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EVALUATION OF ANTI-INFLAMMATORY AND ANTI-DIABETIC EFFECTS OF DIFFERENT FRACTIONS OF *HOLARRHENA PUBESCENS* (BUCH.- HAM.) WALL. SEED EXTRACT

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Keywords:

Anti-diabetic, Anti-inflammatory, *Holarrhena pubescens*, Insulin resistance, Liver glycogen content, Type 2 diabetes

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ABSTRACT: Inflammation is a chemical-mediated defense mechanism of body. Inflammatory process is also associated with development of various diseases like rheumatoid arthritis, psoriasis, atherosclerosis, and diabetes. Diabetes mellitus is a most common and life-threatening health problem in today's date. Several marketed anti-diabetic drugs are characterized with side effects. Hence there is a need to search a safe and effective anti-diabetic drug. In that regard, the present study was carried out to ascertain phytochemical constituents and to evaluate the anti-inflammatory and anti-diabetic effect of an Indian traditional medicinal plant, *Holarrhena pubescens* (seed). The dried and powdered seeds of *H. pubescens* was extracted by ethanol and then fractionated successively. These fractions were evaluated further for anti-inflammatory activity by *in vivo* models for acute and chronic inflammation. The anti-diabetic activity of plant materials was evaluated by oral glucose tolerance test and blood sugar estimation in streptozotocin-induced diabetic rat model. The results of the present study illustrated that the petroleum ether fraction (HPPT) of the plant material at 400 mg/kg dose showed the most potent and significant ($p<0.01$) effect in anti-inflammatory and anti-diabetic studies among all the test drugs. The chloroform fraction (HPC) of the plant material had also shown a significant effect in pharmacological studies. Hence, the findings of the study suggested the anti-inflammatory and anti-diabetic potential of the plant material.

INTRODUCTION: Diabetes mellitus (DM), a metabolic disorder, is characterized by hyper-glycemia and disturbance in the metabolism of carbohydrate, protein, fat [1]. As per the report of WHO, by the year 2040 globally 642 million people will suffer from diabetes [2].

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Diabetes is mainly two types. Type 1 diabetes (T1DM), previously known as insulin-dependent diabetes mellitus (IDDM) develops due to autoimmune destruction of β-cell of islets of Langerhans of the pancreas which is involved in the production of insulin. Type 2 diabetes (T2DM), formerly known as non insulin-dependent diabetes mellitus (NIIDM) is the most common health problem globally in today's date. The development of T2DM is complex and attributed to various reasons like peripheral insulin resistance, defective hepatic glucose production, develops hyperglycemia, and declining β-cell function,

which further leads to the death of cell [3]. Studies had reported that inflammation plays a major role in the impairment of carbohydrate metabolism and development of insulin resistance which leads to the development of T2DM further. The marketed anti-diabetic drugs clinically control hyperglycemia in patients, characterized by several side effects [4]. Hence, there is a need to search for safe and effective anti-diabetic drugs.

In the last few decades, throughout the world extensive research works were carried out to search for safe and potent anti-diabetic drugs from natural sources. Since the existence of human civilization, natural products serve as a source of medicine. India, with a huge resource of natural products and vast ethnobotanical knowledge, always contributes in the development of effective medicines [5,6]. This traditional medicinal knowledge also helped in the evolvement of the modern medicinal system. In this present study, such a traditional Indian medicinal plant, *Holarrhena pubescens* was selected to evaluate its biological efficacy.

Holarrhena pubescens (Buch.-Ham.) Wall. (Apocynaceae) or commonly known as 'kurchi' is a deciduous tree of 0.6–18 m long [7]. Traditionally, the plant has been used for the treatment of asthma, leprosy, diabetes, eczema, colic dyspepsia, dysentery [8, 9]. Previous studies had reported the antiplasmodial, immunomodulatory, antimalarial, febrifuge, anti-dysentric, antidiarrhoeal, anti-diabetic, and anthelmintic properties of the plant [10-13]. The plant has been reported to be contained different classes of chemical constituents like alkaloids, glycosides, steroids and triterpenoids. Isolation of various steroidal alkaloids namely, mokluangins A-D, anti-dysentericine, holaphyllamine, methylhola-phyll-amine, kurchine, kurchinine, pubescinine, holamide, conaine, conessine, from the plant were also reported [14-17]. So far, no studies have been reported for antidiabetic effects of seeds of *Holarrhena pubescens*.

Previously the author had reported anti-inflammatory effect of ethanolic extract of seeds of *Holarrhena pubescens*. In the present study, the plant material was selected to screen the phytoconstituents by preliminary phytochemical study and to ascertain the anti-inflammatory and anti-diabetic activity of various fractions of ethanolic extract of *H. pubescens* seed.

MATERIALS AND METHODS:

Chemicals and Drugs: All the chemicals and reagents used in this study were of analytical grade. Carrageenan, Streptozotocin, and Trichloroacetic acid (TCA) were procured from Hi-Media Research Laboratories Pvt. Ltd., Mumbai. Glibencalamide from Cadila Pharmaceuticals, Ahmedabad, Indomethacin (Indo) from Sigma Aldrich St. Louis, USA, Tween8 0 from S.D. fine Chemicals Pvt. Ltd., Mumbai, Heparin from Gland Pharma Ltd., Hyderabad and Glucose Oxidase/Peroxidase (GOD/POD) Kit from Agappe Diagnostics, Ernakulam was procured. Glucose, Anthrone reagent, and Anaesthetic Ether were procured from Qualigens fine Chemicals, Mumbai.

Plant Material: The seeds of *Holarrhena pubescens* were collected from Mangaluru, Karnataka, during the month of May. It was authenticated by Prof. Dr. Krishna Kumar, Dept. of Applied Botany, Mangalore University, Mangaluru. A voucher specimen (voucher no.D-82) was deposited in the herbarium of NGSM Institute of Pharmaceutical Sciences, Paneer, Deralakatte, Mangaluru, India.

