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MELILOTUS INDICUS TOXICITY PROFILE, HISTOPATHOLOGY AND SCREENING OF HYPOTENSIVE ACTIVITY THROUGH NIBP METHOD ON RATS

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Abstract
The present investigation was carried out to evaluate the effect of *Melilotus indicus* (L.) All. in normotensive and fructose induced hypertensive rats. Aqueous-ethanolic extract of Melilotus indicus in 250, 500 and 750 mg/kg doses was studied in normotensive and fructose induced hypertensive rats using the non-invasive technique. The results obtained showed that the extract has significantly ($p < 0.001$) decreased the blood pressure and heart rate in dose dependent manner. The dose 750 mg/kg of the extract produced the maximum antihypertensive effect and was selected for further experiments. The extract was found to prevent the rise in blood pressure of fructose fed rats as compared to control group in 08 weeks study. The LD50 of the plant extract was more than 2000mg/kg b.w. in rats and acute toxicity study showed that there was no significant alteration in the blood chemistry of the extract treated rats. Furthermore, histopathological study revealed no injury to heart, liver and kidney tissues. It is conceivable, therefore, that the aqueous-ethanolic extract of *Melilotus indicus* has exerted considerable antihypertensive activity in rats and has duly supported traditional medicinal use of plant in hypertension.

Key words: *Melilotus indicus*, Toxicity, Histopathology, Screening, Hypotensive activity

Introduction

Hypertension remains a major risk factor for stroke, cardiovascular disease, renal disease, and ultimately death. By 2025, the number of people living with hypertension is expected to reach 1.56 billion people. Dietary factors that increase blood pressure (BP) are of interest to public health authorities, and recent attention has focused on fructose [1]. High fructose consumption has shown greater adverse effects on metabolism and vascular health than glucose consumption in several animal studies, Obesity, type 2 diabetes, and cardiovascular diseases were shown to be linked to fructose consumption in large amounts [2]. Many studies indicated that the mechanism of BP elevation due to

excessive fructose consumption falls into three broad categories: chronic stimulation of the sympathetic nervous system (SNS), dysfunction of the endothelium, and upregulation of salt absorption [3]. Previous studies revealed that development of hypertension is linked to the function of the SNS in fructose fed rats [4]. Information in the sympathetic and parasympathetic nerves is transmitted by renal afferent fibers. The information converges at the nucleus tractus solitarii (NTS), part of the brain which integrates signals from the peripheral carotid sinus and aortic arch to regulate systemic blood pressure, decreases production of nitric oxide (NO) during fructose-induced hypertension, the primary site of BP and sympathetic nerve activity (SNA) modulation [5, 6] Experimental lesions in the NTS cause a loss of baroreflex control of BP and sympathetic activation, and evoke severe hypertension in animals [7]. Chen *et al.*, suggested that excessive fructose intake increased fructose concentrations in the CSF and led to the development of high BP caused by sympathetic hyperactivity, suggesting that the CNS played a critical role in elevating BP through reduction of NO in the NTS, as well as impaired baroreflex sensitivity [8].

The *Melilotus indicus* family *Fabaceae* and is widely distributed in Asian and European countries, is one of the overwhelming annual herb in this era and its phytochemical analysis showed the active phytoconstuitents including coumarins, phenolic acids, flavonoids, steroids, saponins, volatile oils, triterpenes, carbohydrates, sugar, anthraquinonoid glycosides, mucilage, tannin, bis hydroxycoumarin, choline, alcohols, chicoric acid and many other functional groups [9]. This herb formerly known for its ethnomedicinal activities such as antibacterial, anticoagulant, astringent, emollient, laxative and narcotic [10]. Based on these facts, the current study was designed to explore the antihypertensive profile of ethanolic extract of *M. indicus* using fructose induced hypertension model in rats.

Materials and Methods

Drugs and Chemicals

The plant was extracted using distilled water, and ethanol. The supplier of fructose was Sigma Aldrich Pakistan. All chemicals were of analytical grades.

Animals and Treatments

In the specified animal room of the Government College University Faisalabad, Pakistan, healthy adult male Wister rats (weight range 180–220 g, 10–12 weeks) were retained in suitably ventilated polypropylene cages under standard conditions (temperature 25 ±2 °C, 12 h of light and dark, and 55–60% humidity), and given standard commercial pelleted rat feed and water ad libitum. The animal cages' bedding was changed every 48 hours. Before the investigation, the animals were acquainted for a week and divided into six groups at random (n = 6). For the toxicity investigation and fructose induced hypertension model, the oral route was used.

