2.16.6 Analgesic activity

AS₁ was evaluated for analgesic activity using tail immersion test at doses of 2, 5 or 10 mg/kg, and results have been shown in figure 14.

![Graph showing analgesic activity of AS₁](image)

**Figure 14:** Analgesic activity of AS₁ in tail immersion test. The data is expressed as Mean ± S.E.M.; n = 5; *P<0.05 vs. control; ANOVA followed by Studentized Tukey’s test.

3 DISCUSSION

Anxiety disorders are serious medical illnesses that have affected 1/8th of total population worldwide irrespective of gender, age, religion, nationality and profession (NIMH, 2002). Anxiety Disorders Association of America (ADAA) described anxiety disorders as the most common mental illness in the US, that have affected 19.1 million (13.3%) of the adult (18-54 years) US population (ADAA, 2003). A study commissioned by ADAA on ‘The Economic Burden of Anxiety Disorders’ revealed that anxiety disorders cost the US more than $42 billion a year, almost one-third of the $148 billion total mental health bill for the
US. In India, prevalence rate for all mental disorders is 65.4 per 1000 population, and that for anxiety neurosis is 18.5 per 1000 population (Madhav, 2001). The Global Research on Anxiety and Depression (GRAD) network, a consortium of world’s leading psychiatric epidemiologists and clinical researchers, during the 154th annual meeting of ‘American Psychiatric Association’ (APA) has observed that, “a significant number of world’s population is plagued by chronic and excessive anxiety, also known as generalized anxiety disorder (GAD), which is more serious than those of lung disease, sleep disorders and major depression, and affects more than 5% of the world population” (GRAD, 2001).

In the light of adverse effects associated with the synthetic drugs, researchers have been exploring natural resources to find out safer and effective drugs. Investigating plants, based on their use in traditional systems of medicine, is a sound, viable and cost effective strategy to develop new drugs (Dhawan, 1995). A survey of indigenous market on herbal formulations, homoeopathic mother tinctures and Ayurvedic preparations being used for a variety of CNS depressant effects, especially to combat stress and anxiety, revealed that about 50 such formulations are in use. As many as 121 plants are involved in the preparation of these formulations. Though these plants have a long tradition of use in the treatment of various ailments, still no systematic phytochemical and pharmacological work has ever been carried out on most of these. Among such plants, based on frequency of use, *T. aphrodisiaca* has been shortlisted for present investigation.

### 3.1 Authentication of *T. aphrodisiaca*

*T. aphrodisiaca* has been used in traditional system of medicine of various countries as aphrodisiac, nerve tonic, diuretic, laxative, and in kidney, menstrual and pregnancy disorders. Despite a long tradition of use in various ailments, the plant has never been subjected to systematic phytochemical and biological investigations as is evident from sporadic phytochemical and pharmacological reports on *T. aphrodisiaca*.

Authentication of plant material is an indispensable prerequisite before using it as research material or as medicine. Therefore, it was planned to establish
pharmacognostic standards for *T. aphrodisiaca* so as to have reliable parameters to authenticate the plant.

In India, *T. aphrodisiaca* has not been reported from wild sources. It is cultivated to meet the demands of the pharmaceutical industry, especially those manufacturing homoeopathic drugs. However, *T. ulmifolia* grows wild in the eastern and north-eastern parts of India. This plant, although morphologically different from *T. aphrodisiaca*, has the potential of being used as an adulterant or substitute of *T. aphrodisiaca*. Therefore, it was planned to develop pharmacognostic standards for *T. ulmifolia* as well so that *T. aphrodisiaca* could easily be distinguished from the former.

*T. aphrodisiaca* and *T. ulmifolia* were procured from a cultivated source and their identity was further confirmed through Botanical Survey of India. The positively identified plant material was used to generate pharmacognostic standards so that the latter could serve as reference for authentication of the two plants.

Both *T. aphrodisiaca* and *T. ulmifolia* were subjected to qualitative and quantitative microscopic studies. Transverse sections of various parts as well as the powdered aerial parts of both the plants were studied for microscopic characters. Further, safranin+fast green were applied to differentiate between lignified and non-lignified cells. While safranin stains lignified cells red, cellulosic parenchyma is stained green with fast green.

Transverse sections of stem of *T. aphrodisiaca* and *T. ulmifolia* (Plate 2, pp 74-76) showed similar microscopic characters. Outermost layer consists of a single layer of tangentially elongated epidermal cells with a well defined cuticle bearing few unicellular warty covering trichomes. Inner to epidermis are present 4-5 layers of collenchymatous hypodermis followed by 6-8 layers of parenchymatous cortex. Next zone comprises 8-10 layers of parenchymatous pericycle with patches of sclerenchyma towards the inner side. This is followed by closely aligned groups of collateral vascular bundles consisting of phloem towards outer and xylem towards the inner side. Pith has parenchyma with intracellular spaces, and scattered clusters of calcium oxalate crystals. Remarkable difference in the stem of *T. aphrodisiaca* and *T. ulmifolia* was that of oil cells. Latter are present in the cortical region of *T. aphrodisiaca*, and are absent in *T. ulmifolia*.
Transverse sections through the midrib region of leaves of *T. aphrodisiaca* and *T. ulmifolia* (Plate 3, pp 77-79) revealed similar structures. Leaves of both the plants have dorsiventral structure. Epidermal cells are tabular and covered with cuticle. Mesophyll is composed of single row of palisade cells and spongy parenchyma. Adjoining the lower epidermis, in the midrib region, are present 3-4 layers of collenchyma. The arc shaped vascular bundles are composed of xylem on the lower and phloem on the upper side. Striking differences in the leaves of *T. aphrodisiaca* and *T. ulmifolia* were the trichomes and distribution of cluster crystals of calcium oxalate. Leaves of *T. aphrodisiaca* have abundant unicellular warty trichomes while those of *T. ulmifolia* have abundant stellate trichomes. Although leaf laminae of both the plants have scattered calcium oxalate crystals, latter are aligned, in the midrib region, between the arc shaped vascular bundles and the lower epidermis in *T. aphrodisiaca*, and adjoining the arc shaped vascular bundles in *T. ulmifolia*.