Extraction and Fractionation of Plant Material: The shade-dried powdered seed (6 kg) was extracted with ethanol (95%) by the cold maceration method for four times. The extract was concentrated by reduced pressure to yield 400g of crude ethanolic extract. The ethanolic extract was suspended in distilled water (1:3, v/v) and successively partitioned with petroleum ether (60 – 80°C, 8×500ml), chloroform (8×500ml), n-butanol (8×500ml) and methanol (8×500ml). The organic layers were brought to dryness to yield petroleum ether (27g), chloroform (40g), n-butanol (48g), and methanol (65g) fractions.

Preliminary Phytochemical Screening: The preliminary phytochemical screening of petroleum ether, chloroform, n-butanol, and methanol fractions (HPPT, HPC, HPNB, and HPM, respectively) of ethanolic extract of seeds of *H. pubescens* was carried out for investigating the presence of alkaloids, carbohydrate, protein, flavonoids, glycosides, triterpenoids, resins, saponins, steroids, tannins and starch using the standard methods and procedures [18].

Preparation of Drug Materials: The fractions of ethanolic extract (HPPT, HPC, HPNB, and HPM) of seeds of *H. pubescens* standard drug (indomethacin and streptozotocin) were used as a suspension in 1% solution of tween 80 in water to screen biological activities. The solution of tween 80 (1%) in water was served as vehicle alone in studies.

Animals: Studies were carried out by using Albino wistar rats (150–200 g) of either sex. All animals were obtained from K.S. Hegde Medical Academy (KSHEMA), Deralakatte, Mangaluru. Animals were grouped and housed in polyacrylic cages and kept at ambient temperature (25 ± 2) ºC, relative humidity (60 ± 5) %, and 12 h light and dark cycle. They had been given a standard pellet diet (Hindustan Lever Limited, Mumbai, India) and water *ad libitum* throughout the course of the study. The study protocols were approved by Institutional Animal Ethical Committee (KSHEMA/AEC/077/ 2008).

Acute Toxicity Study: Acute toxicity study was conducted to determine the median lethal dose (LD_{50}) of test material, HPPT, HPC, HPNB, and HPM in adult female albino wistar rats (nulliparous and non-pregnant) by following up and down procedure of OECD guideline no. 425 [19]. Animals were administered the extract preparations orally and observed at half-hour intervals for 4 h, then after 24 h. Test materials (HPPT, HPC, HPNB and HPM) were found to be safe up to an oral dose of 2000 mg/kg. Based on the study, three dose levels of extract were selected of which middle dose was approximately one-tenth of the LD_{50}, low dose was half of that one-tenth dose, and a high dose was twice of that one-tenth dose, so, that was 200mg/kg, 100mg/kg and 400mg/kg respectively, for of all the test materials (HPPT, HPC, HPNB and HPM) to carry out the *in vivo* biological activity studies.

***In-vivo* Anti-Inflammatory Activity Studies:** The anti-inflammatory activity of test drugs was studied by carrageenan-induced rat paw edema, and cotton pellets induced granuloma formation in rat models.

Carrageenan Induced Rat Paw Edema: Acute anti-inflammatory activity of HPPT, HPC, HPNB, and HPM were evaluated by carrageenan-induced rat paw edema according to the method described earlier [20]. Paw edema was induced by injecting 0.1 ml of 1% (w/v) carrageenan suspension in 0.9% (w/v) sterile saline into the plantar tissue of the left hind paw of all animals. Different groups of animals (n=6) were respectively treated orally with vehicle (tween 80, 3 ml of 1% solution), indomethacin (Indo 10 mg/kg body weight.) and all test drugs, *i.e.*, HPPT, HPC, HPNB and HPM (100, 200 and 400 mg/kg body weight) at 1 h prior to the injection of carrageenan. The right paw served as reference to measure the degree of inflammation in the left one. Increase in the paw volume was measured by plethysmograph at hourly intervals for up to 4 h after carrageenan injection. The percentage inhibition of edema volume was calculated by using following formula [21],

$$\text{Percentage Inhibition} = (1\text{-}V_t / V_c) \times 100$$

Where, V_t is the average paw edema volume of extracts and indomethacin treated groups; and V_c is the average paw edema volume of the control group that only received the vehicle.

Cotton Pellets Induced Granuloma In Rats: The granuloma in albino wistar rats was induced by implanting cotton pellets [22]. All animals were anaesthetized with ether after shaving the fur and 10 mg of sterile cotton pellets were inserted, one in each axilla. All the test drugs (HPPT, HPC, HPNB and HPM) at 100, 200 and 400 mg/kg body weight dose, standard drug (Indo 10mg/kg) and vehicle were administered orally to animals of respective groups (n=6) for seven consecutive days. On the eighth day animals were anesthetized again to remove cotton pellets surgically and made free from extraneous tissues. The moist pellets were weighed, dried at 60°C for 24 h and then re-weighed. Increment in dry weight of pellets was taken as measure of granuloma formation. The percentage inhibition of cotton pellet weight was evaluated.

***In-vivo* Anti-Diabetic Activity Studies:** Anti-diabetic activity of petroleum ether fraction (HPPT), chloroform fraction (HPC), an n-butanol fraction (HPNB), and methanol fraction (HPM) of ethanolic extract of seeds of *Holarrhena pubescens* were evaluated by *in-vivo* models, oral glucose tolerance test and effects on body weight, estimation of blood glucose and liver glycogen

content in streptozotocin-induced diabetic rats model.

Oral Glucose Tolerance Test: The oral glucose tolerance test was performed in overnight fasted normal rats. Rats were divided into thirteen groups (n=6). The first group was kept as control and received 1 ml of 1% tween 80 in water, and the remaining groups received the treatment of test drugs (HPPT, HPC, HPNB, and HPM) at 100, 200, and 400 mg/kg body weight dose, respectively. Glucose (2 g/kg) was administered orally to all animals after 30 min of the treatment of respective test drugs [23]. Blood was withdrawn from the retro-orbital sinus at 0, 30, 60, 120 and 180 min of administration of respective test drugs. Fasting blood glucose levels were estimated by the GOD-POD method.

Streptozotocin Induced Type 2 Diabetes in Rat:

Group Design: Albino rats of either sex weighing 150- 200 g were used for the study. The rats were divided into 15 groups (n=6) as following

Group I: Animals served as normal control and received vehicle orally (1 ml of 1% solution of tween 80).

