INVESTIGATION OF ANTINOCICEPTIVE ACTIVITY OF LESPEDEZA CUNEATA ROOT EXTRACT IN EXPERIMENTAL ANIMALS

Prateek Pandey*[1], Dindayal Patidar[1], Naveen Gupta[1], Dharmendra Singh Rajput[1]

[1]Patel College of Pharmacy, Madhyanchal Professional University, Ratibad, Bhopal-462044 (M.P.)

Corresponding Author:

Mr. Prateek Pandey

Research Scholar

Patel College of Pharmacy

Madhyanchal Professional University, Ratibad, Bhopal-462044 (M.P.)

hifiprateek@gmail.com

Abstract:

The aim of the study was to investigate the antinociceptive activity of methanolic extract of the roots of Lespedeza cuneata (MELC) by hot plate method in rats. Dried powdered roots of Lespedeza cuneata were subjected to solvent extraction by using 70 % methanol. Based on acute oral toxicity study according to Organization for Economic Cooperation and Development (OECD) guidelines No. 423, two doses of the test drug 200 mg/kg and 400 mg/kg was selected and were subjected to antinociceptive activity. The results were analyzed by ANOVA. P value<0.05 was considered as significant showed antinociceptive activity in this experimental model of pain. Its onset of action was similar to that of pentazocine. However, the degree of antinociception was significantly less than that of pentazocine.

Keywords: *Antinociception, Lespedeza cuneata, acute oral toxicity, methanol.*

Introduction:

Intense or harmful stimuli can generate pain, an unpleasant feeling that is essentially defensive in nature and can serve as a sensory modality to identify the presence of tissue injury [1,2]. Pain can have many different forms and origins, but it always refers to a feeling of discomfort, either physical or emotional, that interferes with day-to-day activities. Harmful physiological consequences, such as poor sleep, fatigue, confusion, anxiety, tachycardia, increased myocardial oxygen demand, immunosuppression, and accelerated catabolism, might occur even when pain is relieved [3].

Because pain modulation is a complex process involving numerous mediators and receptors at the peripheral and central levels, the management of pain using currently available analgesics could not fully prosper in relieving pain. A wide range of extracellular mediators regulate the sensitivity of nociceptive neurons. These mediators, which can be either neurotransmitters or neuromodulators, stimulate a wide range of receptor classes, which in turn trigger an abundance of signaling cascades that regulate pain perception [4,7]. We are still learning how this variety of cascades mediates pain and nociceptor sensitization. As a result, efforts are being made globally to pinpoint the elements of this intricate process and create novel agents that interact with these elements. Aside from that, a number of side effects have overshadowed the effectiveness of currently available analgesic medicines, such as opiates and nonsteroidal anti-inflammatory drugs (NSAIDs), making them less effective in all situations. For instance, long-term use of morphine, the medication of choice for treating pain, has been shown to result in tolerance and dependence [8]. Existing analgesics exacerbate the condition by relieving pain as a symptom without addressing its underlying cause [9]. Thus, there is a pressing need to find novel analgesic medications with promising pharmacological effects.

Lespedeza cuneata belongs to the family Fabaceae. *L. cuneata* is a flowering plant commonly grown in Asian countries and has been reported for its antioxidative and anti-inflammatory effects [10], scalp-improving properties [11], prostatic hyperplasia [12], early atherosclerosis [13], and whitening skin [14].

In view of the above collection and utilization of different parts of *Lespedeza cuneata*, an attempt was made to investigate the pharmacological activities of roots of *Lespedeza cuneata* methanolic extract. To the best of our knowledge, this is the first report on antinociceptive property of methanolic extract of root of *Lespedeza cuneata* . In the present study, in order to evaluate the potential antinociceptive effect of the extract of *Lespedeza cuneata*, we investigated said activity in experimental animal models using hot plate test in rats.

Material and Methods:

Drugs and chemicals:

Pentazocine (CAS No.: 359-83-1) purchased from Manus Aktteva Biopharma LLP. All other chemicals and solvents were obtained from local firms (India) and were of the highest purity and analytical grade.

Pharmacognostic study:

Plant materials were subjected to morphological examinations using reported method and the results were then compared with reported characters. Fresh plant material was studied for color, odor and taste, determination of shape, surface characteristics and appearance, etc.