Microscopic study of transverse sections of sepals (Plate 4, pp 80-81), petals (Plate 5, p 82) and ovaries (Plate 6, pp 83-84) of *T. aphrodisiaca* and *T. ulmifolia* exhibited similar structures. Sepals of both the plants have structures similar to their leaves. Epidermis has barrel shaped cells covered with cuticle. Mesophyll has abundant cluster crystals of calcium oxalate. Sepals of *T. aphrodisiaca* have abundant unicellular warty covering trichomes. Contrary to the leaves of *T. ulmifolia* which have stellate trichomes, its sepals have unicellular warty covering trichomes. Petals (Plate 5, p 82) have abundant unicellular warty covering trichomes, cluster crystals of calcium oxalate and vascular tissue. Ovaries (Plate 6, pp 83-84) of both the plants show ovarian wall, ovular integuments, micropyle and funiculii. Ovary wall is composed of epidermis, parenchyma with abundant cluster crystals of calcium oxalate, and vascular tissue.

Transverse sections of *T. aphrodisiaca* and *T. ulmifolia* fruits (Plate 7, pp 85-88) show single layered parenchymatous epicarp, multilayered parenchymatous mesocarp with vascular tissue embedded in it, and endocarp composed of thick walled parenchyma cells. Histomorphology of fruit of *T. aphrodisiaca* differs from that of *T. ulmifolia* with regard to presence of oil cells and sclereids. These are present in the mesocarp of *T. aphrodisiaca* but absent in that of *T. ulmifolia*. The two plants differ in the structure of seed testa.
(Plate 8, pp 89-90) as well. Latter in case of *T. aphrodisiaca* consists of lignified sclereidal cells while that of *T. ulmifolia* comprises parenchyma cells.

Similar structures observed during the microscopic studies of powdered aerial parts of *T. aphrodisiaca* and *T. ulmifolia* included pericyclic fibres (Plate 11, p 94), abundant cluster crystals of calcium oxalate aligned mainly along the veins in the leaf lamina (Plate 12, p 95) and anomocytic stomata (Plate 14, p 97). The stomata were present only on the lower epidermii. Upper epidermii were devoid of stomata. Differentiating characters included covering trichomes (Plate 9, p 92; unicellular warty in *T. aphrodisiaca* and stellate in *T. ulmifolia*), lignified tracheidal vessels (Plate 10, p 93; pitted in *T. aphrodisiaca* and spiral in *T. ulmifolia*) and starch (Plate 13, p 96; absent in *T. aphrodisiaca*, and both simple and compound present in *T. ulmifolia*).

In order to identify more parameters to differentiate between *T. aphrodisiaca* and *T. ulmifolia*, quantitative microscopic studies were undertaken. Leaf constants viz., stomatal number, stomatal index, vein-islet number, veinlet termination number and palisade ratio (Table 6, p 99) were determined using leaves of *T. aphrodisiaca* and *T. ulmifolia*. Although all these parameters were different for the two plants yet vein-islet number and veinlet termination number were strikingly different for the two. Vein-islet number and veinlet termination number of *T. aphrodisiaca* were observed to be about 5 and 3 times, respectively, more than that of *T. ulmifolia*. Length and width of trichomes and vessels, and width of pericyclic fibres (Table 7, p 100) of *T. aphrodisiaca* and *T. ulmifolia* offer striking difference and can be very useful in differentiating the two plants.

Presence of excess moisture in the plant acts as an adulterant and can cause decomposition in the plant material as it promotes microbial growth. Thus, it should be determined and controlled. Moisture content of air dried aerial parts of *T. aphrodisiaca* and *T. ulmifolia* was found to be 9.56% and 11.33% v/w, respectively (Table 8, p 102). Moisture content of the aerial parts was accounted for calculating values of other physicochemical parameters on dry weight basis.

Determination of ash is useful for detecting adulteration with spurious, exhausted drugs, and excess of sandy and earthy matter. Most drugs contain calcium oxalate crystals, sometimes in large and variable amounts. The acid insoluble ash is determined to remove all the variable constituents of the ash using
dilute hydrochloric acid. The water soluble ash is used to detect the presence of material exhausted with water. Total ash of *T. ulmifolia* was found to be slightly higher than that of *T. aphrodisiaca* (Table 8, p 102). The total ash was about 8 times more than the acid insoluble ash in *T. aphrodisiaca* and about 3 times more in *T. ulmifolia*, indicating the presence of large number of calcium oxalate crystals or other acid soluble inorganic matter in *T. aphrodisiaca*. The water soluble ash was about 4 times less than total ash in *T. aphrodisiaca* and 6 times less in *T. ulmifolia*. Amongst various ash values, acid insoluble ash is significantly different for the two plants.

Ethanol and water were used to evaluate the extractable constituents in the aerial parts of *T. aphrodisiaca* and *T. ulmifolia* in terms of extractive value. Ethanol- and water-soluble extractive values of *T. aphrodisiaca* were found to be 3 and 2 times, respectively, in comparison to those of *T. ulmifolia* (Table 8, p 102).

