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Biochemical evaluation and hypoglycemic activity of ethanolic extract of *Xanthium strumarium* L. on normoglycemic and alloxan-induced diabetic mice

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Abstract
The aim of present study was to evaluate the hypoglycemic activity of some fractions of ethanolic extracts in normal and diabetic rats as a step toward activity directed isolation of the hypoglycemic component. Spectral analysis were used for structural determination of isolated compound (II).Oral administration of ethanolic extract and isolated compound-II (300 and 500 mg/kg bw) for 14 days resulted in a significant reduction in blood sugar level. The effect was compared with 10mg/kg (i.p.) Glibenclamide. Alloxan-induced hyperglycemic rats were used for the evaluation of antidiabetic activity. The effect of ethanolic extracts and compound-II on normal and alloxan-induced hyperglycemic rats were compared. The results shows Antihyperglycemic activity of ethanolic extract and compound-II were in dose dependent manner. The activity was more for 500mg/kg bw dose in comparision with 300mg/kg bw for both and compound-II showed better reduction of blood glucose level compared to ethanolic extracts.

Keywords: *Xanthium strumarium*, compound-II, ethanolic extracts, glibenclamide

Introduction
In India, Diabetes mellitus is on challenging condition as compared to other developed countries. By the estimation of WHO, the number of diabetic cases in India in 2000 were 31,705,000 and is expected to increase to 79,441,000 by 2030. India has more diabetics than any other country in the world, according to the International Diabetes Foundation (IDF), although more recent data suggest that China has even more. The diseases affect more than 50 million Indians. 7.1% of the nation's adults and kills about 1 million Indians a year. The high incidence is attributed to a combination of genetic susceptibility plus adoption of a high-calorie, low-activity lifestyle by India's growing middle class.

So, there is a big challenge to investigate new effective medicine for the treatment of diabetes mellitus with less side effects and relatively low cost. Though different types of oral hypoglycemic agents are available along with insulin for the treatment of diabetes mellitus. There is an increasing demand by patients to use the natural products with antidiabetic activity. Insulin cannot be used orally and continuous use of the synthetic drugs cause side effects and toxicity. Herbal drugs are prescribed widely even when their biologically active compounds are unknown because of their effectiveness, less side effects and relatively low cost. (Venkatesh *et al.* 2003). Management of diabetes without any side effect is still a challenge for the health sector. This leads to an increasing search for improved antidiabetic drugs. Since antiquity, diabetes has been treated with plant medicines. Previous ethnobotanical studies of traditional herbal remedies used for diabetes around the world have identified more than 1,200 species of plants with hypoglycemic activity (Babu *et al.* 2006) [1]. Natural medicines used in the traditional Chinese medical system for therapy of diabetes mellitus (Li *et al.* 2004) [12]. In India, indigenous remedies have been used in the treatment of DM since the time of Charaka and Sushruta (6th century BC) (Grover and Vats, 2001) [5]. Ayurveda and other Indian traditional medicine use plants in treatment of diabetics (Chopra *et al.*1986) [4]. However, traditional knowledge, derived empirically, has to be supported by scientific testing. and their complications. *Xanthium strumarium* L. (XS) plant species is related to asteraceae family (Sravani *et al.* 2010) [16] and used as an antidiabetic agent but the pharmacology of this plant was not investigated thoroughly.

The aim of the present work related to the isolation, identification and antidiabetic evaluation of compounds derived from *X. strumarium* L.

Materials and Methods

Collection and Identification of plant material

For the present study, fresh mature leaves of *X.strumarium* L. was collected in October-November 2008 from the local surroundings of Betul and Bhopal District of M.P., India. The plant was identified and authenticated by Professor Dr. Jagrati Tripathi, Department of Biotechnology, Unique College, Bhopal (M.P.) and voucher specimen (No. AST/613) was deposited in the herbarium of the same department.

Preparation of the plant extract

The fresh mature leaves of *X.strumarium* was first washed well with tap water and kept for drying in shade at room tempreture and thoroughly air dried plant matrial was grinded to powder (40-60 mesh) weighted and stored. The powder was extracted with ethanol by soxhlet extraction method. The percentage yields were 10.92% in ethanol.

Preliminary phytochemical screening

The qualitative phytochemical screening (Yadav and Agarwala 2011) [18] of ethanolic extract of *X.strumarium* plant contains alkloides, amino acids, carbohydrates, gums, flavonoids, tannins and terpanoids (Sesquiterpene lactones). Which are presented in Table-1.