Approval from the Animal Ethics Committee or Institutional Review Board

Experimentation was conducted after receiving Institutional Review Board (IRB) approval from GC University and receiving the allowed reference number GCUF/ERC/183. All experimental protocols adhered to the National Research Council, Commission on Life Sciences University, and Institute of Laboratory Animal Resources guidelines (1996).

Plant Collection, Authentication, and Extract Preparation

In April 2019, Faisalabad, Pakistan's aerial parts of *Melilotus indicus* underwent collection. A taxonomist recognized and verified Dr. Mansoor Hameed of the Department of Botany from the University of Agriculture Faisalabad, Pakistan, and a voucher specimen with the number 258-1-22 was retained in their herbarium for reference.

After coarse grinding and sifting to achieve the fine powder, the plant was dried under shade for two months. 2 kg of *M. indicus* powder was macerated in 5 L of ethanol and water in 70: 30 ratio for 7 days while being sometimes stirred to produce the aqueous ethanolic *Melilotus indicus* extract (AEMI). To obtain the semisolid extract, filtration was done and mixture was subjected to a rotary evaporation process at 40 °C. By following formula, percentage yield was calculated

$$\text{Extract yield percentage (\%)} = (\text{weight of pure extract/weight of powder macerated}) \times 100$$

Procedure of Acute oral toxicity study

Rats that were female, not pregnant, and nulliparous were chosen for this investigation. The procedure complied OECD (TG) 425 recommendations [11]. Five rats each were included in the control and treatment groups. When the animal survived after receiving highest dose of 2000 mg/kg, four additional rats received the same treatment. Animals in the control group received the same level of care as those in the treatment groups.

All of the animals in both groups were closely monitored for the first 30 min following the oral administration of the extract, followed by hourly observations until the fourth hour after dosing, then periodically monitored for the first 24 h, and then daily afterwards, for 14 days, to access any behavioral changes or signs of toxicity listed in table 1.

Weekly weight records of the body were kept. The animals were slaughtered humanely, and their essential organs were taken out for histopathological analysis, on the fifteenth day. So, blood samples via heart puncture were taken for biochemical and hematological analysis.

Determination of acute hypotensive effect of plant by non-invasive method

Study in normotensive rats

Three sets of six rats each were created using rats whose blood pressure was normal. The aq-ethanolic extract of *Melilotus indicus* (AEMI) was administered in doses of 250, 500, and 750 mg/kg to the test animals in groups I, II, and III, respectively. The non-invasive tail cuff method was used to measure the heart rate and blood pressure at intervals of 0, 2, 4, 6 and 8 hours [12, 13].

Briefly stated, each animal was fitted with an NIBP restrainer, a sensor-equipped cuff put on its tail, and was inflated to a pressure of approximately 250 mmHg, or higher than the expected SBP, before being released. The pulses were recorded using the power lab. SBP, MAP, and heart rate (HR) were recorded directly from trace recordings; however, DBP was calculated using the formula.

$$\text{DBP} = (3\text{MAP} - \text{SBP})/2$$

Study in fructose-fed rats

The protocol set by Mamikutty *et al.*, is followed with slight modification [14], Rats were randomly divided into three groups each containing 6 animals and were given fructose solution (20 %) in water for a period of eight weeks. On the 56^{th} day, rats in were given following treatment. Group I: AEMI 250mg/kg, Group 2: AEMI 500mg/kg, Group 3: AEMI 750mg/kg. Blood pressure was measured with NIBP apparatus on 56^{th} day for 0 to 8 hrs.

Statistical analysis

The data of experimental research were established as mean ± SEM, (n = 6). The statistical significance to define intergroup variation was estimated by one and two-way ANOVA (analysis of variance) plotted by Tukey multiple comparison and Bonferroni post-test, respectively, through GraphPad prism 5 software. The results showing $p < 0.05$ were considered as statistically significant**.**

Results

Acute toxicity study

Effect on behavioral changes and weight gain in rats of control and AEMI extract treated groups during acute toxicity study

Acute toxicity test was performed at single oral dose of 2000mg/kg aqueous ethanolic *Melilotus indicus* extract. There was no change perceived in behavioral or psychological parameter neither any mortality was noticed in whole duration of 14 days as shown in Table1. The body weight comparison between normal group and extract treated group showing a statistically non-significant difference (Table 2) that was ascertaining safety profile of the plant which supports the claim that LD^{50} of the plant is more than 2000mg/kg.