Plant material and extraction:

The roots of *Lespedeza cuneata* were collected from Uttarakhand region of India. The plant material was taxonomically identified and authenticated by the Department of Plant Science, MJP RohilKhand University, Bareilly (U.P.), India, The samples were cleaned, dried under

shade, powdered by a mechanical grinder and stored in an airtight container. Methanol was used as solvent in the extraction process. Methanol was used as a solvent and the mixture was shaken occasionally for 48 hrs. Then the extract was filtered before drying using Whatman's filter paper no.2 on a Buchner funnel and the solvent was removed by vacuum rotary evaporator at 40^{o}C [15].

Physicochemical analysis:

Physicochemical analysis was performed for loss on drying, total ash, water soluble ash, acid insoluble ash, alcohol soluble extractive value [16].

Preliminary phytochemical investigation:

Preliminary phytochemical investigation revealed the presence of carbohydrates, proteins alkaloids, tannins, flavonoids and glycosides, steroids, tannins and phenolic compounds in methanolic root extract of plant *Lespedeza cuneata* (MELC) [16].

Determination of oral acute toxicity study:

The lethal median dose (LD_{50}) determination was performed in rats by Organisation for Economic Co-operation and Development (OECD) Guideline 423. A single dose of the extract (i.e., 5 mg/kg, 50 mg/kg, 300 mg/kg, or 2000 mg/kg) in the appropriate quantity of water was administered orally by gavage to different groups of rat (three rat in each group). However, all the animals were deprived of food for 2 hours prior to dosing and 4 hours after dosing. For the first 12 hours, the animals were initially monitored continuously for any adverse effects for 4 hours and then monitored at 1-hour intervals. They were later monitored twice daily for any abnormal changes throughout the study period (which lasted 14 days) [17]. The lethal median dose (LD_{50}) of the 70% methanolic root extract of plant *Lespedeza cuneata* (MELC) was 2000 mg/kg. One-tenth of the maximum dose of the extract tested for acute toxicity was selected for antinociceptive activity (i.e., 200 mg/kg and its double strength of 400 mg/kg of body weight).

In-Vitro Antioxidant Activity:

DPPH scavenging activity:

The molecule 1, 1-diphenyl-2-picrylhydrazyl (a,a-diphenyl-bpicrylhydrazyl; DPPH) was characterized as a stable free radical by virtue of the delocalization of the spare electron over the molecule as a whole, so that the molecule does not dimerize, as would be the case with most other free radicals. The delocalization of electron also gave rise to the deep violet color, characterized by an absorption band in ethanol solution centered at about 517 nm. When a solution of DPPH was mixed with that of a substrate (AH) that can donate a hydrogen atom, then this gave rise to the reduced form with the loss of this violet color.

In order to evaluate the antioxidant potential through free radical scavenging by the test samples, the change in optical density of DPPH radicals was monitored. The sample extract (0.2 ml) was diluted with methanol and 2 ml of DPPH solution (0.5 mM) was added. After 30 min, the absorbance was measured at 517 nm. The percentage of the DPPH radical scavenging was calculated using the equation as given below:

$$\textbf{\% inhibition of DPPH radical} = ([A_{br} - A_{ar}) / A_{br}] \times 100$$

Where A_{br} is the absorbance before reaction and A_{ar} is the absorbance after reaction has taken place [18].

Hydrogen peroxide scavenging (H_2O_2) assay:

Human beings are exposed to H_2O_2 indirectly via the environment nearly about 0.28 mg/kg/day with intake mostly from leaf crops. Hydrogen peroxide may enter into the human body through inhalation of vapor or mist and through eye or skin contact. H_2O_2 is rapidly decomposed into oxygen and water and this may produce hydroxyl radicals (OH^-) that can initiate lipid peroxidation and cause DNA damage in the body.

The ability of plant extracts to scavenge hydrogen peroxide was estimated by the solution of hydrogen peroxide (40 mM) which was prepared in phosphate buffer (50 mM pH 7.4). The concentration of hydrogen peroxide was determined by absorption at 230 nm using a spectrophotometer. Extract (20–60 μg/mL) in distilled water was added to hydrogen peroxide and absorbance at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging was calculated as follows:

$$\% \text{ scavenged } (H_2O_2) = [(A_i - A_t)/A_i] \times 100$$

Where A_i is the absorbance of control and A_t is the absorbance of test [19].

Ethical Considerations:

The study was carried out in compliance with CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) principles, having been approved by the Institutional Animal Ethics Committee (IAEC).

