ISOLATION AND ESTIMATION OF ANXIOLYTIC PRINCIPLE FROM MEDICAGO SATIVA (LINN.)

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ABSTRACT

Background: Medicago sativa Linn. (Family - Leguminosae) has been used traditionally for the treatment of a variety of CNS disorders. Our previous work inferred that methanol extract of M. sativa aerial parts exhibited significant anti-anxiety activity on elevated plus-maze (EPM) model of anxiety. Objective: The current study has been designed to evaluate the anxiolytic activity of different fractions of bioactive methanol extract of the aerial parts of M. sativa. An attempt has been made to isolate the bioactive (anxiolytic) principle by resorting to bioactivity directed fractionation and column chromatographic technique. Materials and Methods: The bioactive methanol extract was fractionated by solvent partitioning, and each fraction was evaluated for the anxiolytic activity using the EPM model in mice. Further, bioactive fraction was subjected to column chromatography, and evaluated for the anxiolytic activity. Finally, the anxiolytic activity of isolated compound was further confirmed using light/dark test and hole-board test model of anxiety, and characterization and quantification of isolated compound was done by spectroscopic techniques and TLC densitometric method respectively. Results: Anti-anxiety activity guided fractionation of sub-fraction F₄ of ethyl acetate fraction of methanol extract led to isolation of 4',5,7-trihydroxyflavone (apigenin) which showed significant (p<0.05) anxiolytic activity by increasing the average time spent, and number of entries in open arms in EPM model of anxiety at a dose of 2 mg/kg, p.o., in mice with respect to the standard drug diazepam. Further, the anti-anxiety activity of isolated compound was confirmed using light/dark test and hole-board test. Conclusions: On the
basis of findings of the present study, it is concluded that the isolated compound - apigenin is responsible for anxiolytic activity of *M. sativa*.

**Keywords:** *Medicago sativa*; Anxiolytic activity; Apigenin; Elevated plus-maze.

**INTRODUCTION**

Anxiety disorders are among the most common mental, emotional, and behavioral problems \[1,2\]. In a modern society these disorders have relatively high prevalence affecting between 10 to 30% of the general population \[3,4\]. In addition to the high prevalence, anxiety disorders account for major expenditure for their management, and have a substantial negative impact on quality of life \[1\]. In the clinical treatment of anxiety benzodiazepines, GABA\(_A\) receptor agonist and buspirone, 5-HT\(_{1A}\) receptor agonist, are mainly prescribed as first choice treatment. Chronic administration of benzodiazepines, however result in physical dependence such as sedation, myelorelaxation, ataxia, amnesia and pharmacological dependence \[5\]. Moreover, buspirone also results in dizziness, headache, nervousness, paresthesia, diarrhea and sweating as adverse effects \[6,7\]. Therefore, research has been conducted to identify safer, more specific medications possessing anxiolytic effect without any complications. In the past few years, several herbal medicines have been used for the management of anxiety in the world \[8\].

*Medicago sativa* Linn. (Leguminosae), commonly known as ‘Alfalfa’ has a long tradition of use as an Ayurvedic and Homoeopathic medicine in variety of central nervous system (CNS) disorders \[9\]. Traditionally, *M. sativa* is used to improve the memory, as a rejuvenator, antidiabetic, antioxidant, anti-inflammatory, anti-asthmatic, antimicrobial, galactagogue, and in a variety of CNS disorders \[9,10,11\]. The plant contains many important phytococonstituents including saponins, sterols, coumarins, flavonoids, phenolics, vitamins, proteins, minerals, and other nutrients \[12,13\]. Pharmacological studies revealed that *M. sativa* have been used as hypocholesterolemic and antiatherosclerotic \[14\]. It is beneficial in diabetes \[15\], cardiovascular complications \[16\], convalescence and debility and also used as a tonic after blood loss and during anemia \[17\]. Recently, the authors have proven that the plant exhibits significant antioxidant and neuroprotective effects against ischemia and reperfusion insult in mice \[18\].