Volatile oil studies also help in identification of plant material. *T. ulmifolia* was devoid of volatile oil, while *T. aphrodisiaca* was found to contain 0.44% v/w of volatile oil. Values of various quantitative chemical parameters viz., acid value, saponification value, ester value, hydroxyl value, iodine value, peroxide value, unsaponifiable matter and acetyl value for the volatile oil of *T. aphrodisiaca* have been generated (Table 9, p 107).

Amongst various chromatographic techniques, thin layer chromatography is a handy technique for studying separation pattern of volatile oil and various extracts of plant material. TLC fingerprint profiles are useful for the identification/authentication of plant material. Seven solvent systems (Table 10, p 108) were tried for the TLC fingerprint profile of volatile oil of *T. aphrodisiaca*. Out of these the best resolution was obtained with toluene:ethyl acetate as the mobile phase in the ratio of 93:7. Seven spots having Rf values – 0.28 (violet), 0.38 (black), 0.47 (red), 0.53 (black), 0.66 (violet), 0.77 (yellow) and 0.89 (black) were observed (Plate 16, p 111; Table 14, p 110) when visualized by spraying with 0.5% anisaldehyde solution followed by heating at 110°C for 10 minutes.

In order to prepare qualitative TLC fingerprint profiles of petroleum ether, chloroform and methanol extracts of aerial parts of *T. aphrodisiaca* and *T. ulmifolia*, the plant materials were subjected to a standardized extraction
procedure wherein petroleum ether, chloroform and methanol extracts were obtained by direct extraction with petroleum ether, chloroform or methanol. Standard solutions of the extracts were prepared and loaded quantitatively on silica gel TLC plates. Nine mobile phases (Table 11, p 108) were tried to obtain desired separation of petroleum ether extracts. Petroleum ether extracts could be best resolved using hexane:dichloromethane (1:1) as the mobile phase employing 0.5% anisaldehyde as the visualizing agent. Plate 17 (p 112) shows the photographs of TLC of petroleum ether extracts of *T. aphrodisiaca* (9 spots) and *T. ulmifolia* (6 spots). R$_f$ values of various spots in the TL chromatograms of petroleum ether extracts of *T. aphrodisiaca* and *T. ulmifolia* are shown in table 14 (p 110). Thirteen solvent systems were tried for resolving the chloroform extracts (Table 12, p 109). TLC of chloroform extracts, when visualized with 0.5% anisaldehyde, showed eleven spots for *T. aphrodisiaca* and seven spots for *T. ulmifolia* using toluene:ethyl acetate:glacial acetic acid (35:4:1) as the mobile phase (Plate 18, p 113; Table 14, p 110). Despite trying as many as 17 mobile phases (Table 13, pp 109-110), methanol extracts of *T. aphrodisiaca* and *T. ulmifolia* could not be resolved satisfactorily. The two plants, thus, can easily be distinguished on the basis of TLC profiles of their petroleum ether and chloroform extracts.

Further, these two medicinally important plants of *Turnera* can easily be distinguished on the basis of biochemical parameters. Leaves of *T. ulmifolia* have maximum amount of proteins, about 3 times in comparison to *T. aphrodisiaca* (Table 15, p 116). Aerial parts of *T. aphrodisiaca* contain slightly less proteins in comparison to *T. ulmifolia* aerial parts. Aerial parts of *T. aphrodisiaca* have about 3 times more carbohydrates than *T. ulmifolia* aerial parts (Table 16, p 118). Thin layer chromatography of water extracts of *T. aphrodisiaca* and *T. ulmifolia* showed seven and five spots, respectively, using 0.2% ninhydrin solution as visualizing agent (Plate 19, p 120; Table 17, p 119). *T. aphrodisiaca* has seven amino acids which were identified, using reference amino acids, as histidine monohydrochloride, serine, aspartic acid, proline, glutamic acid, methionine and tyrosine, while *T. ulmifolia* possesses histidine monohydrochloride, serine, glutamic acid, methionine and tyrosine. *T. ulmifolia* was found to be devoid of aspartic acid and proline.
Phytochemical screening of various extracts of *T. aphrodisiaca* and *T. ulmifolia* viz., petroleum ether, chloroform, methanol and water was carried out using standard procedures. Petroleum ether extract of *T. aphrodisiaca* showed presence of steroids, whereas chloroform extract showed presence of alkaloids, steroids and flavonoids, methanol extract gave positive tests for alkaloids, cyanogenic glycosides, steroids, flavonoids, tannins, carbohydrates and proteins, and water extract indicated the presence of cyanogenic glycosides, saponins, tannins, carbohydrates and proteins (Table 19, p 121). Phytochemical screening of various extracts of *T. ulmifolia* showed similar profile as that of *T. aphrodisiaca* except for the presence of flavonoids in chloroform extract, and tannins in methanol and water extracts.

Typical parameters that have been found to be very useful for differentiating *T. aphrodisiaca* from *T. ulmifolia* are presented in table 36.