Experimental animals:

Albino male Wistar rats (125-200 g) were used for the experiments. The animals were maintained under standard environmental conditions for at least one week in the laboratory animal room prior to testing. Food and water were given ad libitum unless otherwise specified. The animals were housed for at least one week in the laboratory animal room prior to testing. All experimental protocols were approved by the institutional animal ethics committee.

Animal housing:

Rats were kept in groups of four in a typical large polypropylene cage that measured 40 x 27.5 x 13.5 cm and had a wire mesh top with a place for pellets and drinking water. Each cage's bedding was made of corn cobs. The temperature ($25^oC \pm 5^oC$), relative humidity (55 ± 10%), and light/dark cycle (12/12) were all kept at standard levels. Not only were food and water provided on a daily basis, but rats were also left alone.

Screening for Antinociceptive Activity:

Hot plate method:

Wistar albino rat weighing between 125 and 200 grams were taken for study. In order to measure the rat's reaction time in seconds, the assessment comprised placing it on a heated surface (produced by UGO Basile, Italy, Model No. DS-37) that was set to a temperature of 55±1°C. Reaction time was measured at the beginning of actions like jumping or paw licking. Before and after the delivery of MELC along with pentazocine, these observations were taken at intervals of 0, 30, 60, 90, 120, 150, and 180 minutes. There was careful adherence to a maximum exposure restriction of 15 seconds to avoid any injury to the rat. The rats were divided into four groups, each with six rats, for the purpose of the study [20].

Investigation material:

a) Methanolic extract of root of *Lespedeza cuneata* (MELC)
b) Pentazocine (Standard drug)

Dose selection:

a) *Lespedeza cuneata* methanolic root extract (MELC): 200 mg/kg body weight
b) *Lespedeza cuneata* methanolic root extract (MELC): 400 mg/kg body weight
c) Vehicle control (normal saline): 5 ml/kg (p.o.)
d) Standard (Pentazocine): 5 mg/kg body weight (i.p.)

Statistical analysis:

The standard error of the mean (SEM) for pharmacological experiment outcomes was given as the mean ±. Graph Pad Prism 5.0 (Graph Pad, San Diego, CA, USA) was the program used for the analysis. The following parameters were examined: body weight, paw withdrawal delay, pain threshold, mechanical nociceptive threshold, temperature changes, paw volume, and post hoc Bonferroni test for multiple comparisons. Two-way Analysis of Variance (ANOVA) was used for the analysis. Dunnett's test was performed after one-way Analysis of Variance (ANOVA) was used to assess data on hematological, serum biochemical, and antioxidant parameter values. A p-value of less than 0.05 ($P<0.05$) was considered to be statistically significant.

Results:

Pharmacognostic Study:

The study examined the root of *Lespedeza cuneata*. Morphological traits such as colour, odour, taste, size, and form were also examined. The plant have a distinct distinctive scent and range in colour from light to dark green. The roots of *Lespedeza cuneata* are known for their bitter flavour. The accompanying table provides a summary of the macroscopic analysis results.

Table 1: Macroscopical features of plant material of root of *Lespedeza cuneata*

S.No.	**Parameters**	***Lespedeza cuneata (root)***
1.	Colour	Greenish
2.	Odour	Characteristic
3.	Taste	Slightly bitter
4.	Size	2.1 m long
5.	Shape	Dumbbell or bottle shaped

Preliminary Phytochemical Investigation

Ash Values:

Lespedeza cuneata showed loss on drying 14.5% w/w, total ash of 19.56% w/w, acid-insoluble ash of 2.90% w/w, water-soluble ash of 4.80% w/w. These findings shed light on the mineral makeup and quality of the plant materials under investigation.

Extractive Values:

13.8% was discovered to be the alcohol-soluble extractive value of *Lespedeza cuneata* root. These percentages show how much plant material is soluble in alcohol.

Loss on Drying:

Lespedeza cuneata had a loss on drying of 14.5% w/w, which suggests a comparatively low moisture content.