Group II: Animals served as diabetic control and received vehicle orally (1 ml of 1% solution of tween 80).

Group III, IV and V: Diabetic animals were respectively received 100, 200, 400mg/kg body weight, p.o. dose of petroleum ether fraction of ethanolic extract of seeds of *Holarrhena pubescens* (HPPT) as a suspension in 1% solution of tween 80.

Group VI, VII and VIII: Diabetic animals were respectively received 100, 200, 400mg/kg body weight, p.o. dose of chloroform fraction of ethanolic extract of seeds of *Holarrhena pubescens* (HPC) as a suspension in 1% solution of tween 80.

Group IX, X and XI: Diabetic animals were respectively received 100, 200, 400mg/kg body weight, p.o. dose of n-butanol fraction of ethanolic extract of seeds of *Holarrhena pubescens* (HPNB) as a suspension in 1% solution of tween 80.

Group XII, XIII and XIV: Diabetic animals were respectively received 100, 200, 400mg/kg body weight, p.o. dose of methanolic fraction of ethanolic extract of seeds of *Holarrhena pubescens* (HPM) as a suspension in 1% solution of tween 80.

Group XV: Diabetic animals were administered with standard drug Glibenclamide at a dose of 1 mg/kg body weight, p.o, per day as a suspension in 1% solution of tween 80.

Study Design: A freshly prepared solution of streptozotocin (50 mg/kg body weight) in 0.1 M citrate buffer (pH 4.5) was injected intraperitoneally in a volume of 1 ml/kg body weight to animals in groups II to XV [24]. Streptozotocin (STZ) induced diabetes in rats was confirmed by measuring the fasting blood glucose level. Those rats with a fasting blood glucose level of >300 mg/dl after 48 hrs were considered to be diabetic and were used in the experiment.

The treatment as mentioned in the group design was given to the animals of each group for 28 days. Blood samples were withdrawn from the retro-orbital sinus of the overnight fasted animals using heparinized capillaries on days 0, 7^{th}, 14^{th}, $21^{st,}$ and 28^{th}. The serum was obtained by centrifuging the blood samples at 3000 rpm for 10 min and serum glucose levels were estimated by GOD\POD method using a corresponding kit from Agappe Diagnostics Pvt. Ltd and the intensity of red coloured quinone imine complex formed after treating with the GOD\POD reagents were estimated at 505nm in an autoanalyzer [25].

The animals were sacrificed on the 28^{th} day after the blood collection, and the liver was removed for glycogen estimation. The excised livers were homogenized, and the homogenates were subjected to glycogen estimation, which was carried out by anthrone method [26].

Statistical Analysis: Values were expressed as mean ± S.E.M. Statistical significance of weight or volume change was determined by ANOVA, followed by Dunnet's *t*-test; values with $P<0.05$ and $p<0.01$ were considered as statistically significant. GraphPad Prism version 4.0, GraphPad Software Inc., was used for statistical analysis.

RESULTS:

Preliminary Phytochemical Screening: The preliminary phytochemical tests showed the

presence of various phytoconstituents like alkaloids, carbohydrate, glycosides, triterpenoids, and steroids in the test drugs, *i.e.*, petroleum ether, chloroform, n-butanol, and methanol fractions (HPPT, HPC, HPNB, and HPM, respectively) of ethanolic extract of seeds of *Holarrhena pubescens*, as shown in **Table 1**.

TABLE 1: PRELIMINARY PHYTOCHEMICAL STUDY OF PLANT MATERIALS

Constituent	Test	Inference			
		Pet. Ether Extract (HPPT)	Chloroform Extract (HPC)	N-butanol extract (HPNB)	Methanol extract (HPM)
Alkaloids	a) Dragendorff's test	-ve	+++ve	+ve	-ve
	b) Hager's test	-ve	+++ve	+ve	-ve
	c) Wagner's test	-ve	+++ve	+ve	-ve
	d) Mayer's test	-ve	+++ve	+ve	-ve
Carbohydrates	a) Anthrone test	+ve	+ve	+ve	+ve
	b) Benedict's test	+ve	+ve	+ve	+ve
	c) Fehling's test	+ve	+ve	+ve	+ve
	d) Molisch test	+ve	+ve	+ve	+ve
Proteins	a) Biuret test	-ve	-ve	-ve	-ve
	b) Millon's test	-ve	-ve	-ve	-ve
Flavonoids	a) Shinoda's test	-ve	-ve	-ve	-ve
Glycosides	a) Molisch test	-ve	+ve	+ve	+ve
	b) Borntrager's test	-ve	-ve	-ve	-ve
	c) Modified Borntrager's test	-ve	-ve	-ve	-ve
Triterpenoids	a) Liebermann-Burchard's test	+++ve	++ve	++ve	+ve
Resins		-ve	-ve	-ve	-ve
Saponins		-ve	-ve	-ve	-ve
Steroids	a) Liebermann-Burchard's test	+++ve	++ve	++ve	+ve
	b) Salkwoski test	+++ve	+ve	+ve	+ve
Tannins		-ve	-ve	-ve	-ve
Starch		-ve	-ve	-ve	-ve

Acute Toxicity Study: The acute toxicity study of test drugs (HPPT, HPC, HPNB, and HPM) showed no mortality and significant behaviour changes in rats up to the dose level of 2000 mg/kg. Hence, the test materials were found to be safe up to 2000 mg/kg oral dose, and for the further studies, 100, 200, and 400 mg/kg body weight per oral dose were selected for the test materials (HPPT, HPC, HPNB, and HPM).

