5. RESULTS AND DISCUSSION

The plant material of *Barleria prionitis* Linn. of family Acanthaceae was collected during August to September 2008 and of *B. cristata* Linn. var. *dichotoma* (White flower variety), *B. cristata* Linn. (Blue flower variety), *B. cristata* Linn. (Pink flower variety) and *B. lupulina* Lindl. during February to March 2009 from the Medicinal Plants Garden of University Institute of Pharmaceutical Sciences, Panjab University, Chandigarh. The authenticity of the samples was duly confirmed by National Institute of Science Communication and Information Resources (NISCAIR), New Delhi. The phytochemical studies were carried out on the whole plant material of *B. prionitis* with an aim of isolating the three major and targeted markers. Different extracts and plant parts of various species/varieties of *Barleria* were then subjected to analytical study using three different techniques viz., HPTLC, HPLC and UPLC. Further, comparative chemical and biological studies were performed on the different species/varieties of *Barleria*. These studies were characteristic enough to explore the active extracts/active compounds and identify the right *Barleria* specie/variety which could be useful to the chemotaxonomists, herbal industry and in drug discovery programmes.

As the data generated was expected to be of both scientific and commercial value, so the present study was planned to be carried out in following phases on five selected species/varieties of *Barleria*:

- Phytochemical studies
- Qualitative studies - TLC
- Quantitative studies - HPTLC, HPLC and UPLC
- Biological studies
  - Toxicity study (Up and Down procedure)
  - *In vivo* pharmacological studies -
    - Anti-inflammatory activity (acute, sub-acute and topical models),
    - Analgesic activity (central and peripheral models),
    - Antiulcer activity (Pyloric ligation induced gastric ulcer model), and
    - Antiarthritic activity (CFA induced arthritic model)

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5.1. PHYTOCHEMICAL STUDIES

The phytochemical screening showed the presence of flavonoids, steroids and triterpenoids, anthraquinones, carbohydrates and iridoids in *B. prionitis* and *B. cristata* (table 39). The TLCs of the methanolic extract of whole plant of five different *Barleria* species/varieties were generated with a view to have a clear profile of the presence/absence of the three targeted iridoid markers. Based on their presence and availability of plant material, *B. prionitis* was selected for detailed phytochemical investigation. The collected shade dried whole plant material (3.5 kg) of *B. prionitis* was coarsely powdered and extracted thrice with methanol (5, 3, 3 L) each time for 48 h at room temperature. The resultant extract was filtered and the solvent was removed under vacuum to obtain a viscous residue (200 g). The total methanolic extract, (ME-189 g) was suspended separately in 300 ml of water and extracted successively with solvents of increasing polarity to obtain respective partitioned fractions viz., hexane soluble (HE-28 g), butanol soluble (BE-44 g) and left aqueous extract (LAE-87 g).

The phytochemical studies were aimed at isolation of three major components as observed from TLC for further use in analytical and biological studies. The most intense spots of the desired components were observed in butanol extract of *B. prionitis* (plate 6). Hence the butanol extract (40 g) of *B. prionitis* was subjected to various chromatographic techniques like column chromatography, medium pressure liquid chromatography and preparative thin layer chromatography to yield two iridoids namely B-1 (80 mg) and B-2 (154 mg). B-1 was eluted at 75 % ethyl acetate in hexane and B-2 was obtained with varying ratios from 50-57 % ethyl acetate in hexane as an eluent. Another targeted component B-3 was obtained when 12 g of the left aqueous extract was subjected to gravity columns. Fractions eluted at 40 % methanol in chloroform gave 2.14 g of the pure crystalline isolate B-3 as white needles and recrystallization was done in chloroform-methanol mixture.

The iridoid nature of these isolates was confirmed by blue colour obtained upon chemical treatment with Trim Hill reagent. The spectroscopic data confirmed the
Results and Discussion

identity of isolates B-1, B-2 and B-3 as barlerin, acetyl barlerin and shanzhiside methyl ester respectively. Further co-TLC with the reference markers obtained from the repository of Pharmacognosy laboratory, University Institute of Pharmaceutical Sciences, Panjab University, confirmed their identity.

5.2. QUALITATIVE STUDIES

5.2.1. Comparison of TLC Fingerprint Profile of Various Species and Varieties of Barleria

A comparative study was made for the qualitative chemical evaluation of various species and varieties of Barleria based on the TLC fingerprint profile. A large number of solvent systems were tried and many of them gave good resolution but only for specific extract/plant. Finally, a solvent system (ethyl acetate : methanol : formic acid :: 7.5 : 2 : 0.5) was developed which gave optimally good resolution of the targeted compounds for all plants/extracts to make the comparative study simple for evaluation. This was a significant success in the present study which was further applied covering various aspects as follows:

(i) Comparison of different plant parts - The whole plant of all the five species/varieties of Barleria was taken up for the generation of TLC fingerprint profile (plate 6). The whole plant of B. lupulina was found to have maximum intensity of acetyl barlerin followed by B. prionitis and B. cristata var. dichotoma. The intensity of barlerin was observed to be same in B. prionitis and B. lupulina followed by B. cristata var. dichotoma and the sequence of shanzhiside methyl ester content was B. prionitis > B. lupulina > B. cristata var. dichotoma. However, all the three markers viz., acetyl barlerin, barlerin and shanzhiside methyl ester were absent in B. cristata (Pink flower variety) and B. cristata (Blue flower variety). In case of three other species/varieties, viz., B. cristata (Pink flower variety), B. cristata (Blue flower variety) and B. lupulina because of paucity of the plant material only the methanolic extract was taken up to generate the comparative TLC profile. Further the leaf, stem and root of three selected plants, viz., B. prionitis, B. cristata var. dichotoma and B. lupulina were taken up for comparison. The other two plants, viz.,
Results and Discussion

*B. cristata* (Pink flower variety) and *B. cristata* (Blue flower variety) were not taken up for the different parts because of very less quantity of the available material and more so because the three markers were found to be absent in the methanol extract of whole plant. The leaf of *B. lupulina* was found to be enriched in acetyl barlerin and stem of *B. prionitis* was found to possess more intense spots of barlerin as well as shanzhiside methyl ester. The solvent system used was ethyl acetate : methanol : formic acid :: 7.5 : 2 : 0.5. All the results of comparative profile of different specie/plant parts are given comprehensively in table 122.