Table 2: Preliminary Phytochemical Investigation for *Lespedeza cuneata* root

Parameters	**Results**
	Lespedeza cuneata (root)
Ash values: 1.Water Soluble ash 2. Acid insoluble ash 3. Total Ash	 4.80% w/w 2.90 % w/w 19.56 % w/w
Extractive values: Alcohol soluble extractive value	 13.8% w/w
Loss on drying	14.5 % w/w

Estimation of total tannin content:

Saponins and polyphenolic derivatives outweigh the chemical constituents of active medicinal ingredients in herbal preparations. Polyphenols and tannins have numerous pharmacological uses, including anthelmintics, antioxidants, antivirals, antimicrobials, and cancer treatment. While tannins can be found in almost all plant foods, medicinal plants are the primary suppliers of tannins that are utilized as active ingredients in pharmaceutical goods. However, the structures of these polyphenolic compounds vary widely throughout plant species and are often poorly described. The results of the examination of the total tannin content are shown in the below table 3:

Table 3: Total tannin content in methanolic extract of root of *Lespedeza cuneata*

S.No.	**Extract of Plant material**	**Total tannin (%)**
1.	*Lespedeza cuneata* root	7.23

Estimation of total phenolic Content:

In recent times, phenolic chemicals, which are present in medicinal herbs, have garnered more interest due to their potent antioxidant properties. Numerous studies have shown how important these compounds are in stopping the growth of various ailments. Particularly well-known for their beneficial effects on human health are polyphenols,

which include the potential to reduce the risk of heart disease by blocking the oxidation of low-density lipoproteins. All phenolic compounds have anti-inflammatory and cancer-preventive properties. Moreover, it is well known that some phenolic compounds, like flavonoids, have the ability to scavenge reactive oxygen species, which makes them promising treatments for illnesses associated with free radicals.

Thus, assessing the polyphenolic content of herbs especially in connection to plant-based medicines becomes crucial for assessing their potential health benefits. Gallic acid is commonly used as a standard in the quantification of total phenolic compounds. The results are shown in the linked table 4, together with the standard curve and the proportion of total phenolic compounds in each of the several plant sources.

Table 4: Total phenolic content in methanolic extracts of root of *Lespedeza cuneata*

Plant	**Intensity absorbance**	**Dilution factor**	**Concentration µg/ml**	**Final concentration**
Lespedeza cuneata	0.065	10.00	2.352	51.21%

Preliminary phytochemical screening:
Qualitative phytochemical tests showed presence of carbohydrate, proteins, glycosides, tannins, alkaloids, flavanoids, saponins.

Table 5: Chemical Constituents present in methanolic extract of root of *Lespedeza cuneata (MELC)*

S.No.	**Phytochemicals**	**Test performed**	**MELC**
1.	Alkaloid	Dragendorff's test, Mayer's test, Wagner's test	+
2.	Glycosides	Killer-Killiani test, Legal test	+
3.	Carbohydrate	Benedict's test, Fehling's test	+
4.	Steroids	Salkowski test	+
5.	Proteins	Biuret test, Xanthoprotein test, Lead acetate test	+
6.	Tannins	Lead acetate test, Ferric chloride test	+
7.	Flavonoids	Alkaline reagent test, Lead acetate Test	+
8.	Saponins	Foam test	+
9.	Cynogenetic glycoside	Guignard sodium picrate test	-

In-vitro antioxidant activity:

Table 6: Free radical scavenging activity of methanolic extract of root of *Lespedeza cuneata (MELC)*

Drug	Concentration (µg/ml)	DPPH scavenging activity (% inhibition)	Hydrogen peroxide scavenging (H_2O_2) assay (% inhibition)
MELC	10	2.18%	5.20%
MELC	50	12.92%	15.45%
MELC	100	20.87%	30.66%
MELC	250	59.76%	55.40%
MELC	500	67.45%	65.75%
MELC	1000	75.54%	70.23%
BHT	10	20.07%	22.55%
BHT	50	38.17%	35.80%
BHT	100	47.71%	50.25%
BHT	250	90.25%	91.55%
BHT	500	95.22%	95.45%
BHT	1000	97.21%	97.00%