In-vivo Anti-Inflammatory Activity Studies:

Carrageenan Induced Rat Paw Edema:

TABLE 2: EFFECT OF PLANT MATERIALS ON RAT PAW EDEMA

Groups	Treatment	Dose/kg b.w., p.o.	Increase in paw volume (ml)			
			1h	2h	3h	4h
I	Control	-	0.38±0.13	0.49±0.24	0.78±0.14	1.16±0.11
II	Indo	10 mg	0.25±0.11 (34.21)	0.32±0.18 (34.69)	0.2±0.17 (74.35)**	0.18±0.16 (84.48)**
III	HPPT	100 mg	0.37±0.19 (2.63)	0.45±0.17 (8.16)	0.71±0.14 (8.97)	0.85±0.13 (26.72)
IV	HPPT	200 mg	0.35±0.16 (7.89)	0.4±0.15 (18.36)	0.52±0.11 (32.33)	0.66±0.17 (43.10)
V	HPPT	400 mg	0.31±0.25 (18.42)	0.38±0.19 (22.44)	0.33±0.16 (57.69)*	0.32±0.13 (72.41)**
VI	HPC	100 mg	0.37±0.16 (2.63)	0.47±0.07 (4.08)	0.72±0.18 (7.69)	0.88±0.14 (24.13)
VII	HPC	200 mg	0.35±0.14 (7.89)	0.43±0.12 (13.24)	0.53±0.2 (32.05)	0.71±0.23 (38.79)
VIII	HPC	400 mg	0.33±0.1	0.41±0.16	0.43±0.13	0.35±0.18

			(13.15)	(16.32)	(44.87)	(69.82)**
IX	HPNB	100 mg	0.37±0.18	0.48±0.13	0.75±0.15	0.94±0.16
			(2.63)	(2.04)	(3.84)	(18.96)
X	HPNB	200 mg	0.36±0.26	0.46±0.26	0.66±0.19	0.82±0.11
			(5.26)	(6.12)	(15.38)	(29.31)
XI	HPNB	400 mg	0.34±0.13	0.44±0.2	0.62±0.17	0.73±0.14
			(10.52)	(10.20)	(20.51)	(37.06)
XII	HPM	100 mg	0.37±0.18	0.48±0.15	0.76±0.13	0.98±0.21
			(2.63)	(2.04)	(2.56)	(15.51)
XIII	HPM	200 mg	0.36±0.12	0.48±0.13	0.71±0.22	0.91±0.06
			(5.26)	(2.04)	(8.97)	(21.55)
XIV	HPM	400 mg	0.35±0.17	0.46±0.19	0.68±0.27	0.77±0.15
			(7.89)	(6.12)	(12.32)	(33.62)

All the result are expressed in term of Mean ± S.E.M., n=6 animals in each group; number in parenthesis indicates percentage inhibition in increase in paw volume. Statistical significance was determined by ANOVA, followed by Dunnet's t-test. *p<0.05, ** p<0.01, statistically significant.

The effect of test drug on carrageenan-induced rat paw edema is showed in **Table 2**. Carrageenan showed most inflammation in animals of control group at third and fourth hour after injection. In the study all the test materials showed dose-dependent response. HPPT and HPC 400mg/kg b.w., p.o. doses respectively exhibited significant 72.41% (p<0.01) and 69.82% (p<0.01) inhibition of rat paw edema at 4 h **Fig. 1**. During 3 h HPPT 400mg/kg p.o., b.w. dose inhibited edema by 57.69% (p<0.05). Though HPNB and HPM at 400mg/kg b.w., p.o. dose showed 37.06 and 33.62% rat paw edema inhibition at 4 h respectively, the results were not found to be significant. The standard drug indomethacin showed the significant 84.48% (p<0.01) and 74.35% (p<0.05) inhibition of rat paw edema at 4 and 3 h, respectively in the present study, as shown in **Fig. 1**. Thus, HPPT 400mg/kg dose showed potent effect to inhibit rat paw edema among all these test materials.

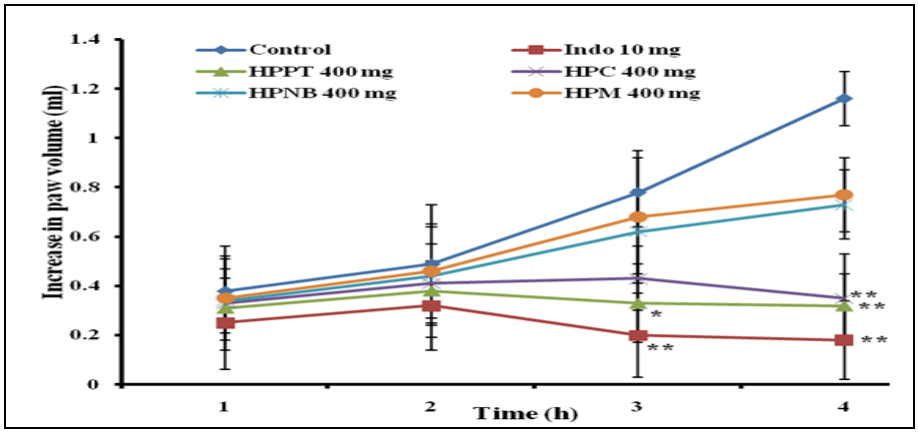

FIG. 1: ANTI-INFLAMMATORY EFFECT OF PLANT MATERIALS. Each value represents as mean ± S.E.M. *p < 0.05 and **p < 0.01 as compared with the control group (one-way ANOVA followed by Dunnet's *t*-test).

Cotton Pellets Induced Granuloma in Rats: As shown in **Table 3**, test materials showed inhibition of cotton pellets induced granuloma formation in dose-dependent manner. The standard drug indomethacin (Indo) 10 mg/kg dose showed significant 63.69 and 72.06% (p<0.01) inhibition of moist and dry cotton pellet weight, respectively, shown in **Fig. 2**. HPPT and HPC 400mg/kg b.w., p.o. doses respectively inhibited 56.98 and 49.86% (p<0.01) weight of moist cotton pellet as compared to control group, as shown in **Fig. 2**. HPNB and HPM 400 mg/kg b.w., p.o. doses showed 21.30% (p<0.05) and 15.87% inhibition of weight of moist cotton pellet, respectively. HPPT 400mg/kg b.w., p.o. dose showed significant 55.81% (p<0.01) inhibition of dry cotton pellets weight (Fig.2). Whereas, HPC 400mg/kg b.w., p.o. dose exhibited significant 47.63% (p<0.01) inhibition of weight of dry cotton pellets. Though, HPC, HPNB and HPM showed inhibition of weight of cotton pellet at 400mg/kg b.w., p.o. dose, but the most potent effect was shown by HPPT at that same dose among all these test materials.