Table 1: Effect of Aq-ethanolic extract of *Melilotus indicus* (AEMI 2000mg/kg) on behavioral changes

Parameters	Observations of control and Aqueous ethanolic AEMI extract treated group									
	30 minutes		4 h		24 h		7 days		14 days	
	Control	AEMI treated	Control	AEMI treated	Control	AEMI treated	Control	AEMI treated	Control	AEMI treated
Eye colour	N	N	N	N	N	N	N	N	N	N
Pupil size	N	N	N	N	N	N	N	N	N	N
Salivation	N	N	N	N	N	N	N	N	N	N
Skin	N	N	N	N	N	N	N	N	N	N
Fur	N	N	N	N	N	N	N	N	N	N
Urine colour	N	N	N	N	N	N	N	N	N	N
Feces consistency	N	N	N	N	N	N	N	N	N	N
Convulsions & tremors	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Itching	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Coma	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Sleep	N	N	N	N	N	N	N	N	N	N
Somatomotor activity & behavior pattern	N	N	N	N	N	N	N	N	N	N

N: normal, **N/A:** not applicable, **++:** increased, **AEMI:** Aqueous ethanolic Melilotus indicus extract

Table 2: Effect of Aq-ethanolic extract of *Melilotus indicus* (AEMI 2000mg/kg) on body weight (g) of rats in acute toxicity study

Days	Control	Treatment (AEMI 2000mg/kg)
	Body weight (g)	
1	183.6±1.36	187.2±1.40 ^{ns}
7	191.2±1.98	194.2±0.86 ^{ns}
14	200.4±1.60	199.4±1.03 ^{ns}

Values exhibited as mean ± SE (n = 6)

Effect on hematological and biochemical parameters during acute toxicity study

In acute toxicity testing, the CBC analysis revealed non-significant alteration between control and extract treated groups except significant variation in platelets count as shown in Table 3. The result was presented in normal range had supporting the safety profile of the plant. There was a noteworthy ($p<0.001$) difference found in cholesterol level of control and extract treated group (Table 4) which auxiliary the statement that plant have anti-hyperlipidemic activity. Analysis of liver and kidney function tests revealed non-significant variance of extract treated group in appraisal to normal control group except a difference of $p<0.05$ significance level detected in ALT level. This value again remained in normal range and indicating no harm to the treated animal.

Table 3: Effect of AEMI extract (2000mg/kg) on hematological in acute toxicity study

Parameters	Units	Normal Control	Treatment (AEMI 2000mg/kg)
Haemoglobin	g/dl	12.94±0.298	12.88±0.306^{ns}
TLC	$\times10^9$/l	12.0±0.70	11.7±0.57 ^{ns}
Total RBC	$\times10^{12}$/l	6.20±0.19	6.48±0.16 ^{ns}
HCT (PCV)	%	43.5±0.58	35.8±0.86 ^{ns}
MCV	Fl	61.0±1.82	53.2±1.77 ^{ns}
MCH	pg	17.6±1.20	18.0±0.70 ^{ns}
MCHC	%	32.6±0.65	35.0±1.00 ^{ns}
Platelets	$\times10^9$/l	568.8±5.81	628.4±1.6***
Neutrophils	%	36.6±2.44	44.4±1.29 ^{ns}
Lymphocytes	%	27.2±1.65	39.0±1.22 ^{ns}
Monocytes	%	2.80±0.73	3.4±0.81 ^{ns}

Values are exhibited as means ± SEM, (n = 5), *** $p < 0.001$, *$p > 0.05$ and ns (non – significant) in comparison to control group.

Table 4: Effect of AEMI extract (2000mg/kg) on lipid profile in acute toxicity study

Parameters	Units	Control	Treatment (AEMI 2000mg/kg)
Cholesterol	mg/dL	81.4±2.01	70.6±2.16***
Triglycerides	mg/dL	104.6±4.0	107.2±2.2ns
HDL	mg/dL	52.8±1.50	56.4±3.50 ns
LDL	mg/dL	73.6±3.25	68.2±1.93 ns
VLDL	mg/dL	18.6±1.03	23.8±1.56 ns

Values are exhibited as means ± SEM, (n = 5), *** $p < 0.001$ and ns (non – significant) in comparison to control group.