(ii) **Comparison of various extracts of the whole plant of two most commonly available species** - A comparison of various extracts based on TLC fingerprint profile was made for the two most commonly and abundantly growing species of *Barleria*, viz., *B. prionitis* and *B. cristata* var. *dichotoma*. Different extracts, viz., mother/methanol, hexane, chloroform, ethyl acetate and butanol, of varying polarity were prepared for both the species and the solvent system used was ethyl acetate : methanol : formic acid :: 7.5 : 2 : 0.5. The methanolic and butanol extract of both the species viz. *B. prionitis* and *B. cristata* var. *dichotoma* were found to possess all the three markers - acetyl barlerin, barlerin and shanzhiside methyl ester and the results of comparative profile of different specie/extracts are given comprehensively in table 122.
Results and Discussion

Table 122: Qualitative analysis of three iridoids - acetyl barlerin (AB), barlerin (B) and shanzhiside ethyl ester (SME) in Barleria species/varieties by TLC

<table>
<thead>
<tr>
<th>Barleria species/varieties</th>
<th>AB</th>
<th>B</th>
<th>SME</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. prionitis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wp*</td>
<td>/</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>Lf*</td>
<td>/</td>
<td>/</td>
<td>/</td>
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<tr>
<td>St*</td>
<td>-</td>
<td>/</td>
<td>/</td>
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<tr>
<td>Rt*</td>
<td>-</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>ME*</td>
<td>/</td>
<td>/</td>
<td>/</td>
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<tr>
<td>HE*</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CE*</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EAE*</td>
<td>/</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>BE*</td>
<td>√</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>TAE*</td>
<td>-</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>B. cristata</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WP*</td>
<td>/</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>Lf*</td>
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<td>/</td>
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<tr>
<td>St*</td>
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<td>Rt*</td>
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<td>ME*</td>
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<tr>
<td>HE*</td>
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<tr>
<td>CE*</td>
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<tr>
<td>EAE*</td>
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<tr>
<td>BE*</td>
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<td>/</td>
</tr>
<tr>
<td>TAE*</td>
<td>-</td>
<td>/</td>
<td>-</td>
</tr>
<tr>
<td>B. cristata (Pink flower variety)</td>
<td>WP/ME*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B. cristata (Blue flower variety)</td>
<td>WP/ME*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B. lupulina</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WP/ME*</td>
<td>/</td>
<td>/</td>
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<tr>
<td>Lf*</td>
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<tr>
<td>St*</td>
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<tr>
<td>Rt*</td>
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<td>/</td>
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<tr>
<td>ME*</td>
<td>/</td>
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<tr>
<td>HE*</td>
<td>-</td>
<td>-</td>
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<td>CE*</td>
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<tr>
<td>EAE*</td>
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</tr>
<tr>
<td>BE*</td>
<td>/</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>TAE*</td>
<td>-</td>
<td>/</td>
<td>-</td>
</tr>
</tbody>
</table>

* where √: Present and the tick number indicates the intensity of bands, Wp: Whole plant, ME: Mother/Methanol extract, Lf: Leaf, St: Stem, Rt: Root, HE: Hexane soluble fraction of methanol extract, CE: Chloroform soluble fraction of methanol extract, EAE: Ethyl acetate soluble fraction of methanol extract and BE: Butanol soluble fraction of methanol extract

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5.2.1.1. Comparative TLC fingerprint profile of different plant parts of *Barleria*

5.2.1.1.1. *B. prionitis*

Comparative TLC fingerprint profile of methanolic extract of whole plant, leaf, stem and root of *B. prionitis* (plate 7/table 40), showed two pink colored spots of shanzhiside methyl ester and barlerin at Rf 0.42 and 0.52, in whole plant, leaf, stem and root under white light. However, a pink colored spot of acetyl barlerin at Rf 0.65, was detected only in whole plant and leaf of *B. prionitis* under white light.

5.2.1.1.2. *B. cristata* var. *dichotoma*

Comparative TLC fingerprint profile of methanolic extract of whole plant, leaf, stem and root of *B. cristata* var. *dichotoma* showed a pink colored spot of shanzhiside methyl ester at Rf 0.42 in whole plant, leaf and stem of the plant when observed under white light and was absent in root (plate 8/table 41). A pink colored spot of barlerin at Rf 0.54 was detected in all parts under white light while a pink spot of acetyl barlerin at Rf 0.60 was observed only in whole plant, leaf and root of the plant.

5.2.1.1.3. *B. lupulina*

Comparative TLC fingerprint profile of methanolic extract of whole plant, leaf, stem and root of *B. lupulina* showed pink colored spots of shanzhiside methyl ester and barlerin at Rf 0.48 and 0.58 respectively in all parts of the plant under white light (plate 9/table 42). However, a pink colored spot of acetyl barlerin was detected at Rf 0.72 in whole plant, leaf and root but absent in stem.

5.2.1.2. Comparative TLC fingerprint profile of various extracts of whole plant of *Barleria*

As stated earlier [section 5.2.1 (ii)], a comparison of various extracts of the whole plant of two most commonly growing species of *Barleria*, viz., *B. prionitis*, *B. cristata* var. *dichotoma* was made. Different extracts of varying polarity, viz., mother/methanol, hexane, chloroform, ethyl acetate and butanol were prepared and the solvent system used was ethyl acetate : methanol : formic acid :: 7.5 : 2 : 0.5. However, for the remaining three species/varieties viz., *B. cristata* (Pink and
Results and Discussion

Blue flower variety) and *B. lupulina* because of the paucity of the material, only methanolic extract of whole plant was taken up.

5.2.1.2.1. *B. prionitis*

A comparative TLC fingerprint profile of various extracts, *viz.*, methanol, hexane, chloroform, ethyl acetate and butanol soluble and total aqueous extracts of the whole plant of *B. prionitis* was prepared. A critical look at plate 10 &12/table 43, showed that all the three markers, *viz.*, shanzhiside methyl ester, barlerin and acetyl barlerin were observed at Rf 0.37, 0.47 and 0.60, in methanol, ethyl acetate and butanol extracts both under UV light at 254 and 366 nm before derivatization and under white light after derivatization with 0.5 % anisaldehyde in conc H$_2$SO$_4$. In total aqueous extract barlerin and shanzhiside methyl ester was observed at Rf 0.51 and 0.40. However, all the three shanzhiside markers were not observed in hexane and chloroform extracts.