**Table 36:** Characteristic parameters for differentiating *T. aphrodisiaca* from *T. ulmifolia.*

<table>
<thead>
<tr>
<th>Parameter</th>
<th><em>T. aphrodisiaca</em></th>
<th><em>T. ulmifolia</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Stem/Fruit Oil cells</td>
<td>present</td>
<td>absent</td>
</tr>
<tr>
<td>Leaf Covering trichomes</td>
<td>unicellular, warty</td>
<td>stellate</td>
</tr>
<tr>
<td>Seed Testa</td>
<td>lignified sclereidal</td>
<td>parenchymatous</td>
</tr>
<tr>
<td>Aerial parts Starch</td>
<td>absent</td>
<td>present</td>
</tr>
<tr>
<td>Vein-islet number</td>
<td>54.62</td>
<td>11.77</td>
</tr>
<tr>
<td>Veinlet termination number</td>
<td>24.81</td>
<td>8.27</td>
</tr>
<tr>
<td>Covering trichomes Mean length</td>
<td>212.20 μm</td>
<td>78.8 μm</td>
</tr>
<tr>
<td>Mean width</td>
<td>9.99 μm</td>
<td>5.7 μm</td>
</tr>
<tr>
<td>Vessels Mean length</td>
<td>261.3 μm</td>
<td>520.2 μm</td>
</tr>
<tr>
<td>Mean width</td>
<td>12.7 μm</td>
<td>24.2 μm</td>
</tr>
<tr>
<td>Pericyclic fibres Mean width</td>
<td>16.4 μm</td>
<td>9.1 μm</td>
</tr>
<tr>
<td>Acid insoluble ash</td>
<td>1.28% w/w</td>
<td>3.85% w/w</td>
</tr>
<tr>
<td>Ethanol soluble extractive value</td>
<td>18.75% w/w</td>
<td>5.53% w/w</td>
</tr>
<tr>
<td>Water soluble extractive value</td>
<td>25.00% w/w</td>
<td>12.55% w/w</td>
</tr>
</tbody>
</table>
3.2 Anxiolytic activity screening of *T. aphrodisiaca* and *T. ulmifolia*

Anti-anxiety activity of various extracts of *T. aphrodisiaca* and *T. ulmifolia* aerial parts was evaluated employing a widely used model, i.e., EPM. The model was chosen since it is effective, cheap, simple, less time consuming, and 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 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 are exhibited by decrease in motor activity, which is measured by the time spent, in the open arms, by the animal.

Petroleum ether, chloroform, methanol and water extracts of *T. aphrodisiaca* and *T. ulmifolia* were prepared by successively extracting their aerial parts with petroleum ether, chloroform, methanol and water. Maximum extractives were obtained with methanol (Table 18, p 119). Dried petroleum ether, chloroform, methanol and water extracts of *T. aphrodisiaca* and *T. ulmifolia* aerial parts, separately suspended in the suitable vehicle, were administered orally to mice. The activity was compared with that observed in the control group as well as with the group treated with the standard anxiolytic drug diazepam. Complete manifestation of anxiety in mice of the control group is evident from the minimum mean time spent in the open arms of EPM by these animals (Tables 20 and 21, pp 122-123). Among various extracts of *T. aphrodisiaca* tested, maximum anxiolytic

<table>
<thead>
<tr>
<th>TLC</th>
<th>Petroleum ether extract</th>
<th>Chloroform extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>9 spots</td>
<td>6 spots</td>
</tr>
<tr>
<td></td>
<td>11 spots</td>
<td>7 spots</td>
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<table>
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<tr>
<th>Protein content</th>
<th>Stem</th>
<th>Leaves</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10.95% w/w</td>
<td>4.65% w/w</td>
</tr>
<tr>
<td></td>
<td>12.36% w/w</td>
<td>34.47% w/w</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Carbohydrate content</th>
<th>Aerial parts</th>
<th>Leaves</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8.21% w/w</td>
<td>3.22% w/w</td>
</tr>
<tr>
<td></td>
<td>6.53% w/w</td>
<td>2.74% w/w</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Amino acids in water extract</th>
<th>7</th>
<th>5</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Phytoconstituents</th>
<th>Tannins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>present</td>
</tr>
<tr>
<td></td>
<td>absent</td>
</tr>
</tbody>
</table>
activity was observed in the methanol extract at a dose of 25 mg/kg which was at par with that of diazepam as evident from statistical equivalence between the results of this dose and that manifested by diazepam (Table 20, p 122). However, the activity decreased at higher doses which might be due to mild sedation. Petroleum ether, chloroform and water extracts of *T. aphrodisiaca* aerial parts were found to be devoid of anxiolytic activity. None of the extracts of *T. ulmifolia* exhibited significant anxiolytic activity (Table 21, p 123).

In an attempt to separate the contents of bioactive methanol extract of *T. aphrodisiaca*, exhibiting significant anxiolytic activity, it was fractionated by shaking with petroleum ether, chloroform and *n*-butyl alcohol. Together, the three solvents could separate only about 19% of the constituents of the methanol extract (Table 22, p 124). The *n*-butyl alcohol fraction (10 mg/kg) and the RME (75 mg/kg) exhibited significant anxiolytic activity using EPM model (Table 23, p 125). Petroleum ether and chloroform fractions did not exhibit anxiolytic activity. From these observations, it was inferred that *n*-butyl alcohol takes up a good quantity of anxiolytic constituent from methanol extract. This is evident from the fact that in order to exhibit anxiolytic activity comparable to the original methanol extract (25 mg/kg), RME had to be administered at a higher dose (75 mg/kg). Flavonoids/alkaloids, present in *n*-butyl alcohol fraction, may be responsible for the anxiolytic activity. Since *n*-butyl alcohol could not selectively remove whole of the bioactive constituents from the methanol extract, original methanol extract was used for bioactivity-guided fractionation in order to avoid the loss of bioactive constituent(s).