TABLE 3: ANTI-PROLIFERATIVE EFFECTS OF PLANT MATERIALS

Groups	Treatment	Dose/kg b.w., p.o.	Moist cotton pellet		Dry cotton pellet	
			Weight (mg)	% inhibition	Weight (mg)	% inhibition
I	Control	-	295.63±14.53	-	84.37±3.00	-
II	Indo	10 mg	107.32±6.38	63.69**	23.57±2.63	72.06**
III	HPPT	100 mg	263.75±10.27	10.78	71.32±3.48	15.46
IV	HPPT	200 mg	196.34±28.51	33.58*	52.77±1.64	37.45**
V	HPPT	400 mg	127.16±22.37	56.98**	37.28±1.09	55.81**
VI	HPC	100 mg	268.31±9.46	9.24	72.61±2.33	13.93
VII	HPC	200 mg	226.17±23.62	23.49*	66.15±2.87	21.59*
VIII	HPC	400 mg	148.22±17.43	49.86**	44.18±2.21	47.63**
IX	HPNB	100 mg	277.31±26.7	6.19	78.46±3.83	7.01
X	HPNB	200 mg	246.83±29.11	16.50	76.13±2.04	9.76
XI	HPNB	400 mg	232.64±34.18	21.30*	69.74±1.16	17.34
XII	HPM	100 mg	287.22±17.26	2.84	81.32±3.27	3.72
XIII	HPM	200 mg	266.74±11.53	9.77	78.64±2.83	6.79
XIV	HPM	400 mg	248.69±14.82	15.87	73.47±3.12	12.91

All the result are expressed in term of Mean ± S.E.M. n=6 animals in each group; Statistical significance was determined by ANOVA, followed by Dunnet's t-test. * p<0.05, ** p<0.01, statistically significant

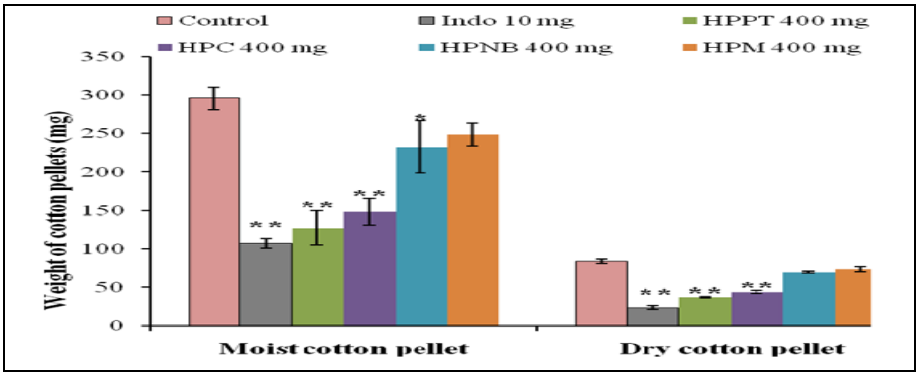

FIG. 2: EFFECTS OF PLANT MATERIALS ON GRANULOMA FORMATION IN RATS: Each value represents as mean ± S.E.M. *p < 0.05 and **p < 0.01 as compared with the control group (one-way ANOVA followed by Dunnet's *t*-test).

In-vivo Anti-diabetic Activity Studies:

Oral Glucose Tolerance Test:

TABLE 4: EFFECT OF PLANT MATERIALS ON ORAL GLUCOSE TOLERANCE TEST

Groups	Treatment	Dose/kg b.w., p.o.	Blood Glucose Level (mg/dl)				
			0 min	30 min	60 min	120 min	180 min
I	Control	---	85.31±1.16	119.7±1.3	109.43±1.12	97.55±1.09	95.6±1.23
II	HPPT	100 mg	85.24±1.18	110.33±1.51	98.38±1.66	90.57±1.06	84.41±1.29
III	HPPT	200 mg	88.83±1.06	105.96±1.88	93.28±1.61	86.11±1.04	80.93±1.68
IV	HPPT	400 mg	84.25±1.73	98.11±1.04	84.79±1.63*	81.35±1.18*	76.13±1.54*
V	HPC	100 mg	87.61±1.03	113.48±1.74	100.84±1.13	92.35±1.52	85.73±1.24
VI	HPC	200 mg	86.36±1.85	108.15±1.34	96.71±1.49	89.43±1.28	82.12±1.7
VII	HPC	400 mg	83.18±1.39	102.67±1.15	85.91±1.73*	83.16±1.35*	79.24±1.06*
VIII	HPNB	100 mg	86.44±1.21	113.85±1.46	104.93±1.02	95.17±1.29	92.59±1.81
IX	HPNB	200 mg	85.19±1.10	111.26±1.69	102.47±1.93	93.18±0.82	90.46±1.13
X	HPNB	400 mg	85.32±1.77	108.9±1.83	98.86±1.14	89.37±1.45	84.79±0.26
XI	HPM	100 mg	87.86±1.65	115.74±1.53	108.61±1.03	97.12±0.61	93.87±1.78
XII	HPM	200 mg	87.15±1.33	114.38±1.62	104.32±1.19	95.63±1.69	91.05±1.0
XIII	HPM	400 mg	85.47±1.51	112.19±1.87	99.87±1.35	91.73±1.21	86.88±1.53

All the result are expressed in term of Mean ± S.E.M., n=6 animals in each group; Statistical significance was determined by ANOVA, followed by Dunnet's *t*-test. * p<0.05, statistically significant.

This method was carried out to evaluate the effect of the extract on glucose loaded rats. As shown in **Table 4**, at 0 min all the groups showed normal blood glucose level. The test groups showed the effect of reducing blood glucose level in dose dependent manner. During 30 min all the groups

showed an elevation of blood glucose level but test drugs showed the reduction of blood glucose level compared to control group. HPPT 400 mg/kg dose had shown a significant ($p<0.05$) 25.58% decrease in the blood glucose level (76.13mg/dl) compared to the control group in 180 min. HPPT 400 mg/kg dose also showed a significant ($p<0.05$) decrease in the blood glucose level to 84.79 and 81.35 mg/dl in 60 and 120 min respectively, compared to the control group. HPPT 100mg/kg and 200 mg/kg dose showed reduction in blood glucose level but the results were not found to be significant when compared to the control group. HPC 400 mg/kg dose also had shown a significant ($p<0.05$) 20.65% decrease in the blood glucose level (79.24 mg/dl) compared to the control group in 180 min. Other treatment groups didn't show significant result in reduction of blood glucose level.