Isolation and Identification of the active compound

Six gram of the pure XS leaves extract was admixed with 10g silica gel (60-120 mesh),dried for uniform mixing and the admixture was loaded in a column (5 cm diameter x 50 cm height) packed with silica gel (150g) using ethyl acetate as the solvent. The supply of the solvent and combination of the solvent was replenished from a separating funnel. The various fractions thus obtained were collected in small glass vials using Benzene: Chloroform (1:1) and n-Haxane: Chloroform (3:1) solvent system. The active fraction (Fr-II) eluted at n-Haxane: Chloroform. The compound was obtained as bluish semisolid. The fraction was characterized by spectroscopy techniques like ^{1}H NMR, IR, UV and MS. Melting points were determined using a Mitamura melting point apparatus and were uncorrected.

Experimental Animals

Swiss albino mice (24-35g) were allowed free access to standard pellet diet and water. The experimental protocol was approved by the IAEC (Institutional Animal Ethical Committee) of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animal) (Reg.No.1283/C/09/CPCSEA).

Acute Toxicity Studies

Acute toxicity study was carried out on plant extracts using female and male Swiss albino mice. The mice were fasted overnight and the weight of each mouse was recorded just before use. Animals were divided randomly into a control and three treatment groups, each group consisting of four mice (2 male and 2 female). Control group received only the vehicle and each treatment group received orally the 70% ethanol and aqueous extract of the studied plant in a dose of 1000, 2000 and 5000mg/kg. (Burger *et al.* 2005, Latha *et al.* 2010) [3, 11] and then they were observed daily for three days for any change in general behavior and physical activities.

Body weight

The change in the body weight of control and experimental groups of mice treated with crude ethanol extract and compound II isolated from plant material (*X. strumarium*) is shown in Table 2.

Oral Glucose Tolerance Test

After 2 weeks of treatment with the plant extract, the animals were made to fast for 12- 14 hours. Their body glucose level were measured and glucose solution (2g/kg body weight) was administered orally and in a volume of 1 ml. Blood samples were collected 30, 60 and 120 minutes after administration of glucose in order to evaluate their blood glucose level (Kumar *et al.* 2006) [10].

Antidiabetic evaluation

Experimental induction of diabetes

Induction of diabetic mellitus :After fasting for 12-14 h,40 rats were injected by intraperitoneally with a single dose of 200mg/kg alloxan monohydrate (Sigma St Louis M.O.,USA) after dissolving it in freshly prepared ice-cold citrate buffer (pH 4.5). After the injection, they had free access to feed and water and were given 5% glucose solution to drink overnight to counter the hypoglycemic shock. The devlopment of diabetes was confirmed after 48 h of the Alloxan monohydrate injection. The rates having fasting blood glucose level more than 200mg/dL were selected for experimentation (Kumar *et al*. 2006) [10]. From the out of 40 animals, 4 animals were died before grouping and 6 animals were omitted from the study, because mild hyperglycemia (below 150mg/dL). From the 30 diabetic mice, they were divided into six groups each having 5 animals (Nagappa *et al*. 2003) [13].

Collection of blood samples and glucose determination

Blood samples were collected by end tail vein cutting method and blood glucose level was determined by using one touch electronic glucometer using glucose strips (Lifescan, Johenson and Johenson Ltd) (Kumar *et al*. 2006) [10].

Experimental protocol

The Group I-consist of 5 normal control animals. The remaining each group consist of 5 alloxan-induced diabetic rates. Group I-consisted of normal rats that neither received alloxan monohydrate nor any drug, Group II-served as positive control (diabetic control), Group III-rates were diabetic and treated with Glibenclamide (10mg/kg p.o.) for 14 days, Group IV&V-diabetic rates received compound-II at the dose of 300 and 500 mg/kg p.o. daily for 14 days respectively, Group VI & VII- diabetic rats received crude ethanolic extract at the dose of 300 and 500 mg/kg p.o. daily for 14 days respectively.

All the group of animals received the treatment by the above schedule for 14 days. Blood samples were collected one hour after drug administration on before treatment and day 01, 07 and 14 th day to determine the blood glucose level by electronic glucometer (Babu *et al*. 2002) [2].

Statistical analysis

Data were statistically evaluated by use of one way ANOVA, followed by post hoc Schiff's test using version 13 of SPSS software and Microsoft Office Excel 2003. The values were considered to be significant if $p<0.05$ was obtained.