Table 5: Effect of AEMI extract (2000mg/kg) on liver and kidney function test in acute toxicity study

Parameters	Units	Normal Control	Treatment (AEMI 2000mg/kg)
Bilirubin	mg/dL	0.35±0.012	0.38±0.017ns
ALT	µ/L	48.8±1.65	56.0±2.43*
AST	µ/L	51.6±1.80	57.8±2.54ns
Alkaline Phosphatase	µ/L	153.0±3.03	148.4±3.35 ns
Protein	g/dL	6.87±0.23	6.24±0.19 ns
Albumin	g/dL	4.44±0.15	3.72±0.09 ns
Globulin	g/dL	2.48±0.122	2.5±0.16 ns
Blood urea	mg/dL	40.0±1.41	39.8±1.65 ns
Serum creatinine	mg/dL	0.69±0.034	0.67±0.025 ns

Values are exhibited as means ± SEM, (n = 5), * $p < 0.05$ and ns (non – significant) in comparison to control group.

Effect of AEMI extract (2000mg/kg) on Histology of heart, liver and kidney in acute toxicity study

Acute administration of aqueous-ethanolic extract of *Melilotus indicus* at 2000mg/kg did not revealed any injury to heart, liver and kidney tissues of the rats in comparison to the normal control group. The photomicrographs representing histology of various organs in both groups of rats shown in figure 1.

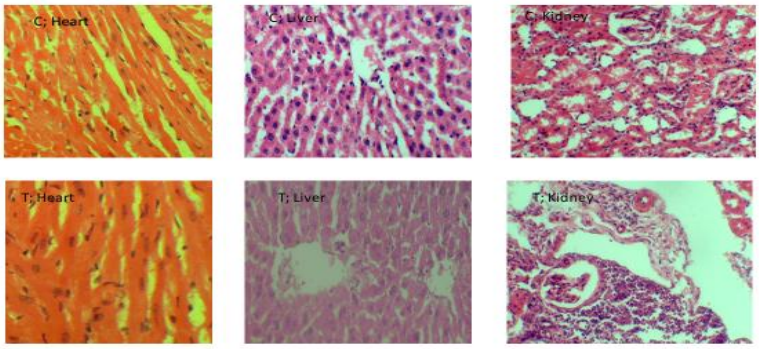

Figure 1: The microscopic photographs of Heart, Liver and Kidney of Control Group (C) and Treatment Group (T) at 40X magnification.

Evaluation of blood pressure and heart rate in normotensive rats

Aq-ethanolic extract of *M. indicus* (AEMI) essentially diminished the systolic blood pressure (SBP), Diastolic blood pressure (DBP), mean arterial blood pressure (MAP) and furthermore Heart rate (HR) of normotensive rodents in all treatment groups in a dose dependent manner. The 750 mg/kg dose was found to deliver greatest diminishing in every one of the three boundaries following 8 hours of experiment though at the 250 mg/kg dose the BP and HR of the experimental animals diminished up to 6 hours, however, at the 500mg/kg dose there was slight decrease in BP and HR all through 8 hours in all treated animals (Figure 2).