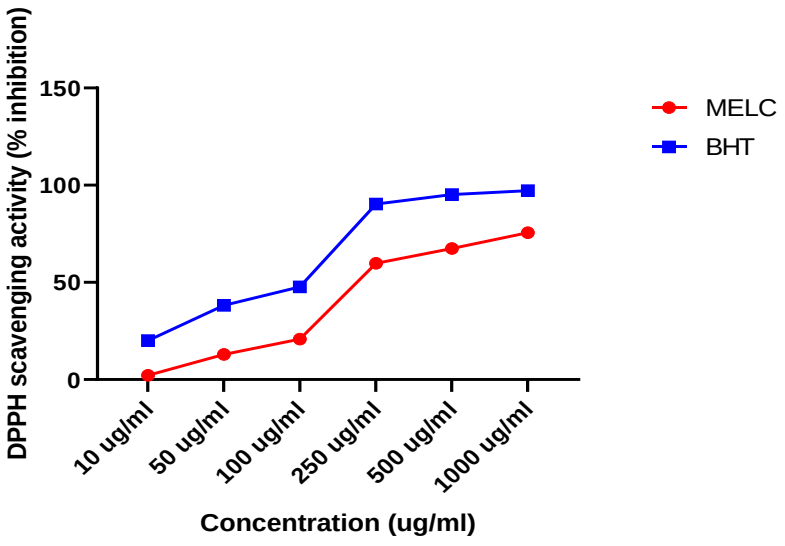

Fig. 1: Graph showing inhibition of DPPH radical by MELC and BHT

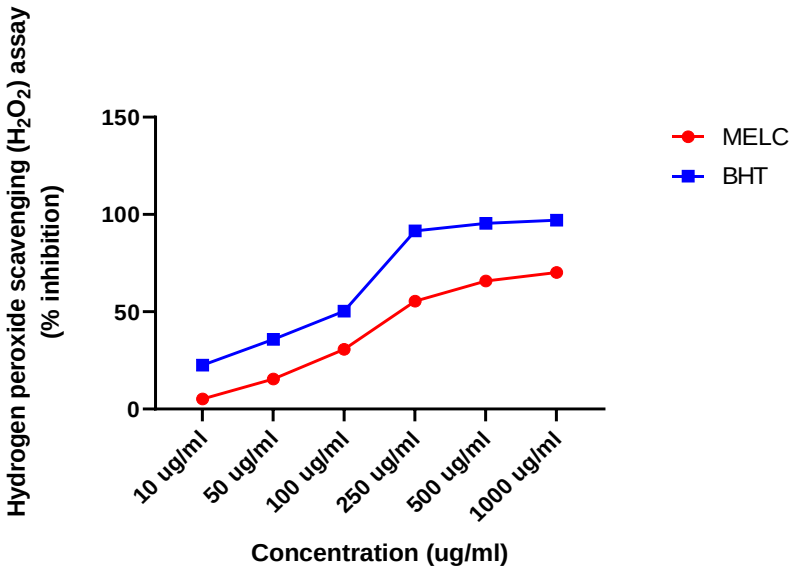

Fig. 2: Graph showing scavenging of hydrogen peroxide (H_2O_2) by MELC and BHT

Screening of antinociceptive activity:

Hot plate method:

Pentazocine (5 mg/kg) injection resulted in a statistically significant increase in reaction times at all time intervals, beginning at 60 minutes, as compared to the control group ($P<0.05$). Refer to table 7 and Fig. 3. Reaction times significantly increased ($P<0.05$) after receiving 200 mg/kg and 400 mg/kg of methanolic root extract of plant *Lespedeza cuneata* in comparison to the control group. It began to act at 60 minutes and continued for up to 120 min. Compared to the rise in reaction times following pentazocine administration, the increase in reaction times with methanolic root extract of plant *Lespedeza cuneata* (MELC) was noticeably smaller.

Table 7: Effect of oral administration of methanolic extract of root of *Lespedeza cuneata* (MELC) on hot plate test in rats

Treatment Groups	**Reaction time (sec.)**						
	0 min.	**30 min.**	**60 min.**	**90 min.**	**120 min.**	**150 min.**	**180 min.**
Vehicle Control	6.02±0.45	5.45±0.44	5.50±0.62	4.83±0.21	5.65±0.55	5.83±0.41	5.68±0.58
Pentazocine (5 mg/kg)	5.65±0.50	5.90±0.38	9.15±0.50***	11.33±0.36***	7.98±0.40**	6.08±0.58	5.87±0.40
MELC (200 mg/kg)	4.73±0.53	5.58±0.57	4.60±0.49	5.30±0.24	5.77±0.58	5.82±0.38	5.78±0.21
MELC (400 mg/kg)	6.50±0.40	4.98±0.51	5.63±0.46	6.15±0.51	5.92±0.32	5.55±0.37	5.22±0.60

Effect of oral administration of methanolic root extract of plant *Lespedeza cuneata* on hot plate test in rats, N=6 and values are expressed as mean±SEM. One way ANOVA followed by Dunnet's test, *p< 0.05, as compared with control.