Recently, authors have reported that methanol extract of *M. sativa* aerial parts at a dose of 100 mg/kg, p.o., exhibited significant anti-anxiety activity on elevated plus maze model \[19\]. Therefore, in continuation of the previous work, in the present study an attempt has been
made to isolate the bioactive fraction by resorting to bioactivity (anxiolytic) directed fractionation of methanol extract of *M. sativa* aerial parts, and quantification of isolated bioactive constituent(s) by TLC densitometric method.

**MATERIALS AND METHODS**

**Plant material**

Aerial parts (leaves and stems) of the plant were procured from Himalaya Herbs Stores, Saharanpur, India. The identity of the plant was confirmed through Dr. H.B. Singh, Scientist F, Head of Raw Material Herbarium and Museum (RHMD), National Institute of Science Communication and Information Resources (NISCAIR), New Delhi, India (Ref. No. NISCAIR/RHMD/Consult/2008-09/1170/202).

**Preparation and fractionation of methanol extract, and evaluation of anxiolytic activity**

The methanol extract was prepared according to our previously described method [19]. The methanol extract 50 g was fractionated by solvent partitioning to produce n-hexane, ethyl acetate and n-butanol. Thus four fractions were obtained namely n-hexane fraction, ethyl acetate fraction, n-butanol fraction and the remaining methanol soluble fraction, and each fraction was evaluated for the anxiolytic activity using the elevated plus maze (EPM) model.

**Animals**

Swiss albino mice of either sex (20-25 g) were employed in the present study. The animals were maintained under standard environmental conditions, and allowed standard laboratory feed and water *ad libitum*. All animals used in the study were naive to the elevated plus-maze test. The approval from the Institutional Animal Ethical Committee of L.R. Institute of Pharmacy, Solan was obtained before carrying out biological studies.

**Vehicle and standard drug**

Distilled water + Tween 80 (5%) were used as vehicle (control) for preparing the suspension of various test doses of different extracts of *M. sativa*. Diazepam (2 mg/kg, p.o.) (Ranbaxy Laboratories Ltd., Mumbai) was used as a standard anxiolytic drug.

**Elevated plus-maze test**

The plus-maze apparatus consisting of two open arms (16 × 5 cm) and two closed arms (16 × 5 × 12 cm) having an open roof, with the plus-maze elevated (25 cm) from the floor was used to observe anxiolytic behavior in animals [20],[21]. Test extracts were administered orally, using
a tuberculin syringe fitted with oral canula. Each mouse was placed at the centre of the elevated plus-maze with its head facing the open arms. During this 5-min experiment, the behavior of the mouse was recorded as: (i) the number of entries into the open arms, and (ii) average time spent by the mouse in the open arms (average time = total time spent in open arms/number of entries in open arms). During the entire experiment, the animals were allowed to socialize. Every precaution was taken to ensure that no external stimuli could invoke anxiety in the animals. Similar observations were recorded for the standard group as well as the control group.

**Light/dark test**
The apparatus consisted of two (20×10×14 cm) plastic boxes: one was dark and the other was transparent. The mice were allowed to move from one box to the other through an open door between the two boxes. A 100-W bulb placed 30 cm above the floor of the transparent box was the only light source in the room. A mouse was put into the light box facing the hole. The transitions between the light and the dark box and time spent in the light box were recorded for 5 min immediately after the mouse stepped into the dark box [22]. The apparatus was cleaned thoroughly between trials.

**The hole-board test**
The apparatus was composed of a gray wooden box (50×50×50 cm) with four equidistant holes 3 cm in diameter in the floor [23]. The centre of each hole was 10 cm from the nearest wall of the box. The floor of the box was positioned 15 cm above the ground and divided into squares of 10×10 cm with a water-resistant marker. An animal was placed in the center of the hole-board and allowed to freely explore the apparatus for 5 min, after 1 h treatment of extract, vehicle or diazepam. The total locomotor activity (numbers of squares crossed), and the number and duration of head-dippings were recorded. A head dip was scored if both eyes disappeared into the hole.