Streptozotocin-Induced type 2 Diabetes in Rat Model:

Effect of Drugs on Body Weight of Streptozotocin Induced Diabetic Rats:

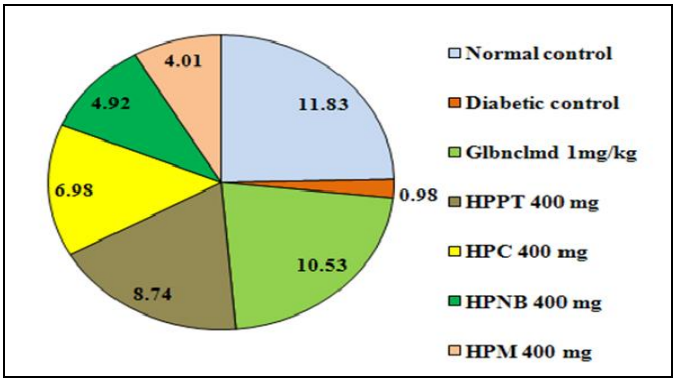

FIG. 3: EFFECT OF PLANT MATERIALS ON BODY WEIGHT OF DIABETIC RATS: Each value represents as percentage of the effect of all groups of the study.

As shown in **Fig. 3**, due to the induction of streptozotocin the rats were shown a severe reduction in body weight. After 28th day the body weight of animals of normal control group were increased up to 11.83%. After 28th day of experiment the result showed only 0.98% increase of the bodyweight of diabetic control group's animals. But an increase in body weight was shown by the animals of test drugs treated groups and glibenclamide group. The glibenclamide (1mg/kg b.w., p.o. dose) treated group showed the highest increase of body weight of animals to 10.53% on 28th day of experiment, which was found to be very significant ($p<0.01$) compared to diabetic control group. HPPT 400 mg/kg dose had shown significant ($p<0.01$) 8.74% increase in body weight of animals on 28th day of experiment compared to diabetic control group and the result was found to be closed to the effect shown by the standard drug (Glibenclamide). HPC 400 mg/kg dose had shown 6.98% increase in body weight of animals on 28th day of experiment which was also found to be significant ($p<0.05$) compared to diabetic control group. HPPT 200 mg/kg dose had shown significant 5.58% ($p<0.05$) increase in body weight of animals on 28th day of experiment. Other treatment groups didn't show significant increase in body weight of animals.

Effect of Drugs on Liver Glycogen Content and Blood Glucose Level of Streptozotocin Induced Diabetic Rats: The results showed the effect of test drugs and standard drug (Glibenclamide) on liver glycogen and blood glucose level of streptozotocin induced diabetic rats. As shown in **Table 5** and **Fig. 4**, the glycogen content in rat liver of diabetic control group was reduced to 10.21 mg/g on 28th day of experiment whereas, the glycogen content in rat liver of normal control group was 43.98 mg/g. The standard drug (Glibenclamide 1mg/kg b.w., p.o. dose) treatment showed significant ($p<0.01$) 73.50% (38.54mg/g) increase of liver glycogen content compared to diabetic control group **Fig. 4**. In this study, also HPPT 400 mg/kg dose had shown a significant ($p<0.01$) 69.44% (33.47mg/g) increase in liver glycogen content compared to diabetic control group and the effect was found to be most potent among all the test drugs. The HPC 400 mg/kg dose had also shown a significant ($p<0.05$) 65.16% (29.31mg/g) increase in liver glycogen content compared to diabetic control group, as shown in **Fig. 4**.

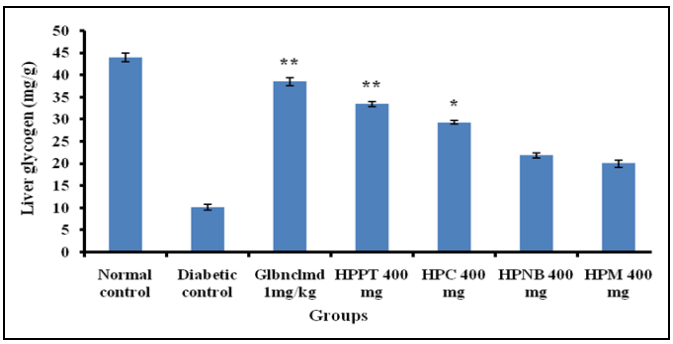

FIG. 4: EFFECTS OF PLANT MATERIALS ON LIVER GLYCOGEN OF DIABETIC RATS. Each value represents as mean ± S.E.M. *p < 0.05 and **p < 0.01 as compared with the control group (one-way ANOVA followed by Dunnet's t-test)

Test drugs had shown a dose-dependent effect on blood glucose level in streptozotocin-induced diabetic rats **Table 5** and **Fig. 5**. Among the test materials, HPPT 400 mg/kg b.w., p.o. dose showed the highest decrease in blood glucose level on day 28 to 148.71 mg/dl ($p<0.01$), which was also found to be closed to the result showed by standard drug Glibenclamide (129.71 mg/dl, $p<0.01$) on the same day. Glibenclamide (1mg/kg b.w., p.o. dose) treatment also showed a significant ($p<0.01$) decrease in blood glucose level to 193.51 and 164.36 mg/dl respectively on day 14 and 21. Whereas HPPT 400 mg/kg dose has shown a significant decrease in blood glucose level to 215.54 ($p<0.05$) and 177.87 mg/dl ($p<0.01$) on day 14 and 21, respectively **Table 5**. HPC 400 mg/kg b.w., p.o. dose showed a significant decrease in blood glucose level on day 28 to 162.16 mg/dl ($p<0.01$) **Fig. 5**. HPPT and HPC 200 mg/kg dose also showed a significant ($p<0.05$) decrease of blood glucose level to 185.47 and 191.22 mg/dl respectively on day 28, as shown in Fig.5. Other test materials showed a decrease in blood glucose level in streptozotocin-induced diabetic rats, but the results were not found to be significant when compared to the diabetic control group.