Table 1: Qualitative phytochemical screening of ethanolic extract of *X. strumarium*

S. NO.	Chemical constituents	Tests	Results
01.	Test for Alkaloids	Mayer's Test	+
		Wagner's Test	+
		Dragendorff's Test	+
		Hager's Test	+
02.	Test for Amino acids	Million's Test	+
		Ninhydrin Test	+
03.	Test for Corbohydrates	Benedict's Test	+
		Fehling's Test	+
		Molisch's Test	+
04.	Test for Glycosides	Keller killani Test	+
05.	Test for Gums	Molisch's Test	+
06.	Test for esters	Zeisel Test	-
07.	Test for Flavonoids	Shinoda Test	+
08.	Test for Saponins	Foam Test	-
09.	Test for Steroids	Liebermann-Burchard Test	-
		Sulphuric acid Test	-
10.	Test for Tannins	Ferric chloride Test	+
11.	Test for Terpanoids	Liebermann-Burchard Test	+
		Salkowski Test	+

Key: +: Positive, - : Negative

Results

Identification of the compound

After isolation and chromatographic purification of the ethanol extract, a bluish semisolid was obtained from fraction-II. Structural determination of the Compound-II was done using spectroscopy technique and it was confirmed as a sesquiterpene lactone. The % yields of sesquiterpene lactone was 0.81% in *X.strumarium* mature leaves powder. The Compound-II was identified based on the following evidence:

HPLC: HPLC analysis of column purified fraction–II fifteen peaks were found at 15.45 min.

IR spectra: 3409 cm^{-1}, 2930 cm^{-1}, 2850 cm^{-1}, 1749 cm^{-1}, 770 cm^{-1}

^{1}H NMR spectra: δ 2.08 (3H, s, 2X $COCH_3$), δ 2.09 (3H, s, 2X $COCH_3$), δ 2.27 (1H, br, d, J=17.4 Hz, H-4), δ 2.40 (1H, dd, J=18.0, 4.2 Hz, H-3), δ 2.84 (1H, m, H-4), δ 2.97 (1H, dd, J=18.0, 10.2 Hz, H-3), δ 3.02 (1H, m, H-3a), δ 4.22 (1H, d, J = 12.0 Hz, 6a- CH_2OAC), δ 4.43 (1H, d, J=12.1 Hz, 6a-CH_2OAC), δ 4.63 (1H, d, J=13.5 Hz, 6-CH_2OAC), δ 4.80 (1H, d, J=13.8 Hz, 6-CH_2OAC), δ 6.07 (1H, brs, H-5).

Mass spectra: Concentrated mass major peak were found of molecular weight 194 m/z.

UV spectra: Active Fr. II maxima found at 210 nm and 280 nm.

On the basis of spectral analysis, the molecular formula of Compound-II may be $C_{11}H_{16}O_4$ by mass analysis (m/z 194).

Melting Point of compound (II): 89 ^{0}C-90 ^{0}C

Acute Toxicity Studies

Acute toxicity studies conducted revealed that the administration of graded doses of both the crude petroleum either and 70% ethanol extracts(up to a dose of 5000mg/kg body weight)of *X.strumarium* leaves extracts up to 2000mg/kg did not produce significant changes in behaviors such as alertness, motor activity, breathing, restlessness, diarrhea, convulsions, coma and appearance of the animals. No death was observed up to the 2g/kg body weight for the crude extracts of plant. These effects were observed during the experimental period (72 hrs).The results showed that in single dose, the plant extracts had no adverse effect, indicating that the medium lethal dose (LD50), could be greater than 2g/kg body weight in mice.

Body weight

The change in the body weight of control and experimental groups of mice treated with ethanolic extract and compound-II isolated from *X.strumarium* is shown in Table-2. Alloxan-induced (200mg/kg body weight) mice showed loss in body weight (From 6.15 to 7.95%), which was reversed by oral administration of compound-II and ethanol extract of *X.strumarium*. The body weight of the normal control mice (Negative control), which took the vehicle only, did not show any significant difference, i.e. a 0.55% change on the 14th day. However, the body weight of diabetic control mice (Positive control) showed a 4.15% decrease in their body weight after two weeks. In the untreated diabetic control group out of the five animals one mouse died on the tenth day. A dose-dependent body weight improvement was observed starting from day 1 in diabetic mice treated with both compounds-II and ethanol extracts. The effect was more pronounced in case of the compound-II treated mice (2.60-2.92%) as compared with the respective dose of the crude ethanol extract (2.34-2.60%) effect during the experimental period. During a 14-day treatment, the compound-II at a dose of 500 mg/kg showed a significant increase in the body weight of the mice from 30.50 ± 0.64g on day 1 to 31.39 ± 0.34g (2.92% increment) on day 14.