**Column chromatography of ethyl acetate fraction and evaluation of anxiolytic activity**
The bioactive ethyl acetate fraction (5.5 g) was subjected to column chromatography using silica gel (60-120). Elution was done with n-hexane, n-hexane-ethyl acetate, ethyl acetate or ethyl acetate-methanol as mobile phase. The different collected sub-fractions were pooled on the basis of similar TLC profiles. Six sub-fractions (F₁-F₆) were generated. The anxiolytic activity of each fraction was evaluated using the EPM model.
Characterization of bioactive phytoconstituent
Bioactive phytoconstituent was subjected to UV (Perkin Elmer Hitachi 330), IR (Perkin Elmer FT IR spectrometer using KBr pellet method), $^1$H NMR and $^{13}$C NMR (Bruker spectrometer at 300 MHz, using DMSO as NMR solvent to dissolve constituents) spectroscopy.

Quantitative determination of bioactive constituent MS$_1$ in *M. sativa* using TLC densitometric method
Preparation of test samples
Direct method
Dried powder of *M. sativa* (2 g), packed in a filter paper sachet, and were defatted by refluxing in 250 ml round bottom flask on boiling water bath with 3×50 ml quantity of petroleum ether (1 h each). The marc obtained was air dried and refluxed under similar conditions with 3×50 ml quantity of methanol. The methanol extracts were pooled, filtered and concentrated under reduced pressure. Dried methanol extract was reconstituted in methanol, in a volumetric flask, and its volume was made up to 10 ml.

Acid hydrolysis
Following the above mentioned procedure, methanol extract was prepared. Dried methanol extract, thus obtained, was heated in a 50 ml round bottom flask with 6% aqueous hydrochloric acid (25 ml) for 45 minutes on water bath in order to hydrolyze flavonoid O-glycosides $^{[24]}$. Aglycones, precipitated on cooling the solution, were removed by filtration, and dissolved in methanol. The last traces of aglycones were removed from filtrate by extracting with 3×20 ml quantity of diethyl ether. Latter were combined, dried over anhydrous sodium sulphate, and evaporated under reduced pressure. The diethyl ether extract, thus obtained, was pooled with methanol solution of aglycones. Finally, volume was made up to 10 ml with methanol in a volumetric flask.

Phytochemical screening
All the extracts/fractionations were screened for different classes of phytoconstituents using specific standard reagents $^{[25],[26]}$.

Statistical analysis
All data are expressed as mean ± SEM. Statistically significant differences between groups
were calculated by the application of an analysis of variance (ANOVA) followed by post hoc Tukey’s multiple range tests. The groups treated with extract were compared with the respective control (vehicle) group; p values <0.05 were considered statistically significant.

RESULTS

Anti-anxiety activity of various fractions obtained from methanol extract of *M. sativa*

Among the four fractions viz. *n*-hexane fraction, ethyl acetate fraction, *n*-butanol fraction or remaining methanol soluble fraction, only ethyl acetate fraction at a dose of 50 mg/kg, p.o., exhibited the significant (*P* < 0.05) anxiolytic activity by increasing both the mean time spent, and number of open arm entries of EPM. Diazepam (2 mg/kg) was used as standard drug.

Column chromatography of ethyl acetate fraction, and evaluation of its anxiolytic activity

Table 1 summarizes the various sub-fractions from ethyl acetate fraction using column chromatography. The eluants were pooled on the basis of similar TLC profiles. Six sub-fractions (F₁ - F₆) were generated.

Table 2 shows the anti-anxiety activity of various bioactive ethyl acetate sub-fractions (F₁ - F₆). Only the bioactive sub-fraction F₄ (20 mg/kg, p.o.) showed the significant (*P* < 0.05) anxiolytic activity in EPM test.

