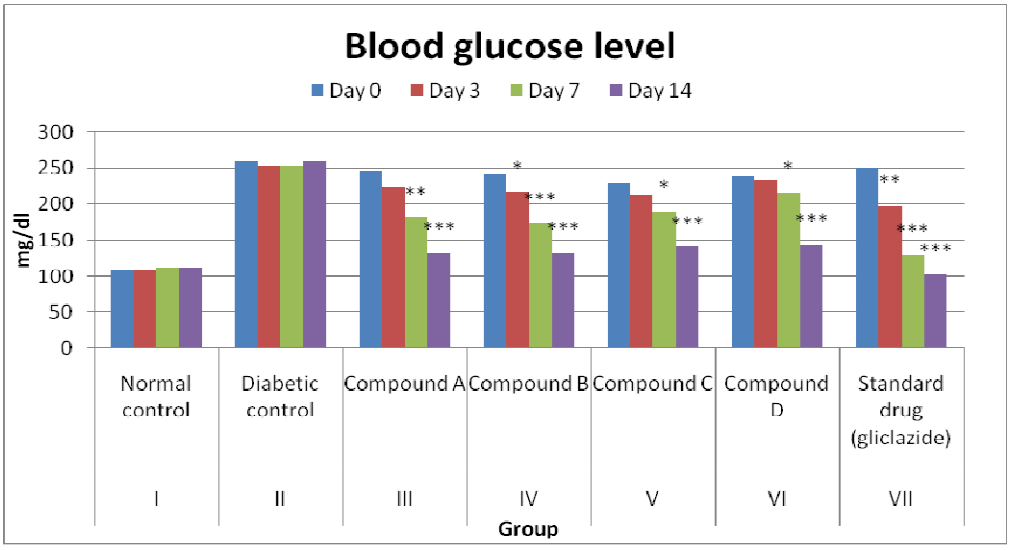

Figure 5: Blood glucose level between days of different groups

4.3. Statistical Analysis

The data obtained in the studies was subjected to one way analysis of variance (ANOVA) for determining the significance. P-value <0.05 will be considered for significance and expressed mean ± SEM. [9,10]

One way analysis of variance (ANOVA) were performed (Table No. 8-9). Values are expressed as mean ± SEM; n = number of animals. P>0.05 was considered as non-significant, *P<0.05 was considered as significant.**P<0.005 was considered highly significant ***P<0.0005 was considered as very highly significant. # Shows % reduction of blood glucose level

RESULTS AND DISCUSSION

It was found that the Marmelosin possessed highest significant reduction in blood glucose level as compared to synthesized compounds against Alloxan induced diabetic rats, after daily administration of synthesized compounds for 14 days.

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