Table 2: Showing change in body weight of mice treated with compound-II and ethanolic extract of *X.strumarium*

Groups	Doses	Days of treatment			% change1	% change2
		Day 0	Day 1	Day 14		
Crude-II(Ethanol)	300 mg /kg	34.04±0.43	31.58±0.54	32.32±0.58	-7.23	2.34
Crude-II(Ethanol)	500 mg /kg	34.20±0.97	31.48±1.10	32.30±0.78	-7.95	2.6
Compound-II	300 mg /kg	30.50±1.72	28.46±1.63	29.20±1.55	-6.69	2.6
Compound-II	500 mg /kg	32.50±0.73	30.50±0.64	31.39±0.34	-6.15	2.92
Normal Control (Negative)	1 ml (vehicle)	25.00±0.96	25.38±0.99	25.52±0.96	1.52	0.55
Diabetic Control (Positive)		31.92±1.35	29.66±1.09	28.62±1.33	-6.45	-4.15
Glibenclamide	10 mg /kg	30.74±1.18	28.96±1.27	30.20±0.65	-5.79	4.28

Each result is with a mean of 5 mice. % change1 indicates the change between day 0 (before alloxan-induction) and day 1 (after alloxan-induction). % change2 indicates the change between day 1 and day 14.

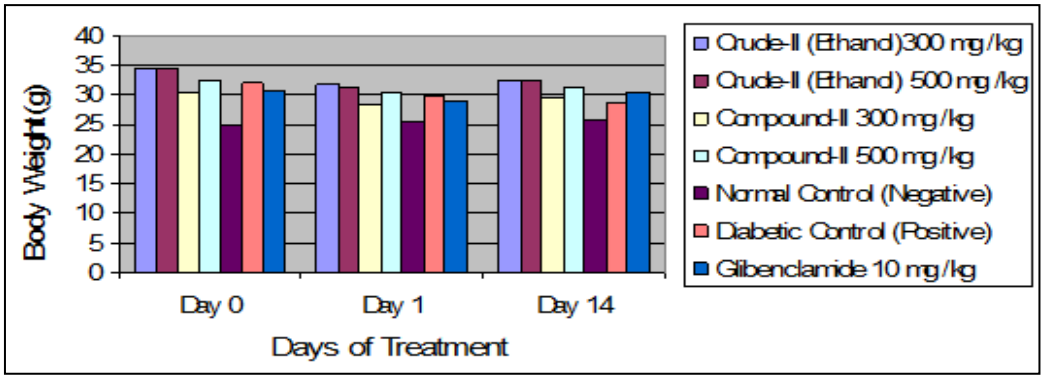

Fig 1: Showing 40 35 with Compound-II change in body weight and Ethanolic extract of mice treated of *X.strumatium*

Antidiabetic activity

The blood sugar levels measured in normal and experimental rats in initial and at the 01, 07 and 14 th days of treatment are given in table-3.Alloxan induced diabetic rates show significant increase in the levels on blood glucose as compared to normal rates. Oral administration of compound-II and crude ethanol extract (300 and 500 mg/kg) showed significant decrease (p<0.05) in blood glucose level. The isolated compound-II, Sesquiterpene lactone may be xanthanolide type (Kamboj and Saluja 2010) [8] at a dose level of 500mg/kg showed better reduction (p<0.05) in blood glucose level compared to similar dose of crude ethanol extract. The standard drug Glibenclamide decreased blood glucose level in 14 days treatment. On treatment with compound -II (300 and 500 mg/kg), the fasting mean blood glucose levels on day-1 (after being diabetic), i.e. 276.80±22.62 mg/dl reduced to 174.60±12.66mg/dl and 335.60±31.89 mg/dl reduced to 198.80±23.89 mg/dl respectively. This reduction accounts for 36.92% and 40.76%, respectively and treatment with ethanolic extract (300 and 500 mg/kg), the fasting mean blood glucose levels on day-1 (after being diabetic), i.e. 349.80 ±29.32 mg/dl reduced to 259.00±17.28 mg/dl and 343.80±20.22 mg/dl reduced to 248.30±12.85 mg/dl respectively. This reduction accounts for 25.95% and 27.77%, respectively the fasting mean blood glucose level of diabetic mice treated with Glibenclamide showed a reduction of 51.05% as compared with diabetic control (Positive control) mice on day 14.The improvement in blood glucose homeostasis was in dose dependent manner after 14 days treatment. The effect of the compound-II at a dose of 500 mg/kg body weight showed significantly better reduction as compared with the respective compound-I at a dose of 300 mg/kg and also with that of ethanol extract. This study indicated that the reduction of blood glucose level in compound-II and crude ethanol extract of *V.cinerea* in alloxan-induced mice were a dose dependent.