Column chromatography of bioactive sub-fraction F₄, and evaluation of its anxiolytic activity

Table 3 summarizes the further fractionation of bioactive sub-fraction F₄. Three sub-fractions (F₄₁ - F₄₃) were obtained. Anxiolytic activity of each sub-fraction was evaluated using EPM model of anxiety (Table 4).

Chromatographic studies of F₄₃

TLC

TLC (chromatograms were developed on pre-coated aluminum-based TLC sheets Merck, Silica gel G, 0.2 mm) of F₄₃, using toluene:ethyl acetate:methanol (7:3:1) as the mobile phase, visualized in UV chamber (254/366 nm), and after spraying with natural products reagent showed two distinct spots indicative of the presence of flavonoids.
Preparative TLC of \( F_{4,3} \) and evaluation of anti-anxiety activity: Repeated preparative TLC of \( F_{4,3} \), using solvent system toluene:ethyl acetate:methanol (7:3:1), yielded two pure isolates \( MS_1 \) (310.24 mg) and \( MS_2 \) (198.51 mg) which were evaluated for anti-anxiety activity at dose levels of 1.0, 2.0 or 5.0 mg/kg, p.o. Anti-anxiety activity profiles of \( MS_1 \) and \( MS_2 \) are reported in Table 5 and Figure 1.

Confirmation of anxiolytic activity of isolated constituents \( MS_1 \)
The anti-anxiety activity of the bioactive compound \( MS_1 \) was further confirmed using light/dark test and hole-board test model of anxiety at a dose of 1.0, 2.0 and 5.0 mg/kg, and activity was compared with that of diazepam.

The results of \( MS_1 \) on exploratory behavior in mice on the light/dark test and hole-board test are shown in Figure 2 and Table 6 respectively.

Characterization of \( MS_1 \)
\( MS_1 \) was subjected to UV, IR, \(^1\)H NMR and \(^{13}\)C NMR spectroscopy, and the results were compared with those of reference apigenin (Sigma, USA). On the basis of spectral data, the structure of \( MS_1 \) was characterized as 4’,5,7-trihydroxyflavone (apigenin).

Quantitative determination of \( MS_1 \) in \( M. \) sativa
A standard graph was plotted against absorbance and amount (ng) of \( MS_1 \). TLC densitometric profiles of \( MS_1 \) isolated from \( M. \) sativa and the acid hydrolyzed methanol extract of \( M. \) sativa are shown in Figures 3 and 4 respectively. Table 7 shows the content of \( MS_1 \) present in \( M. \) sativa.