5.2.1.2.2. *B. cristata var. dichotoma*

A comparative TLC fingerprint profile of various extracts, *viz.*, methanol, hexane, chloroform, ethyl acetate and butanol soluble and total aqueous extracts of the whole plant of *B. cristata var. dichotoma* was prepared. A careful study of the plate 11 &12/table 44, showed that the three markers, *viz.*, acetyl barlerin, barlerin, shanzhiside methyl ester were absent in hexane, chloroform and ethyl acetate extracts when observed under both UV light at 254 and 366 nm before derivatization and under white light after derivatization with 0.5 % anisaldehyde in conc H$_2$SO$_4$. However, pink colored spots of shanzhiside methyl ester, barlerin and acetyl barlerin were observed at Rf 0.55, 0.65 and 0.75 in methanol and butanol extracts and shanzhiside methyl ester at Rf 0.40 in total aqueous extract under UV and white light.

5.2.1.2.3. *B. cristata var. dichotoma* (Pink and Blue flower variety)

The pink colored spots of shanzhiside methyl ester, barlerin and acetyl barlerin were not observed in methanol extracts under UV and white light in any of these two varieties (plate 13 & 14/table 45 & 46).
Results and Discussion

5.2.1.2.4. *B. lupulina*

The pink colored spots of all the three markers viz., shanzhiside methyl ester, barlerin and acetyl barlerin were observed at Rf 0.48, 0.58 and 0.72 in methanol extract under UV and white light (plate 9/table 42).

Thus from the comparative study done using TLC fingerprint profile of five different species and varieties of *Barleria*, following findings were made -

A) A large number of solvent systems were tried and many of them gave good resolution but only for specific extract/plant. Finally such solvent system (ethyl acetate : methanol : formic acid :: 7.5 : 2.0 : 0.5) was developed which gave optimally good resolution for all plants/extracts to make comparative study simple for evaluation. This was a significant success in the present study.

B) Amongst the various species, *B. prionitis* and *B. lupulina* showed the intense spots of the three markers viz., acetyl barlerin, barlerin and shanzhiside methyl ester while their intensity was found to be less in case of *B. cristata* var. *dichotoma*. Further, none of the three markers could be detected qualitatively in two varieties of *B. cristata* viz., pink flower variety and blue flower variety.

C) Certain conclusions were drawn when different plant parts were qualitatively analysed using TLC. Out of the five species worked up in present study, *B. prionitis, B. cristata* and *B. lupulina* appeared to be closely related with respect to the presence of three selected markers as well complete TLC fingerprint profile. However, a significant difference could be seen in the amounts of the three markers in these plants as indicated by intensity and size of the spots/bands. The stem of *B. prionitis* appears to be the richest source of barlerin followed by root of the same plant; leaf of *B. lupulina* is the richest source of acetyl barlerin; and stem of *B. prionitis* appears to be the richest source of shanzhiside methyl ester followed by leaf of the same plant. However, *B. cristata* (Pink flower variety) and *B. cristata* (Blue flower variety) are completely devoid of any of the three targeted markers.
5.3. QUANTITATIVE STUDIES

The quantitative analysis of three shanzhiside esters viz., acetyl barlerin, barlerin and shanzhiside methyl ester was done using the techniques of HPTLC, HPLC and UPLC. The three iridoids were estimated in different plant parts and various extracts of the selected species/varieties of Barleria (table 123).

Table 123: Per cent content of the shanzhiside iridoids - acetyl barlerin (AB), barlerin (B) and shanzhiside ethyl ester (SME) in Barleria species/varieties by HPTLC/HPLC/UPLC

<table>
<thead>
<tr>
<th>Barleria species/varieties</th>
<th>Per cent content of iridoids</th>
<th>HPTLC</th>
<th>HPLC</th>
<th>UPLC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AB</td>
<td>B</td>
<td>SME</td>
<td>AB</td>
</tr>
<tr>
<td><strong>B. prionitis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wp/ME*</td>
<td>0.35</td>
<td>0.81</td>
<td>1.36</td>
<td>1.69</td>
</tr>
<tr>
<td>Lf*</td>
<td>0.95</td>
<td>0.28</td>
<td>2.62</td>
<td>1.11</td>
</tr>
<tr>
<td>St*</td>
<td>ND*</td>
<td>0.97</td>
<td>1.46</td>
<td>ND*</td>
</tr>
<tr>
<td>Rt*</td>
<td>ND*</td>
<td>0.96</td>
<td>1.35</td>
<td>ND*</td>
</tr>
<tr>
<td>HE*</td>
<td>ND*</td>
<td>0.02</td>
<td>ND*</td>
<td>ND*</td>
</tr>
<tr>
<td>CE*</td>
<td>0.28</td>
<td>0.08</td>
<td>0.11</td>
<td>0.66</td>
</tr>
<tr>
<td>EAE*</td>
<td>1.28</td>
<td>1.10</td>
<td>1.00</td>
<td>4.87</td>
</tr>
<tr>
<td>BE*</td>
<td>1.68</td>
<td>2.82</td>
<td>3.92</td>
<td>1.81</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>B. cristata var. dichotoma</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Wp/ME*</td>
</tr>
<tr>
<td>Lf*</td>
</tr>
<tr>
<td>St*</td>
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<tr>
<td>Rt*</td>
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<tr>
<td>HE*</td>
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<tr>
<td>CE*</td>
</tr>
<tr>
<td>EAE*</td>
</tr>
<tr>
<td>BE*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>B. cristata (Pink flower variety)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Wp/ME*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>B. cristata (Blue flower variety)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Wp/ME*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>B. lupulina</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Wp/ME*</td>
</tr>
<tr>
<td>Lf*</td>
</tr>
<tr>
<td>St*</td>
</tr>
<tr>
<td>Rt*</td>
</tr>
</tbody>
</table>

* where ND: Not detected, Wp - Whole plant, ME - Mother/Methanol extract, Lf - Leaf, St - Stem, Rt - Root, HE - Hexane soluble fraction of methanol extract, CE - Chloroform soluble fraction of methanol extract, EAE - Ethyl acetate soluble fraction of methanol extract and BE - Butanol soluble fraction of methanol extract
Table 124: Validation studies of the shanzhiside iridoids - acetyl barlerin (AB), barlerin (B) and shanzhiside methyl ester (SME) by HPTLC/HPLC/UPLC