### 3.3 Anti-anxiety activity screening of marketed formulations of *T. aphrodisiaca*

Anti-anxiety activity of the mother tinctures of *T. aphrodisiaca* marketed by reputed manufacturers of homoeopathic medicines was evaluated using EPM apparatus. A mother tincture is essentially an alcoholic extract prepared as per the procedure described in Homoeopathic Pharmacopoeias (Banerjee and Sinha, 1984). Despite being a homoeopathic formulation, a mother tincture is as good as an alcoholic extract commonly employed by the researchers during phytochemical or biological studies.
Mother tinctures of *T. aphrodisiaca* from three reputed manufacturers – NLK, DWSG and SBL were procured from the market. Dry residues of these, obtained after recovery of the solvent, were evaluated for anxiolytic activity. Significant activity was observed in NLK (50 mg/kg), DWSG (75 mg/kg) and SBL (125 mg/kg) respectively with respect to control as well as diazepam (Table 24, p 126). However, the activity decreased at higher doses, probably due to mild sedation. From these findings, it is clear that all the three mother tinctures of *T. aphrodisiaca* are anxiolytic. DWSG and SBL mother tinctures yielded about similar percentage (about 2%, p 124) of dry residues while NLK mother tincture yielded about one-fourth of them. Despite one-fourth yield, NLK exhibited significant activity at lower dose in comparison to DWSG and SBL indicating that NLK is the most potent among the three mother tinctures. Phytochemical screening showed that all the mother tinctures have similar classes of phytoconstituents. Flavonoids, alkaloids or steroids might be responsible for anxiolytic activity of *T. aphrodisiaca*.

### 3.4 Aphrodisiac activity screening of *T. aphrodisiaca*

Since *T. aphrodisiaca* has also been used in traditional systems of medicine to improve sexual performance, it was considered worthwhile to evaluate the plant for aphrodisiac activity. Petroleum ether, chloroform, methanol and water extracts, and volatile oil of *T. aphrodisiaca* aerial parts along with the total alkaloidal fraction were tested for aphrodisiac activity using mounting behaviour of male mice as the parameter for evaluating aphrodisiac activity. While all the test substances were evaluated after acute administration (single dose), effect of total alkaloidal fraction was also studied after sub-acute administration (twice daily doses for nine days). It is evident from table 25 (pp 127-128) that among the various test substances, chloroform and methanol extracts were found to be active. While chloroform extract exhibited maximum aphrodisiac activity at a dose of 200 mg/kg, methanol extract exhibited similar level of activity at a dose of 50 mg/kg during 2nd hour of test with respect to control. Aphrodisiac activity declined with the passage of time, i.e., during 3rd hour of the test as the bioactive constituent(s) seem to have undergone a metabolic degradation. Mice did not show any activity at higher doses of methanol because
of mild sedative effect produced by the extract at higher dose levels. Volatile oil did not exhibit aphrodisiac activity at any of doses tested (Table 25, pp 127-128). A significant dose dependent (5, 10 or 25 mg/kg) increase in aphrodisiac activity was observed in alkaloidal fraction upon acute administration during 2\textsuperscript{nd} hour of interaction. Male mice showed significant aphrodisiac activity at 5 mg/kg upon sub-acute administration (9 days) of alkaloidal fraction even during 3\textsuperscript{rd} hour of interaction. Sub-acute administration of alkaloidal fraction at higher doses (10, 25 or 50 mg/kg) in male mice showed dose dependent decrease in number of mounts. From these observations, it can be concluded that, true to its name, \textit{T. aphrodisiaca} exhibits aphrodisiac activity upon long term use, but at optimum dose levels.

### 3.5 Isolation of bioactive constituent(s) of \textit{T. aphrodisiaca}

After confirming the anxiolytic activity of \textit{T. aphrodisiaca}, which was observed in methanol extract of the aerial parts, it was decided to isolate the bioactive constituent(s) of the plant. For this purpose, methanol extract of \textit{T. aphrodisiaca} aerial parts was subjected to bioactivity-guided fractionation employing EPM test.

Methanol extract of \textit{T. aphrodisiaca} aerial parts was subjected to column chromatography which yielded 7 fractions F\textsubscript{1}-F\textsubscript{7} (Table 26, p 129). All the 7 fractions were subjected to evaluation of anxiolytic activity using EPM apparatus. Among these, only F\textsubscript{5} exhibited significant anti-anxiety activity at a dose of 10 mg/kg, p.o. (Table 27, pp 130-131). F\textsubscript{5} tested positive for alkaloids and flavonoids. Column chromatography of F\textsubscript{5} yielded 7 sub-fractions F\textsubscript{5.1}-F\textsubscript{5.7} (Table 28, p 131), out of which only F\textsubscript{5.3} exhibited significant anxiolytic activity at a dose of 5 mg/kg, p.o. (Table 29, pp 132-133). Though F\textsubscript{5} tested positive for both alkaloids and flavonoids, F\textsubscript{5.3} gave positive test only for flavonoids. Repeated preparative thin layer chromatography of F\textsubscript{5.3} ultimately led to the isolation of two pure compounds AS\textsubscript{1} (72 mg, yield 0.03%) and AS\textsubscript{2} (13 mg, yield 0.005%). AS\textsubscript{1} exerted significant anxiolytic effect which was comparable to that of the diazepam, at a dose of 2 mg/kg, p.o. (Table 30, p 133). AS\textsubscript{2} was found to be devoid of anti-anxiety activity. Anxiolytic activity of AS\textsubscript{1} declined at 5 mg/kg, p.o. dose due to mild sedation. Thus, it was concluded that AS\textsubscript{1} is the anxiolytic constituent of \textit{T. aphrodisiaca}.
3.6 The bioactive constituent of *T. aphrodisiaca*

3.6.1 Characterization of AS₁

Structure of AS₁ was elucidated by UV, ¹H NMR and ¹³C NMR spectral data (Table 31, p 134), and comparing the same with that of reference standard of apigenin. Thus, AS₁ was characterized as 5,7,4'-trihydroxy flavone or apigenin. Since the quantity of AS₂ (a flavonoid) generated following column and preparative thin layer chromatography was not sufficient to perform its spectral analysis, and since it did not exhibit anxiolytic activity, its structure was not established.