Table 1 Different sub-fractions from ethyl acetate fraction of methanol extract of \( M. \) sativa using column chromatography.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Eluents</th>
<th>Obtained by pooling fractions</th>
<th>Yield (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( F_1 )</td>
<td>( n)-Hexane</td>
<td>01-07</td>
<td>0.03</td>
</tr>
<tr>
<td>( F_2 )</td>
<td>( n)-Hexane + ethyl acetate (9 : 1)</td>
<td>08-18</td>
<td>0.36</td>
</tr>
<tr>
<td>( F_3 )</td>
<td>( n)-Hexane + ethyl acetate (1 : 1)</td>
<td>19-32</td>
<td>0.45</td>
</tr>
<tr>
<td>( F_4 )</td>
<td>Ethyl acetate Ethyl acetate + methanol (99 : 1)</td>
<td>33-37</td>
<td>2.51</td>
</tr>
<tr>
<td>( F_5 )</td>
<td>Ethyl acetate + methanol (19 : 1)</td>
<td>38-50</td>
<td>0.73</td>
</tr>
<tr>
<td>( F_6 )</td>
<td>Ethyl acetate + methanol (98 : 2)</td>
<td>51-62</td>
<td>0.94</td>
</tr>
</tbody>
</table>
Table 2  Anti-anxiety activity of various sub-fractions (F₁ - F₅) obtained from the bioactive ethyl acetate fraction of methanol extract of *M. sativa*.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg, p.o.)</th>
<th>Number of entries in open arms (Mean ± SEM)</th>
<th>Time spent in open arms (sec) (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Vehicle</td>
<td>4.2 ± 0.54 *</td>
<td>4.8 ± 0.65 *</td>
</tr>
<tr>
<td>Diazepam</td>
<td>2</td>
<td>10.0 ± 0.98</td>
<td>15.6 ± 0.82</td>
</tr>
<tr>
<td>F₁</td>
<td>20</td>
<td>4.8 ± 0.82</td>
<td>5.2 ± 0.86</td>
</tr>
<tr>
<td>F₂</td>
<td>20</td>
<td>4.9 ± 0.49</td>
<td>5.5 ± 0.55</td>
</tr>
<tr>
<td>F₃</td>
<td>20</td>
<td>4.9 ± 0.54</td>
<td>5.4 ± 0.48</td>
</tr>
<tr>
<td>F₄</td>
<td>20</td>
<td>10.0 ± 0.45</td>
<td>16 ± 0.61</td>
</tr>
<tr>
<td>F₅</td>
<td>20</td>
<td>4.2 ± 0.66</td>
<td>4.6 ± 0.98</td>
</tr>
<tr>
<td>F₆</td>
<td>20</td>
<td>4.0 ± 0.99</td>
<td>4.0 ± 0.49</td>
</tr>
</tbody>
</table>

All data are expressed as mean ± SEM. n=10. *P<0.05 vs Control; *P<0.05 vs Standard.

Table 3 Fractionation of bioactive sub-fraction F₄ using column chromatography.

<table>
<thead>
<tr>
<th>Sub-fraction</th>
<th>Eluants</th>
<th>Obtained by pooling fractions</th>
<th>Yield (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F₄,₁</td>
<td>Hexane + ethyl acetate (1 : 1)</td>
<td>1-6</td>
<td>0.49</td>
</tr>
<tr>
<td>F₄,₂</td>
<td>Hexane + ethyl acetate (2 : 3)</td>
<td>7-15</td>
<td>0.77</td>
</tr>
<tr>
<td></td>
<td>Hexane + ethyl acetate (1 : 4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F₄,₃</td>
<td>Ethyl acetate + methanol (99 : 1)</td>
<td>16-17</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td>Ethyl acetate + methanol (98 : 2)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4  Results of anti-anxiety activity of various sub-fractions obtained from F₄ using EPM model.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg, p.o.)</th>
<th>Number of entries in open arms (Mean ± SEM)</th>
<th>Time spent in open arms (sec) (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Vehicle</td>
<td>3.9 ± 0.64 *</td>
<td>4.5 ± 0.72 *</td>
</tr>
<tr>
<td>Diazepam</td>
<td>2</td>
<td>9.8 ± 0.58 *</td>
<td>15.1 ± 0.69 *</td>
</tr>
<tr>
<td>F₄,₁</td>
<td>10</td>
<td>4.0 ± 0.63</td>
<td>4.6 ± 0.89</td>
</tr>
<tr>
<td>F₄,₂</td>
<td>10</td>
<td>4.2 ± 0.81</td>
<td>5.5 ± 0.79</td>
</tr>
<tr>
<td>F₄,₃</td>
<td>10</td>
<td>8.5 ± 0.53</td>
<td>13.7 ± 0.55</td>
</tr>
</tbody>
</table>