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HPTLC</th>
<th>HPLC</th>
<th>UPLC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Per cent content of iridoids</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AB</td>
<td>B</td>
<td>SME</td>
</tr>
<tr>
<td>LOD (ng)</td>
<td>71</td>
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<td>50</td>
</tr>
<tr>
<td>LOQ (ng)</td>
<td>212</td>
<td>150</td>
<td>150</td>
</tr>
<tr>
<td>Linearity</td>
<td>1415-4953</td>
<td>278-1665</td>
<td>600-3600</td>
</tr>
<tr>
<td>Specificity</td>
<td>Specific</td>
<td>Specific</td>
<td>Specific</td>
</tr>
<tr>
<td>Accuracy</td>
<td>Accurate</td>
<td>Accurate</td>
<td>Accurate</td>
</tr>
<tr>
<td>Sensitivity</td>
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<td></td>
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</tr>
<tr>
<td>Precision</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intraday</td>
<td>2121 ± 40</td>
<td>556 ± 13.8</td>
<td>1199 ± 28</td>
</tr>
<tr>
<td>Interday</td>
<td>2122 ± 31</td>
<td>557 ± 5.91</td>
<td>1202 ± 28</td>
</tr>
<tr>
<td>Reproducibility</td>
<td>2124 ± 11</td>
<td>558 ± 11.0</td>
<td>1201 ± 2</td>
</tr>
<tr>
<td>Robustness</td>
<td>Robust</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recovery studies</td>
<td>50 %</td>
<td>100%</td>
<td>150%</td>
</tr>
<tr>
<td></td>
<td>97 ± 4.02</td>
<td>98 ± 2.36</td>
<td>98 ± 3.77</td>
</tr>
<tr>
<td></td>
<td>96 ± 4.46</td>
<td>99 ± 1.04</td>
<td>99 ± 1.96</td>
</tr>
</tbody>
</table>
Results and Discussion

5.3.1. High Performance Thin Layer Chromatography (HPTLC)

A simple, sensitive, selective, precise, robust, rapid and reliable high-performance thin layer chromatographic method of analysis for simultaneous determination of acetyl barlerin, barlerin and shanzhiside methyl ester (the major iridoids of Barleria) was developed and validated. To our knowledge, two reports are known where analysis of B. prionitis has been done by TLC densitometry using the solvent system chloroform-methanol (8 : 2, v/v).\(^\text{[190,191]}\) The first report was from our laboratory and this solvent system was developed for the estimation of single iridoid marker barlerin and the method was not validated. Subsequently in a recent publication 10-50 times high per cent content of barlerin (10.03 ± 1.69) along with exceptionally high content of shanzhiside methyl ester (21.55 ± 2.40 per cent) has been reported employing this solvent system. This indicated that the earlier reported method is not specific when more than one markers are desired to be quantified simultaneously. Therefore, in the present study, a battery of solvent systems with varying compositions of chloroform : methanol; ethyl acetate : methanol : formic acid; ethyl acetate : methanol : acetic acid; chloroform : ethyl acetate : methanol : acetic acid etc were tested with an aim -

(i) to resolve the underlying spots interfering with the markers,
(ii) to validate the developed method as per ICH guidelines for the drug quality assurance and
(iii) to obtain the most accurate values of per cent content of the three iridoid markers.

Hence in this study, the optimum solvent system that gave compact spots with most selective resolution of the selected markers was chloroform : ethyl acetate : methanol : acetic acid (3.0 : 3.0 : 3.0 : 1.0, v/v/v/v) with chamber saturation of 10 min at 25°C and solvent migration distance of 80 mm. Further, the desired resolution was obtained with single development having significantly different \(R_i\) values centered around 0.52, 0.61 and 0.71 for shanzhiside methyl ester, barlerin and acetyl barlerin and none of the desired components had any underlying or interfering spots. The optimum resolution was obtained using unmodified silica.
Results and Discussion

layer as stationary phase on precoated plates and slit-scanning densitometry with UV-Visible light as the detection technique. The three iridoid markers were examined directly on the stationary layer. It did not involve any pretreatment or derivatization and optical densitometric scanning was used for the in situ measurement of compounds directly on the layer.

Under the experimental conditions employed, the minimum amount of markers that could be detected was found to be 71 ng for acetyl barlerin and 50 ng for both barlerin and shanzhiside methyl ester. The lowest quantifiable amount of markers was 212 ng/spot for acetyl barlerin, 150 ng/spot for barlerin and shanzhiside methyl ester (table 50). The method was confirmed to be particularly specific as no interference of matrix was observed for any of the markers. The method was also linear in a concentration range of 1415-4953 ng/spot for acetyl barlerin, 278-1665 ng/spot for barlerin and 600-3600 ng/spot for shanzhiside methyl ester (n=6), with respect to peak area. The linear regression data shown in table 51 using SPSS software (version 16) with 95 % confidence limits revealed a good linear relationship over the concentration range studied demonstrating its suitability for analysis. The developed method is simple, sensitive, precise, robust, specific, accurate and reliable for the determination of shanzhiside methyl ester iridoids (table 52 to 56). The low % RSD value indicated the suitability of this method for routine analysis of shanzhiside methyl esters. Thus, this validated HPTLC method was used as a rapid method of analysis for the simultaneous determination of shanzhiside iridoids viz., acetyl barlerin, barlerin and shanzhiside methyl ester in atleast 5 different Barleria species and varieties viz., B. prionitis, B. cristata var. dichotoma, B. cristata (Pink flower variety), B. cristata (Blue flower variety) and B. lupulina without any other component interfering in the analysis (table 123). It is expected that the developed method could easily and successfully be applied with precise results for the estimation of shanzhiside esters in other Barleria species/related plant species, herbal extracts, formulations etc (table 124).