3.6.2 Anxiolytic activity of apigenin – a comment

The observations that AS₁ is apigenin and is the anxiolytic constituent of *T. aphrodisiaca*, are in agreement with those of Viola et al. (1995) and Salguiero et al. (1997) who have reported that apigenin exhibits anxiolytic activity at a dose of 3 mg/kg, i.p. and mild sedative activity at higher doses.

It is interesting to note that methanol extract of *T. aphrodisiaca* aerial parts exhibits significant anxiolytic activity at 25 mg/kg, p.o., dose whereas apigenin, isolated from the same methanol extract, exhibits equivalent anxiolytic activity at a dose of 2 mg/kg, p.o., i.e., at a dose roughly one-twelfth of the methanol extract. This observation when coupled with the yield of apigenin (0.03%) from the methanol extract shows that in order to exhibit anxiolytic activity comparable to the methanol extract (25 mg/kg, p.o.), the dose of apigenin should have been very much less than 2 mg/kg, p.o. This discrepancy between the doses of the methanol extract and apigenin, exhibiting comparable anxiolytic activity, could possibly be due to presence of higher amount of apigenin in the methanol extract (as apigenin glycoside) and/or presence of some constituents in the extract which potentiate the anxiolytic activity of apigenin. It is a known fact that apigenin occurs in plants mainly as glucoside. Apigenin has been isolated, in the present investigation, as an aglycone. Keeping in view the possibility of its occurrence in the plant as glycoside, extraction procedure was modified for preparing the extract for determining apigenin content (Section 2.15.2.2, p 135).
3.7 HPTLC of aerial parts of *T. aphrodisiaca*, and its marketed formulations

Having established apigenin as the anxiolytic constituent of *T. aphrodisiaca*, 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 *T. aphrodisiaca* by HPTLC densitometry. HPTLC 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 was resolved well in test samples using toluene:ethyl acetate (1:4) as the mobile phase. The spots of the chromatogram were scanned under UV at 336 nm (Figure 3, p 136). Linearity of the calibration curve was achieved between 0.05 and 0.25 μg for apigenin (r² = 0.998; Figure 4, p 137).

Standard procedures were adopted to obtain methanol extracts of *T. aphrodisiaca* aerial and segregated parts. 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 hydrolysed, in order to free apigenin from its O-glycoside. Apigenin content in the hydrolysed methanol extract of *T. aphrodisiaca* aerial parts was found to be about 14 times more than methanol extract (Table 32, p 138). From this observation, it can be concluded that most of the apigenin is present in O-glycosidic form in *T. aphrodisiaca* aerial parts. Thus, before HPTLC determinations, methanol extracts obtained from the segregated aerial parts of the plant were acid hydrolysed under optimized extraction conditions. Amongst different plant parts, flowers possessed maximum content of apigenin followed by leaves. Dried market preparations were also acid hydrolysed before the determination of apigenin content. DWSG possessed maximum content followed by SBL and NLK (Table 33, p 138). Content of apigenin in DWSG was found to be about 35 times more than NLK. Despite the presence of very less content of anxiolytic component of *T. aphrodisiaca*, i.e., apigenin, NLK showed maximum anxiolytic activity at lower dose (Section 3.3, pp 154-155). This could be probably due to: (i) addition of anxiolytic compound(s) as no official control is
applicable to these OTC products, and/or (ii) improper selection of the plant material.

The effect of season on content of apigenin in *T. aphrodisiaca* was also observed. Aerial parts of *T. aphrodisiaca* were collected at bimonthly intervals over a period of one year in the months of January, March, May, July, September and November. Apigenin content in all the samples were determined using validated HPTLC densitometric assay, and was found to vary in the range of 0.141-0.199%. The plant material collected in September showed the presence of maximum content of apigenin (Table 34, p 139).

Further, the HPTLC assay used for determining apigenin content in *T. aphrodisiaca* was validated by adding known quantities of reference apigenin to the test samples, and determining its content (Figure 6, p 139; Table 35, p 140). Recovery percentage of apigenin was observed to be very high (98.57%) indicating that the assay procedure developed, in the present investigation, is quite reliable.

### 3.8 Pharmacological profile of apigenin

Apigenin, the anxiolytic constituent of *T. aphrodisiaca*, was evaluated for various CNS-related bioactivities. Taking cue from previous results of apigenin in EPM test, it was further evaluated for anti-anxiety activity at a dose of 2 mg/kg using other well established models of anxiety, i.e., hole board test, light/dark test and mirrored chamber test. Apigenin significantly increased head dipping in hole board test (Figure 7, p 140). Further, apigenin increased latency to leave light zone and the time spent in light compartment of light/dark model of anxiety (Figure 8, p 141). Apigenin also decreased the latency time to enter the mirrored chamber, and increased the total time spent/number of entries in the mirrored chamber with respect to control (Figure 9, p 142). All these observations further confirmed the anxiolytic activity of apigenin. At a higher dose (about 12 fold the anxiolytic dose), apigenin showed mild sedative activity in actophotometer as it decreased activity scores (Figure 10, p 143). The results were statistically significant to that of control group but not comparable to diazepam (10 mg/kg). Apigenin did not exhibit sedative activity at doses of 5 or 10 mg/kg. It (2, 5 or 10 mg/kg) was found to be devoid of anticonvulsant, antidepressant and antistress activity in MES-
induced convulsion test, despair swim test and cold swimming endurance test, respectively (Figures 11, 12 and 13, pp 143-144).