All data are expressed as mean ± SEM. n=10. *P<0.05 vs Control; *P<0.05 vs Standard.
Table 5  Results of anti-anxiety activity of MS₁ and MS₂ isolated from F₄,₃ using EPM apparatus.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg, p.o.)</th>
<th>Number of entries in open arms (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Vehicle</td>
<td>3.5 ± 0.44*</td>
</tr>
<tr>
<td>Diazepam (Standard)</td>
<td>2</td>
<td>8.9 ± 0.48#</td>
</tr>
<tr>
<td>MS₁</td>
<td>1.0</td>
<td>3.9 ± 0.70</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>8.1 ± 0.45#</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>5.1 ± 0.63#*</td>
</tr>
<tr>
<td>MS₂</td>
<td>1.0</td>
<td>3.2 ± 0.61*</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>3.6 ± 0.82*</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>3.2 ± 0.64*</td>
</tr>
</tbody>
</table>

All data are expressed as mean ± SEM (n=10). *P<0.05 vs Control; #P<0.05 vs Standard.

Table 6: Effects of MS₁ on exploratory behavior in mice on the hole-board test.

<table>
<thead>
<tr>
<th>Group</th>
<th>Head-dip counts</th>
<th>Head-dip duration (s)</th>
<th>No. of squares crossing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10 ± 1.2</td>
<td>14.5 ± 1.61</td>
<td>59.6 ± 4.72</td>
</tr>
<tr>
<td>Diazepam (2 mg/kg)</td>
<td>21.2 ± 1.1#</td>
<td>24.8 ± 1.52#</td>
<td>45.1 ± 3.88</td>
</tr>
<tr>
<td>MS₁ (2 mg/kg)</td>
<td>20.5 ± 1.7#</td>
<td>21.1 ± 2.02#</td>
<td>52.5 ± 6.03</td>
</tr>
</tbody>
</table>

All data are expressed as mean ± SEM, n = 10. #P < 0.05 compared with control.

Table 7  MS₁ content in M. sativa aerial parts as determined by TLC densitometry.

<table>
<thead>
<tr>
<th>Methods of estimation of MS₁ in M. sativa aerial parts</th>
<th>MS₁ content (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct method</td>
<td>0.08 ± 0.002</td>
</tr>
<tr>
<td>Acid hydrolysis method</td>
<td>0.22 ± 0.001</td>
</tr>
</tbody>
</table>

Mean ± S.D., n = 3
Figure 1. Results of anti-anxiety activity of MS1 and MS2 isolated from F4.3 using EPM apparatus.

Figure 2. Effects of MS1 in the light/dark test in mice. (A): Time in the light box (s); (B): No. of transitions. Results are expressed as means ± SEM (n = 10). Superscript * indicates p value < 0.05 as compared with control. Statistical analysis was done by one way ANOVA followed by Tukey’s Test.
DISCUSSION AND CONCLUSION

The present study was designed to isolate, characterize and quantify the anxiolytic constituent of *M. sativa*. The elevated plus maze (EPM) model of anxiety was employed in this study. This model was chosen as it is effective, cheap, simple, less time consuming, requires no preliminary training to the mice and does not cause much discomfort to the animals while handling. The model is principally based on the observations that the exposure of animals to an elevated and open maze results in approach-avoidance conflict which is manifested as an exploratory-cum-fear drive. The fear due to height (acrophobia) induces anxiety in the animals when placed on the EPM. The ultimate manifestation of anxiety and fear in the animals is exhibited by decrease in motor activity, which is measured by the time spent by the animal in the open arms [27].
The light/dark box is also widely used in rodents as a model for screening anxiolytic or anxiogenic drugs, based on the innate aversion of rodents to brightly illuminated areas and the spontaneous exploratory behavior of rodents in response to mild stressors, that is, a novel environment and light \cite{28,29} reported that the time spent in the light is a stronger indicator in the study of anxiety, whereas the number of transfers reflects both anxiety and exploration. The hole-board test provides a simple method for measuring the response of an animal to an unfamiliar environment and is widely used to assess emotionality, anti-anxiety and/or response to stress \cite{23}. Recently, authors have reported that among the four extracts viz., petroleum ether, chloroform, methanol and aqueous, prepared from *M. sativa* aerial parts, only methanol extract at a dose of 100 mg/kg, p.o., exhibited significant anxiolytic activity on elevated plus maze model \cite{19}.