Hence comparative chromatograms of various species and varieties of Barleria were prepared and analyzed for the per cent content of three selected iridoids.
Results and Discussion

5.3.1.1. Estimation of shanzhiside esters in different plant parts of B. prionitis, B. cristata and B. lupulina

5.3.1.1.1. Whole plant

a) Acetyl barlerin

The maximum content of acetyl barlerin was found in whole plant of *B. lupulina* followed by *B. prionitis* and minimum in *B. cristata* var. *dichotoma* at 2.2, 0.35 and 0.26 per cent respectively. Acetyl barlerin was not detected in whole plant of *B. cristata* (Pink flower variety) and *B. cristata* (Blue flower variety).

b) Barlerin

The maximum content of barlerin was found in whole plant of *B. prionitis* followed by *B. lupulina* and minimum in *B. cristata* var. *dichotoma* at 0.81, 0.65 and 0.18 per cent respectively. Barlerin was not detected in whole plant of *B. cristata* (Pink flower variety) and *B. cristata* (Blue flower variety).

c) Shanzhiside methyl ester

The maximum content of shanzhiside methyl ester was found in whole plant of *B. prionitis* followed by *B. lupulina* and minimum in *B. cristata* var. *dichotoma* at 1.36, 0.91 and 0.52 per cent respectively. Shanzhiside methyl ester was not detected in whole plant of *B. cristata* (Pink flower variety) and *B. cristata* (Blue flower variety).

5.3.1.1.2. Leaf

a) Acetyl barlerin

The maximum content of acetyl barlerin was found in leaves of *B. lupulina* followed by *B. prionitis* and minimum in *B. cristata* var. *dichotoma* at 3.82, 0.95 and 0.46 per cent respectively. Acetyl barlerin was not detected in leaves of *B. cristata* (Pink flower variety) and *B. cristata* (Blue flower variety).

b) Barlerin

The maximum content of barlerin was found in leaves of *B. lupulina* followed by *B. prionitis* and minimum in *B. cristata* var. *dichotoma* at 0.73, 0.28 and 0.18 per cent respectively. Barlerin was not detected in leaves of *B. cristata* (Pink flower variety) and *B. cristata* (Blue flower variety).
c) **Shanzhiside methyl ester**

The maximum content of shanzhiside methyl ester was found in leaves of *B. prionitis* followed by *B. lupulina* and minimum in *B. cristata* var. *dichotoma* at 2.62, 1.30 and 0.70 per cent respectively. Shanzhiside methyl ester was not detected in leaves of *B. cristata* (Pink flower variety) and *B. cristata* (Blue flower variety).

5.3.1.1.3. Stem

a) **Acetyl barlerin**

The per cent content of acetyl barlerin in stem of *B. lupulina* was 0.43, while it was absent in the stem of rest of the species and varieties of *Barleria*.

b) **Barlerin**

The maximum content of barlerin was found in stem of *B. prionitis* followed by *B. lupulina* and minimum in *B. cristata* var. *dichotoma* at 0.97, 0.59 and 0.23 per cent respectively. Barlerin was not detected in stem of *B. cristata* (Pink flower variety) and *B. cristata* (Blue flower variety).

c) **Shanzhiside methyl ester**

The maximum content of shanzhiside methyl ester was found in stem of *B. prionitis* followed by *B. cristata* var. *dichotoma* and *B. lupulina* at 1.46, 0.89 and 0.80 per cent respectively. Shanzhiside methyl ester was not detected in stem of *B. cristata* (Pink flower variety) and *B. cristata* (Blue flower variety).

5.3.1.1.4. Root

a) **Acetyl barlerin**

The maximum content of acetyl barlerin was found in roots of *B. lupulina* followed by *B. cristata* var. *dichotoma* at 0.72 and 0.16 per cent respectively. It was not detected in roots of *B. prionitis, B. cristata* (Pink flower variety) and *B. cristata* (Blue flower variety).

b) **Barlerin**

The maximum content of barlerin was found in roots of *B. prionitis* followed by *B. lupulina* and minimum in *B. cristata* var. *dichotoma* at 0.96, 0.45 and 0.14 per
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cent respectively. Barlerin was not detected in roots of B. cristata (Pink flower variety) and B. cristata (Blue flower variety).

c) Shanzhiside methyl ester
The maximum content of shanzhiside methyl ester was found in roots of B. prionitis followed by B. lupulina was 1.35 and 1.03 respectively, whereas it was not detected in B. cristata var. dichotoma, B. cristata (Pink flower variety) and B. cristata (Blue flower variety).

The results are summarized as:

**Acetyl barlerin**: Leaf of B. lupulina > Whole plant of B. lupulina > Leaf of B. prionitis > Root of B. lupulina > Leaf of B. cristata var. dichotoma > Stem of B. lupulina > Whole plant of B. prionitis > Whole plant of B. cristata var. dichotoma > Root of B. cristata var. dichotoma.

**Barlerin**: Stem of B. prionitis > Root of B. prionitis > Whole plant of B. prionitis > Leaf of B. lupulina > Whole plant of B. lupulina > Stem of B. lupulina > Root of B. lupulina > Leaf of B. prionitis > Stem of B. cristata var. dichotoma > Whole plant and leaf of B. cristata var. dichotoma > Root of B. cristata var. dichotoma.

**Shanzhiside methyl ester**: Leaf of B. prionitis > Stem of B. prionitis > Whole plant of B. prionitis > Root of B. prionitis > Leaf of B. lupulina > Root of B. lupulina > Whole plant of B. lupulina > Stem of B. cristata var. dichotoma > Stem of B. lupulina > Leaf of B. cristata var. dichotoma > Whole plant of B. cristata var. dichotoma.

5.3.1.2. Estimation of shanzhiside esters in different extracts of B. prionitis and B. cristata var. dichotoma

The quantitative estimation of the three selected iridoids was done in different extracts of only two abundantly growing species of Barleria i.e. B. prionitis and B. cristata var. dichotoma. Due to less quantity of the available material, different extracts could not be taken up for B. cristata (Pink flower variety), B. cristata (Blue flower variety) and B. lupulina.
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a) Acetyl barlerin
Different extracts viz., methanol, hexane, chloroform, ethyl acetate and butanol were prepared of whole plant of *B. prionitis* and *B. cristata* var. *dichotoma*. The maximum content of acetyl barlerin was found in butanol extract of *B. prionitis* followed by ethyl acetate extract of the same plant and then butanol extract of *B. cristata* var. *dichotoma* at 1.68, 1.28 and 0.89 per cent respectively. It was absent in hexane extract of both the species. In the case of chloroform extract, content of acetyl barlerin was 0.28 per cent in *B. prionitis* while it was not detected in *B. cristata* var. *dichotoma*. It was also absent in ethyl acetate extract of *B. cristata* var. *dichotoma*. In case of methanol/mother extract, the content was found to be 0.35 and 0.26 respectively for *B. prionitis* and *B. cristata* var. *dichotoma*.