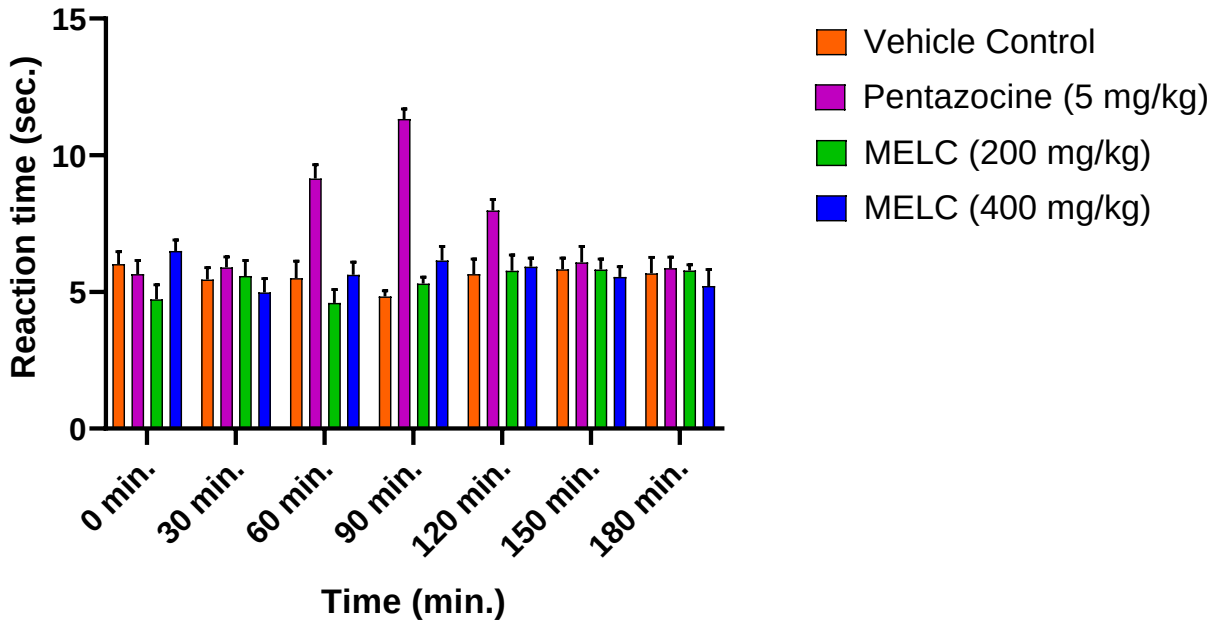

Fig. 3: Effect of oral administration of methanolic root extract of plant *Lespedeza cuneata* on hot plate test in rats

Discussion:

Administration of pentazocine resulted in significant increase in reaction time, which validated the model used. Similarly, administration of methanolic root extract of plant *Lespedeza cuneata* (MELC) 200 mg/kg and 400 mg/kg resulted in significant increase in reaction times at various time interval, suggesting the antinociceptive activity of this drug. The onset, peak and duration of MELC and pentazocine were similar, indicating that MELC can be used at similar time intervals. This study demonstrated that methanolic root extract of plant *Lespedeza cuneata* (MELC) can be used as an adjuvant to the other standard antinociceptive drugs or can be used alone as an antinociceptive.

Conclusion:

In this present study antinociceptive effect of methanolic root extract of plant *Lespedeza cuneata* (MELC) 200 mg/kg and 400 mg/kg was evaluated by using hot plate method from which it is concuded that 400 mg/kg dose of methanolic root extract of plant *Lespedeza cuneata* (MELC) is effective as antinoceceptive. Hot plate method is a thermal method of antinociceptive activity. It shows effective antinociceptive activity at 1 ½ hrs. The 400 mg/kg drug of methanolic root extract of plant *Lespedeza cuneata* (MELC) having similar effect to the standard drug pentazocine at 1 ½ hrs. Pentazocine is agonist-antagonist type of analgesic drug. In hot plate method the 400 mg/kg drug of methanolic root extract of plant *Lespedeza cuneata* (MELC) is effective at the 1 ½ hrs than the 200 mg/kg.

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