In tail immersion test for six hours, apigenin exhibited excellent analgesic activity, which was comparable to that of morphine sulphate (5 mg/kg). Apigenin (2, 5 or 10 mg/kg) showed dose dependent analgesic activity (Figure 14, p 145). Maximum activity was observed 30 minutes after the administration of 10 mg/kg dose of apigenin. The activity remained significant upto the fourth hour of study in a manner similar to that shown by morphine sulphate (Figure 14, p 145). At sixth hour, analgesic activity decreased which might be due to the metabolization of apigenin.

3.9 Conclusion

Various pharmacognostic standards including qualitative and quantitative microscopic characters, various physicochemical parameters, and TLC fingerprint profiles were generated for *T. aphrodisiaca* and *T. ulmifolia* so that these medicinally important plants could be easily differentiated. Preliminary anti-anxiety activity screening of both the plants showed that *T. aphrodisiaca* possesses anxiolytic activity whereas *T. ulmifolia* is devoid of it. Amongst various extracts viz., petroleum ether, chloroform, methanol and water of *T. aphrodisiaca* tested, only methanol extract exhibited significant anxiolytic activity using EPM apparatus. Three marketed formulations of *T. aphrodisiaca* were also screened for anxiolytic activity, and were found to exhibit the same. Methanol extract was shaken with petroleum ether, chloroform, *n*-butyl alcohol, and all shakings and RME were evaluated for anxiolytic activity. The *n*-butyl alcohol fraction and RME showed anxiolytic activity. To avoid loss of bioactive constituent(s), original methanol extract was used for the bioactivity-guided fractionation studies. Various extracts, volatile oil and alkaloidal fraction were evaluated for aphrodisiac activity. Chloroform and methanol extracts, and alkaloidal fraction (acute and subacute administration) exhibited significant aphrodisiac activity. Bioactivity-directed fractionation of methanol extract of *T. aphrodisiaca* aerial parts led to the isolation of bioactive flavonoid apigenin. Quantitative determination of apigenin using HPTLC in different aerial parts of *T. aphrodisiaca*, and its marketed formulations confirmed the presence of maximum
content of apigenin in flowers, and DWSG among various mother tinctures. Seasonal variation studies showed that apigenin content is maximum in the plant in the month of September. Apigenin exhibited anti-anxiety activity in EPM assay as well as hole board, light/dark chamber and double mirrored chamber tests. Apigenin was also subjected to various CNS activities such as sedative, anticonvulsant, antidepressant, antistress and analgesic. At about 10-fold higher dose than anxiolytic dose, apigenin showed mild sedative activity in comparison to standard drug. A dose dependent analgesic activity was also observed in tail immersion method. Apigenin was devoid of any other CNS activity tested.

3.10 Mongraph on Damiana

Damiana consists of dried aerial parts of *Turnera aphrodisiaca* Ward synonym *T. diffusa* Willd. (Family Turneraeae).

**Category:** Aphrodisiac, Anxiolytic

**Dose:** 2 to 4 g as an infusion

**Description:** Green powder; odour, aromatic

**Storage:** Store in air tight container, protected from moisture and against attack of insects, and rodents

**STANDARDS**

Damiana aerial parts contain not less than 0.2 percent apigenin (C\(_{15}\)H\(_{10}\)O\(_5\); molecular wt., 270).

**Identification:** A; *Macroscopy* – stem reddish-brown, woody; leaves pale green or yellow green in color, 10-25 mm long, 4-10 mm broad, broadly lanceolate, short petioled, acute tip, cuneate base, serrate margin, smooth surface, lower surface glabrous with few hairs on ribs, and prominent veins on the under surface; branches have reddish-brown bark; flowers yellow, globose pods, 8-12 mm long with 5 yellowish petals and 5 styles; fruits small capsules.

B; *Microscopy* – stem presents epidermis consisting of single layer of wavy walled epidermal cells, covered with a thick cuticle, bearing unicellular warty
trichomes; hypodermis composed of 4-5 layers of collenchymatous cells followed by cortex; cortex consisting of 6-8 layers of parenchyma cells, oil cells; pericycle composed of 8-10 layers of thick walled parenchyma cells interspersed with groups of lignified sclerenchymatous fibres; vascular tissue composed of closely aligned collateral vascular bundles composed of xylem and phloem; pith consisting of parenchymatous cells with intracellular spaces and scattered cluster crystals of calcium oxalate.

Leaf presents epidermis with parenchymatous tabular cells covered with cuticle on the upper side, unicellular warty trichomes; mesophyll dorsiventral, composed of one layer of palisade cells followed by spongy parenchyma; midrib comprising 4-5 layers of isodiametric parenchyma cells with intracellular spaces, arc shaped meristele.

Sepal presents structures similar to those of leaf. Petal presents abundant unicellular warty trichomes, cluster calcium oxalate crystals, spiral xylem vessels.

Ovary presents ovary wall composed of epidermis, parenchyma containing abundant cluster crystals of calcium oxalate, vascular tissue; ovular integuments; micropyle; funiculus.

Fruit presents epicarp consisting of single layer of thin walled cells; mesocarp with parenchymatous cells, oil cells, sclereids, spiral vessels; endocarp composed of thick parenchymatous cells; seed testa consisting of lignified sclereidal cells.