b) Barlerin
Different extracts viz., methanol, hexane, chloroform, ethyl acetate and butanol were prepared for *B. prionitis* and *B. cristata* var. *dichotoma*. The maximum content of barlerin was found in butanol extract of *B. prionitis* followed by ethyl acetate and methanol extract of the same plant at 2.82, 1.10 and 0.81 per cent respectively. The per cent content of barlerin in butanol extract of *B. cristata* var. *dichotoma* was 0.59 followed by ethyl acetate and methanol extract of the same plant at 0.59, 0.21 and 0.18 per cent respectively. In the case of chloroform extract, content of barlerin was 0.08 per cent in *B. prionitis* while it was not detected in *B. cristata* var. *dichotoma*. Barlerin per cent content in hexane extract of *B. prionitis* was 0.02, whereas it was not detected in *B. cristata* var. *dichotoma*.

c) Shanzhiside methyl ester
Different extracts viz., methanol, hexane, chloroform, ethyl acetate and butanol were prepared for *B. prionitis* and *B. cristata* var. *dichotoma*. The maximum content of shanzhiside methyl ester was found in butanol extract of *B. prionitis* followed by methanol extract of the same plant at 3.92, 1.36 and 1.28 per cent respectively. The per cent content of shanzhiside methyl ester in ethyl acetate and chloroform extract of *B. prionitis* was 1.00 and 0.11 respectively. However, it was absent in chloroform extract of *B. prionitis*.
Results and Discussion

and ethyl acetate extract of *B. cristata* var. *dichotoma* and hexane extract of both the species.

It is significant to note that the observations made in qualitative analysis with respect to presence/absence or high/low amounts of the shanzhiside esters were in partial corroboration with the quantitative results obtained with TLC densitometry. The possible reason for this could be the difference in resolution in qualitative and quantitative profiles. In qualitative studies, more emphasis was laid on the overall fingerprint profile which gives a possibility of overlapping of some spots, while in quantitative analysis strict attention was paid to the three selected iridoid markers taking care that no other component interferes in the analysis.

5.3.2. High Performance Liquid Chromatography (HPLC)

HPLC is an instrumental chromatographic technique used to separate a mixture of compounds with the purpose of identifying, purifying and quantifying the individual components of the mixture and is of special interest to analytical chemists. To our knowledge, no reports have been known regarding the simultaneous analysis of the three shanzhiside esters viz., acetyl barlerin, barlerin and shanzhiside methyl ester using HPLC technique. So, a battery of solvent combinations both gradiently and isocratically and with different flow rates were tried as mentioned in section 4.1.4.4.2. Finally, a precise and feasible HPLC gradient method with following experimental conditions was selected for quantitative analysis of shanziside esters in *Barleria* spp: C18 column (HIBAR), run time 40 min, gradient system, curve 2-water : methanol : acetonitrile (1 ml/min)

0’ 65 : 34 : 1  
5’ 65 : 34 : 1  
10’ 50 : 49 : 1  
15’ 50 : 49 : 1  
20’ 65 : 34 : 1  
35’ End

This solvent system was developed with an aim to get a well resolved chromatogram and to analyse the content of three shanzhiside esters in different
plant parts and extracts of all *Barleria* species/varieties. This method was further validated according to the ICH guidelines (1996).

Under the experimental conditions employed, the minimum amount of markers that could be detected was found to be 6 ng for acetyl barlerin, 4 ng for barlerin and 2 ng for shanzhiside methyl ester; the lowest quantifiable amount of markers was 22 ng/spot for acetyl barlerin, 16 ng/spot for barlerin and 8 ng/spot for shanzhiside methyl ester (table 67 & 124). The method was confirmed to be particularly specific and was also linear in a concentration range of 22-1415 ng/spot for acetyl barlerin, 16-1000 ng/spot for barlerin and 8-1000 ng/spot for shanzhiside methyl ester (n=6), with respect to peak area. The linear regression data shown in table 68 & 122 revealed a good linear relationship over the concentration range studied demonstrating its suitability for analysis. The developed method was precise, specific and accurate for the determination of shanzhiside methyl ester iridoids (table 69, 70 & 124). Statistical evaluation of the results was performed with respect to accuracy and precision which showed low % RSD value indicating the suitability of this method for the analysis of shanzhiside methyl esters. Thus, this developed and validated HPLC method can be used to determine the content of shanzhiside iridoids viz., acetyl barlerin, barlerin and shanzhiside methyl ester in different *Barleria* species and varieties (table 123). The various chromatograms generated using the developed method were analyzed critically and following conclusions were made:

5.3.2.1. Estimation of shanzhiside esters in different plant parts of *B. prionitis, B. cristata and B. lupulina*

5.3.2.1.1. Stem

a) *Acetyl barlerin*

The per cent content of acetyl barlerin in stem of *B. lupulina* was 0.09, while it was absent in the stem of *B. prionitis*. However, the per cent content of 0.004 was detected in the stem part of *B. cristata* var. *dichotoma* while it was not detected when analysed by TLC densitometry.
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b) Barlerin
The maximum content of barlerin was found in stem of B. prionitis followed by B. lupulina and minimum in B. cristata var. dichotoma at 2.15, 1.02 and 0.08 per cent respectively. Although, the maximum content was estimated in B. prionitis and B. lupulina, there is an interference of some other compounds which has given higher value of AUC.

c) Shanzhiside methyl ester
The maximum content of shanzhiside methyl ester was found in stem of B. prionitis followed by B. cristata var. dichotoma and B. lupulina at 2.01, 0.50 and 0.42 per cent respectively.