Table 3: Showing effect of compound-II (purified from *X.strumarium* L.) and ethanolic crude extract on fasting blood glucose level (mg/dl) in normal control and alloxan-induced diabetic mice

Groups	Days Of Treatment			
	Day 0	Day 1	Day 7	Day 14
Normal control (Negative control)	110.00±11.47	105.40 ± 10.99	111.40 ± 10.94	109.80 ±7.49
Diabetic control (Positive control)	123.40± 9.29	371.20 ± 37.20*	391.80 ± 31.26*	405.00±40.97*
Glibenclamide 10mg/kg	116.40 ± 3.97	349.40 ± 27.57	285.00 ± 22.49*	171.00±18.29*
Crude-II 300mg/kg	112.00 ± 6.93	349.80 ±29.32	289.60±23.76*	259.00±17.28*
Crude-II 500mg/kg	107.40 ± 9.21	343.80±20.22	240.40±17.98*	248.30±12.85*
Compound-II 300mg/kg	112.80 ± 9.23	276.80±22.62*	200.00±21.04*	174.60±12.66*
Compound-II 500mg/kg	116.00±10.19	335.60±31.89*	261.20±25.64*	198.80±23.89*

Values are given as mean ± standard deviation for groups of five animals. Values are statistically significant at* p<0.05.

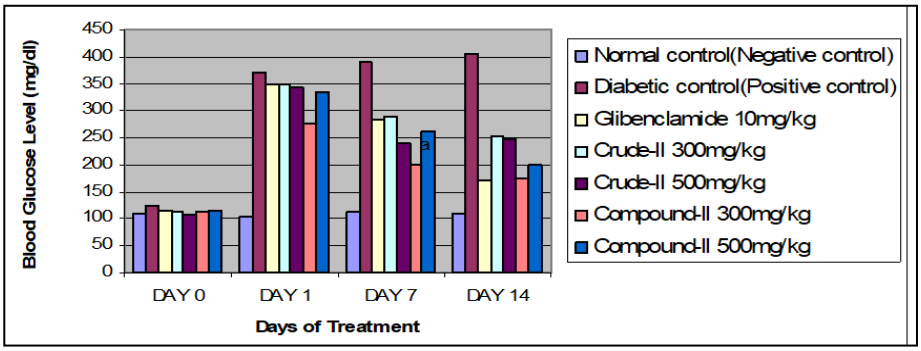

Fig 2: Showing effect of compound-II (purified from *X.strumatium* L) and Ethanol IC crude extract on fasting blood glucose level (mg/di) in normal control and alloxan-induced diabetic mice

Oral Glucose Tolerance Test

On fasting, the blood glucose level of the mice demonstrated basal hyperglycemia (Figure-03). The mean blood glucose value in the normal control (Negative control) mice rose to a peak value 60 min. after glucose load and decreased to near normal level at 120min. In diabetic control (Positive

control) mice, however, the peak increase in mean blood glucose concentration was observed after 60 min and remained high over the next 60 min. The animals that were subjected to oral glucose tolerance test showed a reduction in the mean blood glucose levels after 60 min. load of glucose. At 60 min. the blood glucose level reached the maximum in compound-II treated animals and then significant reduction was observed in the blood glucose level of diabetic treated with glucose loaded mice as compared with diabetic control (Positive control) mice, loaded only glucose. The mean blood glucose level at 120 min. after glucose administration was near to the baseline (fasting) in the compound-II treated as compared with diabetic mice untreated (Positive control). (Table-4)

Table 4: Showing glucose tolerance test of compound-II and ethanolic extract of *X.strumarium* on alloxan-induced diabetic mice