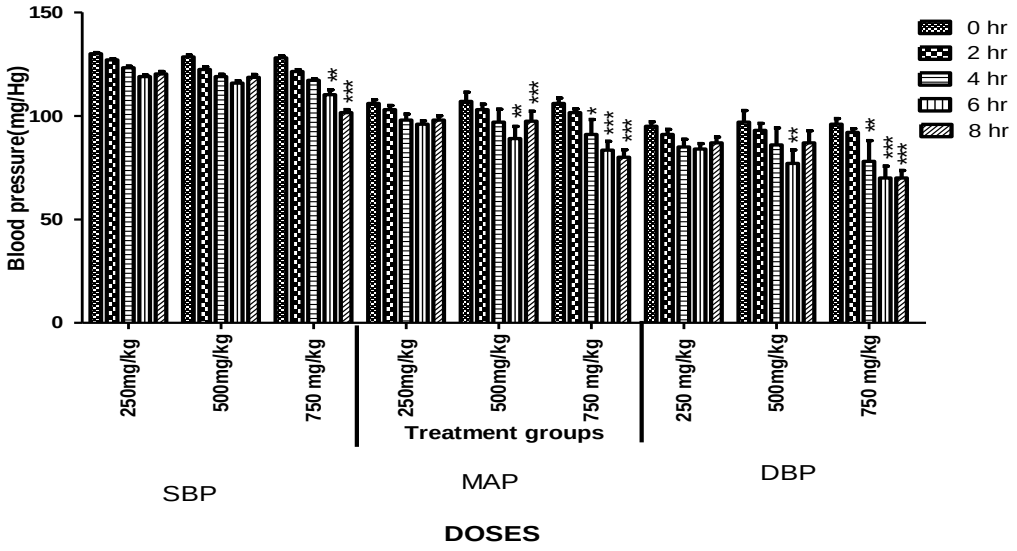

Figure 2: Effects of oral administration of various doses of AEMI extract on SBP, MAP and DBP in normotensive rats.

Results are exhibited as means ± SEM, (n = 5), *** $p < 0.001$, **$p < 0.01$,* $p<0.05$ in comparison to 0 hr.

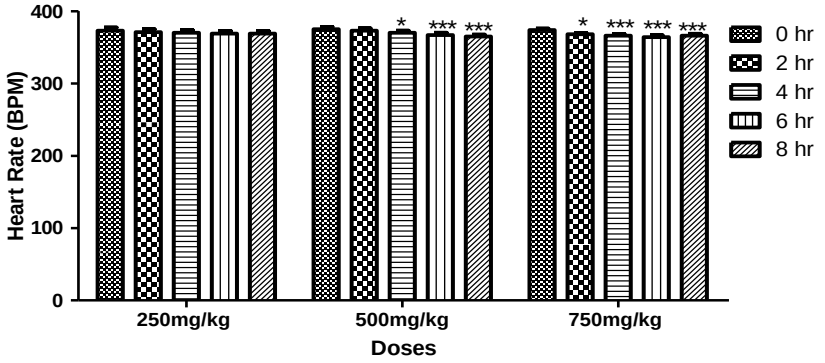

Figure 3: Effects of oral administration of various doses of AEMI extract on heart rate in normotensive rats.

Results are exhibited as means ± SEM, (n = 5), *** $p < 0.001$, **$p < 0.01$,* $p<0.05$ in comparison to 0 hr

Evaluation of acute antihypertensive effect of *Melilotus indicus* extract in Fructose-fed hypertensive rats

Aq-ethanolic extract of *M. indicus* inauspiciously and dose dependently reduced the blood pressure and heart rate of fructose-fed rats from 1 h to 8 hours (Table 6). The 750 mg/kg dose showed most intense hypotensive impact in both normotensive and fructose-fed rats. Accordingly, this dose of the extract was chosen for additional pharmacological experiments in rodents. The result remained

statistically significant $p<0.001$ after 8 hours of treatment when contrasted with 0 hr in totally treated groups.

Table 6: Effects of oral administration of AEMI on SBP, MAP and DBP and HR in Fructose fed rats

Time	Treatment with aqueous-ethanolic Melilotus indicus extract in fructose-fed hypertensive rats											
	250 mg/kg dose				500 mg/kg dose				750 mg/kg dose			
	SBP	MAP	DBP	HR	SBP	MAP	DBP	HR	SBP	MAP	DBP	HR
0 hr	169.8±0.86	145.6±1.575	133.5±1.93	398±1.22	170.6±0.805	155.6 ±1.22	148.2±1.48	400±1.89	171±0.98	151.2±1.40	141±1.75	402±1.25
2 hr	165.5±0.697	140.6±1.61	128.25±2.17	385±6.6	164.3±1.37	139.7±1.26	126.2±1.51	382±1.84	157.3 ±1.02	140.2±1.19	131.5±1.87	380±1.72
4 hr	156.5±0.69*	133±0.78**	121.25 ±0.84**	378±1.53**	152.0±1.25**	121.5±1.63***	105±1.83***	373±1.18**	143.5±1.22***	132.5±1.07***	127±1.65***	371±1.26**
6 hr	148.3±0.77***	127.8±1.04***	117.6 ±1.20***	371±1.36***	141±1.84***	114.2±1.80***	99.4±2.05**	371±1.29***	133.6 ± 1.05***	127.3±0.90***	124.2±1.32***	357±1.58***
8 hr	145.6±1.15***	125.6±0.87***	115.6±0.97***	367±1.36***	137.8±1.04***	109.5 ±1.07***	94.6±1.52***	369±1.20***	127.2±1.06***	113.3 ±1.22***	106.4 ±1.47***	335±2.1***

Results are exhibited as means ± SEM, (n = 5), *** $p < 0.001$, **$p< 0.01$, * $p<0.05$ in comparison to 0 hr.