5.3.2.1.2. Root

a) Acetyl barlerin
The maximum content of acetyl barlerin was found in roots of B. lupulina followed by minimum content of B. cristata var. dichotoma at 0.97 and 0.007 per cent respectively. However, it was not detected in roots of B. prionitis. The pattern regarding the content of acetyl barlerin was found to be same as in HPTLC but due to the higher sensitivity of HPLC, the content in roots of B. lupulina was found to be more than in densitometry analysis.

b) Barlerin
The maximum content of barlerin was found in roots of B. prionitis followed by B. lupulina at 6.05 and 0.78 level and minimum in B. cristata var. dichotoma at 0.04 per cent respectively. The high per cent content of 6.05 in roots of B. prionitis is mainly due to the interference of the compound having very close Rt.

c) Shanzhiside methyl ester
The maximum content of shanzhiside methyl ester found in roots of B. prionitis followed by B. cristata var. dichotoma and B. lupulina was 3.16, 0.31 and 0.27 respectively.
5.3.2.1.3. Leaf

a) Acetyl barlerin
The maximum content of acetyl barlerin was found in B. lupulina followed by B. prionitis and minimum in B. cristata var. dichotoma at 7.30, 0.11 and 0.09 per cent respectively. The content of acetyl barlerin in B. lupulina was observed to be almost double as compared to the content found in HPTLC which could be because of the high sensitivity of HPLC technique as compared to HPTLC method.

b) Barlerin
The maximum content of barlerin was found in B. prionitis followed by B. lupulina and minimum in B. cristata var. dichotoma at 1.46, 1.10 and 0.14 per cent respectively.

c) Shanzhiside methyl ester
The maximum content of shanzhiside methyl ester was found in B. prionitis followed by B. cristata var. dichotoma and minimum in B. lupulina at 4.27, 3.04 and 0.82 per cent respectively. The value of per cent content of shanzhiside methyl ester in B. prionitis and B. cristata var. dichotoma was on the higher side as compared to their per cent content in HPTLC which could be because of some interfering compounds.

5.3.2.1.4. Whole plant

a) Acetyl barlerin
The maximum content of acetyl barlerin was found in whole plant of B. lupulina followed by B. prionitis and minimum in B. cristata var. dichotoma at 1.57, 0.81 and 0.12 per cent respectively. Acetyl barlerin was not detected in whole plant of B. cristata (Pink flower variety) and B. cristata (Blue flower variety).

In spite of getting sharp peaks in chromatogram, the interfering compounds could not get separated in case of B. prionitis and B. cristata var. dichotoma. Hence, the content of acetyl barlerin in whole plant of these two species was found to be more than the other plant parts which in true sense should not exceed.
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b) Barlerin
The content of barlerin was found maximum in *B. lupulina* followed by *B. prionitis*, and minimum in *B. cristata* var. *dichotoma* at 2.20, 1.69 and 0.08 per cent respectively whereas in case of *B. cristata* var. blue flower and *B. cristata* var. pink flower, compounds having different spectra were eluted at the same Rt.

Despite getting the sharp peaks of barlerin in chromatogram, the interfering compounds could not get separated in case of whole plant of all *Barleria* specie/varieties except for *B. cristata* var. dichotoma where the underlying component had resolved. The content of barlerin in whole plant of *B. lupulina* was found to be more than the other plant parts.

c) Shanzhiside methyl ester
The maximum content of shanzhiside methyl ester was found in *B. cristata* var. *dichotoma*, followed by *B. lupulina* and minimum in *B. prionitis* at 1.32, 1.24 and 1.01 per cent respectively.

In the chromatograms of whole plant of *B. cristata* var. *dichotoma* and *B. lupulina*, the interfering compounds could not get resolved despite our several efforts. Therefore, the content of shanzhiside methyl ester in whole plant of *B. lupulina* was found to more than the other plant parts which in real sense should not be higher. In case of *B. cristata* var. *dichotoma*, the content was on the higher side possibly because of the interference of other compounds.

5.3.2.2. Estimation of shanzhiside esters in different extracts of *B. prionitis* and *B. cristata* var. *dichotoma*

Different extracts *viz.*, methanol, hexane, chloroform, ethyl acetate and butanol prepared for *B. prionitis* and *B. cristata* var. *dichotoma* for HPTLC, were also used for HPLC analysis.

a) Acetyl barlerin
The maximum content of acetyl barlerin was found in ethyl acetate extract of *B. prionitis* followed by butanol and methanol extract of the same specie at 4.87, 1.81 and 0.81 per cent respectively. In the case of chloroform extract, content of acetyl barlerin was 0.66 per cent in *B. prionitis* while it was not detected in
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B. cristata var. dichotoma. However, it was present at 0.12 per cent in methanol extract of B. cristata var. dichotoma. It was found to be absent in hexane extract of B. cristata var. dichotoma and was detected in B. prionitis with 0.07 per cent content. Acetyl barlerin in ethyl acetate extract of B. cristata var. dichotoma was not detected whereas in butanol extract it was found to be 0.01 per cent. The content was found to be higher in methanol extract of B. cristata var. dichotoma than in different partitioned fractions due to interference of other compounds.

b) Barlerin
The maximum content of barlerin was found in butanol extract of B. prionitis at 3.01 per cent followed by ethyl acetate and methanol extract of the same species at 1.75 and 1.69 per cent respectively. Barlerin per cent content in butanol extract of B. cristata var. dichotoma was found to be 0.25. This is closely followed by chloroform extract of B. prionitis with 0.21 per cent. The minimum content was detected in hexane extract of B. prionitis at 0.08 per cent.

c) Shanzhiside methyl ester
The maximum content of shanzhiside methyl ester was found in butanol extract of B. prionitis at a level of 1.57 per cent followed by methanol extract of B. cristata var. dichotoma at 1.32 per cent. It was absent in hexane and chloroform extract of both the species. The percent content in methanol extract of B. prionitis was 1.01 while it was found to be present in butanol extract of B. cristata var. dichotoma with 0.94 per cent content. The percent content in ethyl acetate extract of B. prionitis was 0.17 while it was absent in B. cristata var. dichotoma. The content of shanzhiside methyl ester in methanol extract of B. cristata var. dichotoma was found to be higher than the different partitioned fractions. This may be due to the combined area of the interfering peaks which has given higher content.
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5.3.3. Ultra Performance Liquid Chromatography (UPLC)

The ACQUITY UPLC system gives higher chromatographic resolution, sensitivity and greater throughput using sub-2-µm particle technology. It eliminates significant time and cost per sample from analytical process while improving the quality of results. By outperforming traditional or optimized HPLC using 5 µm particle technology, the system allows chromatographers to work at higher efficiencies with a much wider range of linear velocities, flow rates, and backpressures.