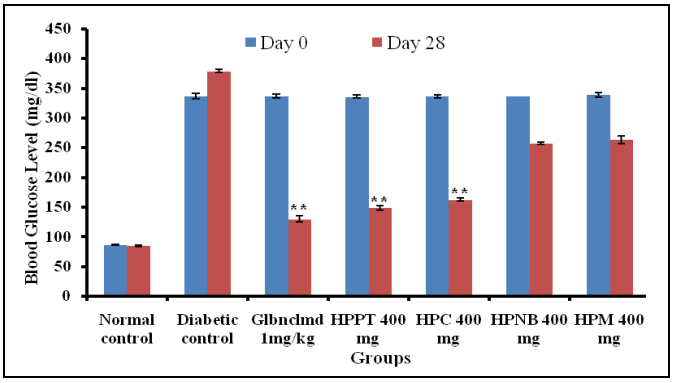

FIG. 5: EFFECTS OF PLANT MATERIALS ON BLOOD GLUCOSE LEVEL. Each value represents as mean ± S.E.M. ***p* < 0.01 as compared with the control group (one-way ANOVA followed by Dunnet's *t*-test)

TABLE 5: EFFECT OF PLANT MATERIALS ON LIVER GLYCOGEN AND BLOOD GLUCOSE LEVEL IN DIABETIC RATS

Groups	Treatment	Dose/kg b.w., p.o.	Liver glycogen (mg/g)	Blood Glucose Level (mg/dl)				
				0 day	7th day	14th day	21st day	28th day
I	Normal control	---	43.98 ±0.94	86.25 ±0.95	85.5 ±1.41	85.66 ±0.99	84.88 ±0.71	84.2 ±0.91
II	Diabetic control	---	10.21 ±0.69	336.33 ±4.8	356.83±2. 49	362.16 ±3.01	367.66 ±2.31	378.83 ±2.58
III	HPPT	100 mg	22.71 ±0.92	339.52 ±2.48	293.43 ±2.15	258.37 ±2.82	228.64 ±2.37	204.73 ±2.18
IV	HPPT	200 mg	26.15 ±0.39*	337.38 ±2.97	276.8 ±3.77	233.18 ±3.34	205.26 ±2.89*	185.47 ±2.65*
V	HPPT	400 mg	33.47 ±0.53**	335.63 ±2.71	263.92 ±2.46	215.54 ±2.31*	177.87 ±3.16**	148.71 ±3.7**
VI	HPC	100 mg	20.21 ± 0.37	338.27 ± 2.54	305.39 ±2.26	289.18 ±2.78	274.68 ±2.53	255.39 ±2.29
VII	HPC	200 mg	24.68 ± 0.26*	336.12 ± 3.61	299.83 ±2.74	272.5 ±3.21	230.82 ±3.43	191.22 ±2.78*
VIII	HPC	400 mg	29.31 ± 0.42*	335.89 ± 2.23	279.31 ±3.0	250.44 ±2.65	209.62 ±2.3*	162.16 ±2.59**
IX	HPNB	100 mg	15.11 ± 1.07	339.43 ±4.5	321.13 ±2.53	308.27 ±3.16	288.42 ±2.63	276.93 ±2.37
X	HPNB	200 mg	18.35 ± 0.26	338.35 ± 4.91	306.4 ±3.53	298.81 ±4.39	283.15 ±2.78	262.33 ±4.86
XI	HPNB	400 mg	21.89 ± 0.57	336.13 ± 3. 74	291.83 ±4.21	279.11 ±5.93	265.72 ±2.54	257.13 ±2.32
XII	HPM	100 mg	13.46 ± 0.7	340.17 ± 3.84	327.2 ±5.84	322.0 ±4.67	303.67 ±2.8	282.88 ±4.47
XIII	HPM	200 mg	16.92 ± 0.68	341.23 ± 5.19	309.85 ±4.7	305.62 ±4.89	291.24 ±6.32	269.27 ±5.14
XIV	HPM	400 mg	20.04 ± 0.81	338.61 ± 3.53	307.43 ±3.64	288.41 ±5.35	274.37 ±4.6	262.96 ±7.12
XV	Glibenclamide (std)	1 mg	38.54 ±0.88**	336.2 ±3.42	239.6 ±2.21*	193.51 ±3.50**	164.36 ±4.36**	129.71 ±5.4**

All the result are expressed in term of Mean ± S.E.M., n=6 animals in each group; Statistical significance was determined by ANOVA, followed by Dunnet's *t*-test. * $p<0.05$ when compared with diabetic control group, ** $p<0.01$, statistically significant when compared with diabetic control group.

The result showed that HPPT 400 mg/kg dose was most effective among these test drugs to decrease the blood glucose level and to increase the liver glycogen content in streptozotocin induced diabetic

rats. Hence, the findings suggest that HPPT 400mg/kg dose exhibits the most potent anti-diabetic effect among all the test drugs.