In continuation of the work, further, the bioactive methanol extract of *M. sativa* was fractionated using *n*-hexane, ethyl acetate and 1-butanol resulting in four fractions namely *n*-hexane fraction, ethyl acetate fraction, 1-butanol fraction and the remaining methanol soluble fraction in order to separate constituents on the basis of polarity.

Among the four fractions, only ethyl acetate fraction at a dose of 50 mg/kg, p.o., exhibited the significant (P < 0.05) anxiolytic activity by increasing both the mean time spent and number of open arm entries of EPM. Bioactive ethyl acetate fraction was further fractionated using column chromatography into six fractions $- F_1 - F_6$ (table 1). From among these six fractions $F_4$, exhibiting maximum anxiolytic activity (table 2) was selected for further processing. $F_4$ was further fractionated using column chromatography into three sub-fractions $- F_{4.1} - F_{4.3}$ (table 3).

Of these, the sub-fraction $F_{4.3}$ demonstrated significant anxiolytic activity comparable to that of diazepam (table 4). Repeated preparative thin layer chromatography of $F_{4.3}$ yielded two pure isolates $MS_1$ and $MS_2$ which were evaluated for anti-anxiety activity. $MS_1$ exhibited significant anti-anxiety activity (fig.1, table 5) whereas $MS_2$ was found devoid of the anti-anxiety activity. The anxiolytic activity of $MS_1$ was confirmed using other well established model of anxiety i.e., light/dark test and hole-board test. $MS_1$ increased latency to leave light zone and the time spent in light compartment of light/dark model of anxiety (fig. 2).

Further, $MS_1$ significantly increased head dipping in hole board test (table 6). All these observations further confirmed the anxiolytic activity of $MS_1$. Thus, $MS_1$ was chosen for
Further analysis. The structure of MS₁ was elucidated by UV, IR, ¹H NMR, and ¹³C NMR spectral data and characterized as 4',5,7-trihydroxyflavone (apigenin) [30,31]. Further, the identity of MS₁ was confirmed by comparison of its spectral data with that of reference standard of apigenin. After having established apigenin as the anxiolytic constituent of *M. sativa*, it was decided to use it as marker to standardize the plant material. Apigenin was used as an external standard for determining its content in *M. sativa* by TLC densitometry. TLC was used as its method development is easy, cost effective, efficient and requires not much clean-up of the test samples. Preliminary TLC studies revealed that apigenin resolved well in test samples using toluene:ethyl acetate:methanol (7:3:1) as the mobile phase. The chromatograms of apigenin and acid hydrolyzed methanol extract of *M. sativa* were scanned under UV at 336 nm (figs. 3 and 4).

Standard procedures were adopted to obtain methanol extracts of *M. sativa*. Two methods were followed for preparing the test samples for determining the apigenin content. First, apigenin was determined in the methanol extract of the plant material and, second, methanol extract was acid hydrolyzed in order to free apigenin from its O-glycoside. Apigenin content in the hydrolyzed methanol extract of *M. sativa* aerial parts was found to be about 3 times more than methanol extract (table 6). From this observation, it can be concluded that most of the apigenin is present in glycosidic form in *M. sativa*. The observations that MS₁ is apigenin and is the anxiolytic constituent of *M. sativa*, are in agreement with those of Viola *et al.* [30] and Salgueiro *et al.* [32] who reported that apigenin exhibits anxiolytic activity. Similarly, in the present investigation, apigenin exhibited significant anxiolytic activity using different model of anxiety.

In conclusion, the findings of current study demonstrate that apigenin isolated from *M. sativa* is responsible for the anxiolytic activity of *Medicago sativa* Linn. The present study also validates the traditional claims of *M. sativa* in the treatment of CNS related disorders.

REFERENCES