Groups	Time Intervals			
	Base line	30 min.	60 min.	120 min.
Compound-II (300mg/kg)	174.6±12.66	205.4±15.70	231.2±17.68	212±20.83
Compound-II (500 mg/kg)	198.8±23.89	256.2±15.92	305.4±14.82	217±8.62
Crude-II ethanol extract (300 mg/kg)	184.5±14.36	209.4±17.21	229±21.34	207.4±18.23
Crude-II ethanol extract (500 mg/kg)	240.8±23.89	313.6±28.37	331.6±23.18	273±28.68
Normal Control	109.8±7.49	151.6±14.96	156.8±7.67	131.4±10.22
Diabetic Control	405±40.97	422±26.65	433.8±30.12	432.6±29.54
Glibenclamide (10 mg/kg)	171±18.29	201.4±18.92	214.8±21.21	173.8±15.31

Values are given as mean ± standard deviation for groups of five animals.

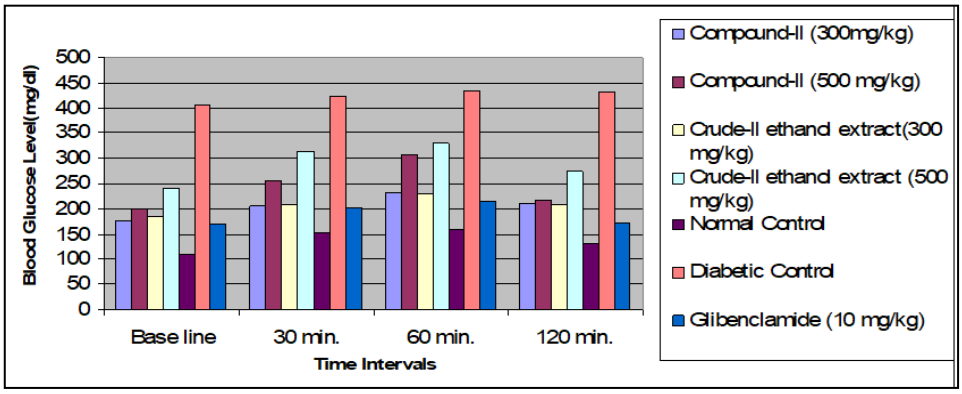

Fig 3: Showing glucose tolerance of conpound-Il and ethanclic extract of *X.drumadurn* on alloxan-educed dialbetic nice

Discussion

The aim of the present study was to evaluate the antidiabetic effect of ethanolic extract of *X.strumarium* plant and isolated compound Sesquiterpene lactone, may be Xanthanolide type (Kamboj and Saluja 2010) [8] against alloxan induced diabetic rates. The continuous treatment of the extracts of *X.strumarium* for a period of 14 days produced a significant reduction in the blood glucose level of mice. These results confirmed the use of *X.strumarium* plant in traditional practice as an antidiabetic agents and for the treatment of various diseases. The standard drug, Glibenclamide has been used for many years to treat diabetes, to stimulate insulin secretion from pancreatic β cells (Kumar *et al.* 2008) [11]. It may be suggested that the mechanism of action of Sesquiterpene lactone (Compound-II) is similar to glibenclamide.

The possible mechanism by which plant extract brings about a decrease in blood sugar level may be by potentiation of the insulin effect of plasma by increasing either the pancreatic secretion of insulin from β cells of the islets of Langerhans or its release from the bound form, A number of other plants have been reported to exert hypoglycemic activity through insulin release stimulatory effects (Gupta SS 1994, Kumar *et al.* 2008) [6, 9].

The ethanolic extracts *X.strumarium* evaluated in this work was phytochemically investigated in parallel, revealing the presence of sesquiterpene lactones (may be xanthanolide type), alkaloids, amino acids, carbohydrates, gums, glycosides, flavonoids, tannins. Plant with hypoglycemic and Antihyperglycemic activities may contain one or more chemical constituents. Some classes of chemicals and compound isolated from plant including caffiec acid, carboxyatractyloside, phenolic compounds are documented to decrease blood glucose level in *X.strumarium* (Kamboj and Saluja 2010) [8] but antidiabetic activity of this plant was not observed thoroughly. Extracts and metabolites from this plant have been known to possess pharmacological properties (Hasan *et al.* 2011, Pandey *et al.* 2012, Sravani and Mohana laxmi 2012) [15].

These results confirmed the use of *X.strumarium* in traditional system of medicine to treat diabetes in India. Further comprehensive chemical and pharmacological investigations are needed to elucidate the exact mechanism of the anti-hyperglycemic effect of *X. strumarium* plant.

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