DISCUSSION: The anti-inflammatory activity of test drugs (HPPT, HPC, HPNB, and HPM) was evaluated by carrageenan-induced rat paw edema and cotton pellets induced granuloma formation in rat models with doses of 100, 200, and 400 mg/kg b.w., p.o. of each drugs, in the present study. The results indicated a potent anti-inflammatory effect of petroleum ether fraction of ethanolic extract of *H. pubescens* (HPPT) with 400 mg/kg dose in both the study. The experimental model carrageenan-induced rat paw edema is an acute inflammation study. The paw edema development by administration of carrageenan is attributed to the release of several chemokines as well as the synthesis of prostaglandins [27, 28]. The anti-inflammatory agents generally act on these mediators to reduce inflammation. As shown in the study, the standard drug indomethacin reduced the inflammation of rat paw by third and fourth hours. Similarly, the test drug HPPT and HPC with the dose of 400 mg/kg dose significantly reduced the rat paw edema at 4th h in the study **Table 2**. HPPT with 400 mg/kg dose also showed the effect to reduce inflammation of rat paw significantly at third hour of the study. Hence the study suggested that the test drug (HPPT and HPC 400 mg/kg dose) was possibly able to control the signature mediators of inflammation to exhibit anti-inflammatory effect. Further, the finding was supported by the results of chronic inflammatory study cotton pellets induced granuloma formation in rat models. The induction of cotton pellet in rat initiates proliferation of granuloma is due to accumulation of macrophages and lymphocytes around the foreign particles [29, 30]. The standard anti-inflammatory drugs are showing the effect by reducing granuloma formation. The reduction of granuloma formation of drug is called as anti-proliferative effect which is measured by percentage inhibition of cotton pellet weight during the study. In this study, indomethacin showed significant ($p<0.01$) inhibition of moist and dry cotton pellet weight **Table 3**. The inhibition of moist cotton pellet indicates the absorption of fluids which accumulate at the site of granuloma formation and reduction of dry weight of cotton pellet is the sign of anti-proliferative effects of the drug [31]. Results of the present study suggested that, among all the test drugs HPPT at 400 mg/kg dose showed potent effect by a significant reduction of both moist and dry cotton pellet weight. Though the HPC with the same dose showed significant reduction but HPPT exhibited a better response. Whereas, other test materials showed the reduction of moist and dry cotton pellet weight compared to control group, but none of the results were found to be significant. Hence, the findings suggested the most potent anti-inflammatory effect of HPPT at 400 mg/kg dose among all the test drugs. The result of the present study also supported the finding of the author's previous study on anti-inflammatory effects of ethanolic extract of seeds of *Holarrhena pubescens* at 400 mg/kg dose [32].

Inflammation plays a major role in β-cell dysfunction and insulin resistance, and these are the prominent pathogenetic mechanisms of type 2 diabetes. Previous study reports suggest that inflammation causes the impaired function of β-cell of the pancreas, which leads to death of the cell. The mechanism also causes the development of insulin resistance. The involvement of pro-inflammatory cytokines like TNF-α, IL-6 in the development of insulin resistance has been reported [33-36]. There are also some studies that have reported the prominent hypoglycemic effect of anti-inflammatory drugs, like salicylate and aspirin [37, 38]. Hence in the present study anti-diabetic effect of the all the test drugs were evaluated as they also showed prominent anti-inflammatory effects in the respective studies. The anti-diabetic activity of the test drugs were evaluated by oral glucose tolerance test and effects on body weight, estimation of blood glucose and liver glycogen content in strepto-zotocin induced diabetic rats model. The results of the present study suggested anti-diabetic effect of test drugs in a dose-dependent manner. In oral glucose tolerance test, both HPPT and HPC at 400 mg/kg dose had shown a significant ($p<0.05$) hypoglycemic effect. But the highest effect was observed by HPPT among all the test drugs, and the result was close to the effect showed by the standard drug Glibenclamide. The effect of drugs in oral glucose tolerance test suggested the possible mechanism of improving insulin sensitivity in animals as the experiment widely practiced for evaluating peripheral insulin resistance [39, 40].

The findings further supported by the study on estimation of blood glucose in streptozotocin induced diabetic rats models where HPPT 400 mg/kg b.w., p.o. dose also showed a significant ($p<0.01$) hypoglycemic effect. The induction of STZ in rats causes hyperglycemia by its cytotoxic effect to β-cell of the pancreas, and the damage leads to glucose intolerance. The possible mechanism of action of anti-diabetic effect of test drug of the study is protecting β-cell from damage and enhancement of insulin-sensitizing in peripheral tissues [41, 42]. Glibenclamide, the well-known hypoglycemic agent used in this study as a standard drug. STZ induced diabetes in rats also causes the degradation of structural proteins, which leads to a reduction in body weight in animals. The treatment with test drugs altered the weight loss in animals, possibly by reducing the hyperglycemia [43]. In the present study, HPPT (400 mg/kg dose) showed significant inhibition of weight loss in animals. STZ induced diabetes is also associated with liver insulin resistance which reduces the level of glycogen synthase and causes the reduction in liver glycogen content [44]. In the present study, the test drug (HPPT at 400 mg/kg dose) treated group showed a significant increase in liver glycogen content.

Hence, the report of the present studies suggested the anti-diabetic effect of the seed of *Holarrhena pubescens* and the petroleum ether fraction (HPPT) of ethanolic extract of the plant material was found to be most potent at 400 mg/kg dose among all the test materials.

The preliminary phytochemical study showed the presence of various phytoconstituents like alkaloids, carbohydrates, glycosides, triterpenoids, and steroids in all the test material. The petroleum ether fraction, which was found to be most potent in anti-inflammatory and anti-diabetic studies, showed the presence of carbohydrate, triterpenoids, and steroids in a preliminary phytochemical study. Another fraction (chloroform fraction) HPC, which was also showed a prominent effect in anti-inflammatory and anti-diabetic studies, showed the presence of alkaloids, carbohydrates, glycosides, triterpenoids, and steroids.

CONCLUSION: The present study was planned to ascertain the type of phytochemicals by preliminary screening, anti-inflammatory, and anti-diabetic activities of petroleum ether, chloroform, n-butanol and methanol fractions (HPPT, HPC, HPNB, and HPM, respectively) of ethanolic extract of seeds of *Holarrhena pubescens*. The results of the present study suggested the anti-inflammatory and anti-diabetic effect of the plant material, which also supported the claim of the traditional approach. Further, petroleum ether fraction (HPPT) of ethanolic extract of the plant material exhibited the most prominent effect at 400 mg/kg dose among all the test drugs. This petroleum ether fraction also showed the presence of triterpenoid and steroids in preliminary phytochemical study. Hence, the present study suggested that triterpenoid and steroid containing petroleum ether fraction (HPPT) were found to be the most potent among all the test drugs in anti-inflammatory and anti-diabetic activities at 400 mg/kg dose. In the future, an in-depth study is needed to be carried out to understand the possible mechanism of action of the anti-diabetic effect of *Holarrhena pubescens*.

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CONFLICTS OF INTEREST: Authors declare that they have no conflicts of interest.

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