Reduce to a moderately fine powder, if necessary. Examine microscopically using chloral hydrate solution. The powder consists of unicellular covering trichomes, pitted tracheidal vessels, pericyclic fibres, cluster crystals of calcium oxalate.

C; Thin layer chromatography – Carry out the method for thin layer chromatography using pre-coated aluminum-based TLC plate (silica gel G; 0.2 mm) for petroleum ether and chloroform extracts of the plant.

TLC of petroleum ether and chloroform extracts: pack separately dried aerial parts of *T. aphrodisiaca* (2 g each) in filter paper sachets, place inside two separate 100 ml round bottom flasks fitted with condenser, allow to macerate for 15 minutes separately, each of 50 ml, with petroleum ether and chloroform, and reflux (1 h each) on a boiling water bath. Recover solvents from the respective
extracts under reduced pressure. Reconstitute dried petroleum ether and chloroform extracts separately up to 5 ml in 5 ml volumetric flasks. Load 10 μl of the standard solution of each extract on TLC plates in the form of band using 2 μl capillary tubes. Develop TLC plate for petroleum ether extract using a mixture of 1 volume of hexane and 1 volume of dichloromethane, and allowing the solvent front to ascend 7 cm above the line of application. Remove the plate, allow it to dry, spray with 0.5% anisaldehyde solution and heat at 110°C for 10 minutes. Observe nine spots with Rf values – 0.14, 0.22, 0.28, 0.36, 0.42, 0.66, 0.70, 0.78 and 0.94. Similarly, develop TLC plate for chloroform extract using a mixture of 35 volumes of toluene, 4 volumes of ethyl acetate and 1 volume of glacial acetic acid, and observe eleven spots with Rf values – 0.06, 0.13, 0.19, 0.27, 0.31, 0.40, 0.45, 0.50, 0.56, 0.71 and 0.88.

**Total ash:** not more than 9.12% w/w (Section 2.4, p 101)

**Acid-insoluble ash:** not more than 1.3% w/w (Section 2.4, p 101)

**Water-soluble ash:** not more than 2.2% w/w (Section 2.4, p 101)

**Ethanol soluble extractive value:** not less than 18% w/w (Section 2.5, pp 101-102)

**Water soluble extractive value:** not less than 25% w/w (Section 2.5, pp 101-102)

**Volatile oil content:** not less than 0.4% v/w (Section 2.6.1, p 102)

**Assay:** Weigh accurately 1 g of the drug, in fine powder, add 50 ml petroleum ether and mix. Place in a water bath and heat under reflux condenser for 1 hour. Repeat the operation in triplicate. Reflux air dry marc under similar conditions with 3 quantity, each of 50 ml, of methanol. Pool methanol extracts, filter, concentrate under reduced pressure. Heat methanol extract with 6% aqueous hydrochloric acid (25 ml) for 45 minutes on boiling water bath. Cool the solution, remove precipitates by filtration and dissolve in 10 ml methanol (A). Extract the filtrate with 3×20 ml quantity of diethyl ether in 250 ml separating funnel, combine diethyl ether extracts, dry over anhydrous sodium sulphate, evaporate under reduced pressure (B). Mix A and B. Finally, make up the volume to 25 ml with methanol in a 25 ml volumetric flask (test sample).

Prepare stock solution of apigenin (1 mg/10 ml) in methanol. Dilute appropriately stock solution with methanol to get four working standard solutions.
of concentration 1 mg, 0.75 mg, 0.5 mg or 0.25 mg/10 ml. Apply 2 µl of the stock solution and the working standard solution of apigenin, in triplicate, on 20x20 cm pre-coated aluminum-based TLC plate (silica gel G; 0.2 mm) using standard 2 µl capillaries. Develop the plate using a mixture of 1 volume of toluene and 4 volumes of ethyl acetate as mobile phase and allowing the solvent front to ascend 5 cm above the line of application. Scan developed plates at 336 nm using HPTLC densitometer. Plot a standard graph against mean area under the peak and apigenin amount (µg).

Apply aliquot (2 µl) of test sample, in triplicate, to the pre-coated aluminum-based TLC plate (silica gel G; 0.2 mm) using standard 2 µl capillaries. Develop the plate using a mixture of 1 volume of toluene and 4 volumes of ethyl acetate as mobile phase and allowing the solvent front to ascend 5 cm above the line of application. Scan the chromatogram at 336 nm using HPTLC densitometer. Calculate the percentage content of apigenin from the regression equation of standard graph.

**VOLATILE OIL OF DAMIANA**

**Description:** Yellowish green liquid; odour, aromatic.

**Solubility:** Freely soluble in alcohol.

**Storage:** Store in well-filled, tightly-closed, light resistant container in a cool place.

**Identification:**

*Thin layer chromatography* – Carry out the method for thin layer chromatography using pre-coated aluminum-based TLC plate (silica gel G; 0.2 mm) for volatile oil.

For volatile oil solution, dissolve 1 volume of volatile oil in 10 volumes of chloroform. Load 2 µl volatile oil solution on TLC plate using standard 2 µl capillaries. Develop the plate using a mixture of 93 volumes of toluene and 7 volumes of ethyl acetate, and allowing the solvent front to ascend 7 cm above the line of application. Remove the plate, allow it to dry, spray with 0.5% anisaldehyde solution and heat at 110°C for 10 minutes. Observe seven spots having *R*<sub>f</sub> values – 0.28 (violet), 0.38 (black), 0.47 (red), 0.53 (black), 0.66 (violet), 0.77 (yellow) and 0.89 (black).