Discussion

Herbal drugs have been used from prehistoric times. In this background, aboriginal drugs considered to be of great importance from both economic and professional point of view [15]. Acute oral toxicity study gives initial vision of a plant's level of toxicity. In acute oral toxicity, dose 2000 mg/kg/b.w. of the aqueous ethanolic extract of *Melilotus indicus* (AEMI) was administered as single dose orally in rats. The results of the present study did not show any detrimental effects or mortality on different hematological and biochemical parameters as shown in Table 3, 4 &5. The histopathological studies on liver, heart and kidney confirms the safety profile of tested plant as shown in Figure 1. Thus, extract of *M. indicus* deliberated non-toxic at the acute level and consequently, the oral lethal dose (LD50) of the aqueous ethanolic extract of *M. indicus* is more than 2000 mg/kg/b.w. However, the efficacy of *M. indicus* as an antihypertensive agent has never been investigated before. On this basis the current investigation was conducted to evaluate the antihypertensive effects of *M. indicus*. The core outcome of this study was that the AEMI was able to decrease blood pressure both in normotensive as well as fructose-treated hypertensive rats. It has been reported that Fructose is responsible to raise BP via increasing uric acid production, which exerts hemodynamic effects, such as increased oxidative stress, endothelial dysfunction, and activation of the renin-angiotensin-aldosterone system as well as sympathetic nervous system activation [16, 17]. In present study, we used fructose-induced hypertension model to investigate antihypertensive effect of aqueous ethanolic extract of *M. indicus* for this purpose 20% fructose solution was used in drinking water for 08 weeks which induced a significant increase in blood pressure of rats. Pharmacological investigation of AEMI showed a dose dependent decrease in the SBP, MBP, DBP and heart rate in both the normotensive and fructose-treated hypertensive rats. The results of screening of different doses such as 250, 500 and 750 mg/kg exhibited that the reductions in all the above parameters were more pronounced at 750 mg/kg and less in 500mg/kg dose in normotensive rats (Figure 2). Effect on heart rate was more pronounced on 750mg/kg doses at 4 to 8 hour interval. In fructose-fed rats a comparative study between different doses 250, 500 and 750mg/kg was made. 750mg/kg dose exhibited more pronounced reduction in blood pressure parameters and heart rate up to 8 hours interval (Table 6). It has been reported that plants rich in polyphenols having an antioxidant effect, which improves endothelial dysfunction through increase NO formation, decrease LDL formation, increase prostacyclin formation, increase endothelium-derived hyperpolarizing factor (EDHF)-mediated vasorelaxation and decrease Endothelin-1 production. So, the polyphenols concerning vasorelaxation beneficial effects have been attributed to blood pressure reducing properties in rodents [18]. The hypotensive effect of extract was more pronounced in fructose-induced hypertensive rats as compared to normotensive rats. This is in line with previous findings that hypertensive rats appear to have more pronounced response to hypotensive agents than normotensive rats [19]. Previously *M. indicus* reported to have β-sitosterol, coumarin, methyl 3-(4-hydroxyphenyl) propionate, kaempferol-3-O-α-L-rhamnoside, rutin, apigenin-7-O-β-D-lutinoside, dibutyl phthalate, robinin, clovin, kaempferol-3-O-rutinoside, isoquerecitrin and medicarpin 3-O-β-glucopyranoside after purification by silica gel column chromatography [20].

Kaempferol-3-O-α-L-rhamnoside has a potential in cardiovascular disease especially in hypertension, dyslipidemia and hyperglycemia. Its major role is reported in the treatment of electrolyte disturbance and renal insufficiencies due to high blood pressure [21]. Kaempferol-3-O-rutinoside has potential to treat cell death include necrosis, apoptosis and autophagy but its potential against metabolic disorders including diabetes, specially hypertension, heart failure and in inflammation and oxidative stress is reported [22]. Thus, the antihypertensive effect of *M. indicus* may be due to the presence of polyphenols having antioxidant effects.

Conclusion

Aqueous ethanolic extract of *Melilotus indicus* possesses antihypertensive effect which may be due to the presence of phytochemical constituents. Moreover, acute toxicity study have shown that plant extract is safe for use. Thus present investigation calls for further activity guided fractionation study of the test plant extract and to investigate exact antihypertensive mechanism.

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