In HPLC analysis, the separation of underlying or interfering components was difficult in few samples. With an aim to have better resolution of all the three markers, another attempt with the more sensitive, sophisticated and advanced technique was made. With the help of selected gradient system of HPLC, different combinations of solvents were tried as mentioned in section 4.1.4.4.2. Finally, highly robust and reproducible gradient method with following specification was developed to get quick analysis and better resolution.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow rate (ml/min)</th>
<th>Water : Acetonitrile : Methanol</th>
<th>Curve</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.4</td>
<td>65 : 1.0 : 34</td>
<td>2</td>
</tr>
<tr>
<td>0.43</td>
<td>0.4</td>
<td>50 : 1.0 : 49</td>
<td>2</td>
</tr>
<tr>
<td>0.85</td>
<td>0.4</td>
<td>50 : 1.0 : 49</td>
<td>2</td>
</tr>
<tr>
<td>1.28</td>
<td>0.4</td>
<td>65 : 1.0 : 34</td>
<td>2</td>
</tr>
<tr>
<td>2.55</td>
<td>0.4</td>
<td>65 : 1.0 : 34</td>
<td>2</td>
</tr>
</tbody>
</table>

The developed method was used to quantify the three shanzhiside esters in different extracts of the selected all Barleria species/varieties. Under the experimental conditions employed, the minimum amount of markers that could be detected was found to be 0.14 ng for acetyl barlerin, 0.15 ng for barlerin and 0.13 ng for shanzhiside methyl ester. The lowest quantifiable amount of markers was 0.59 ng/spot for acetyl barlerin, 0.59 ng/spot for barlerin and 0.51 ng/spot for shanzhiside methyl ester (table 81 & 124). The method was confirmed to be particularly specific and was linear in a concentration range of 0.55-141 ng/spot for acetyl barlerin, 0.59-150 ng/spot for barlerin and 0.51-130 ng/spot for shanzhiside methyl ester (n=6), with respect to peak area. The linear regression data shown in table 82 & 124 revealed a good linear relationship over the concentration range.
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studied demonstrating its suitability for analysis. The developed method was precise, specific and accurate for the determination of shanzhiside methyl ester iridoids (table 83, 84 & 124). Statistical evaluation of the results was performed with respect to accuracy and precision which showed low % RSD value indicating the suitability of this method for the analysis of shanzhiside methyl esters. Thus this validated UPLC method was used as a rapid method of analysis for the determination of shanzhiside iridoids viz., acetyl barlerin, barlerin and shanzhiside methyl ester in at least 5 different Barleria species and varieties viz., B. prionitis, B. lupulina, B. cristata var. dichotoma, B. cristata (Pink flower variety) and B. cristata (Blue flower variety) (table 123). The resulting chromatograms were analyzed carefully to generate the findings which are discussed below.

5.3.3.1. Estimation of shanzhiside esters in different plant parts of B. prionitis, B. cristata and B. lupulina

5.3.3.1.1. Stem

a) Acetyl barlerin
The per cent content of acetyl barlerin in stem of B. lupulina was 0.17 and it was absent in B. prionitis and B. cristata var. dichotoma.

b) Barlerin
The maximum content of barlerin was found in stem of B. prionitis followed by B. lupulina and minimum in B. cristata var. dichotoma at 1.25, 0.57 and 0.02 per cent respectively.

c) Shanzhiside methyl ester
The maximum content of shanzhiside methyl ester was found in stem of B. prionitis followed by B. cristata var. dichotoma and B. lupulina at 1.52, 0.38 and 0.19 per cent respectively.

5.3.3.1.2. Root

a) Acetyl barlerin
The content of acetyl barlerin found in roots of B. lupulina was 0.38 per cent respectively. It was not detected in roots of B. prionitis and B. cristata var. dichotoma.
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b) Barlerin
The maximum content of barlerin was found in roots of *B. prionitis* followed by *B. lupulina* at 2.53 and 0.43 per cent respectively. It was absent in *B. cristata* var. *dichotoma*.

c) Shanzhiside methyl ester
The maximum content of shanzhiside methyl ester was found in roots of *B. prionitis* followed by *B. cristata* var. *dichotoma* and *B. lupulina* and the per cent content was 1.64, 0.13 and 0.10 respectively.

5.3.3.1.3. Leaf

a) Acetyl barlerin
The maximum content of acetyl barlerin was found in *B. lupulina* at 2.65 per cent followed by minimum content of *B. prionitis* and *B. cristata* var. *dichotoma* with 0.09 and 0.05 per cent respectively.

b) Barlerin
The maximum content of barlerin was found in *B. cristata* var. *dichotoma* followed by *B. lupulina* and minimum in *B. prionitis* at 1.13, 1.06 and 0.98 per cent respectively.

c) Shanzhiside methyl ester
The maximum content of shanzhiside methyl ester was found in *B. prionitis* followed by *B. cristata* var. *dichotoma* and minimum in *B. lupulina* at 2.78, 1.93 and 0.75 per cent respectively.

5.3.3.1.4. Whole plant

a) Acetyl barlerin
Despite our several efforts, the compound interfering with acetyl barlerin could not get separated in the whole plant of *B. prionitis* and *B. cristata* Linn. var. *dichotoma*.

The maximum content of acetyl barlerin was found in whole plant of *B. lupulina* followed by *B. prionitis* and minimum in *B. cristata* var. *dichotoma* at 0.71, 0.30 and 0.07 per cent respectively. Acetyl barlerin was not detected in whole plant of *B. cristata* (Pink flower variety) and *B. cristata* (Blue flower variety). In case of
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*B. cristata var. dichotoma*, the amount in whole plant was found to be little higher than its leaf content which possibly could be because of some other underlying component.

**b) Barlerin**
The attempt using UPLC technique was not successful in resolving the interfering component in case of *B. lupulina*, where the per cent content in whole plant was found to be higher than in other plant parts. The content of barlerin was found maximum in whole plant of *B. lupulina* followed by *B. prionitis*, and minimum in *B. cristata var. dichotoma* at 1.55, 1.16 and 0.21 per cent respectively. However barlerin was not detected in whole plant of *B. cristata* (Blue and Pink flower variety) where as in whole plant of *B. cristata* (Pink flower variety) another compound of different spectra was eluted at the same Rt. The content of barlerin in *B. lupulina* was found to be more in whole plant than in different plant parts because of the underlying compounds which could not get resolved from the barlerin peak.

**c) Shanzhiside methyl ester**
The maximum content of shanzhiside methyl ester was found in *B. lupulina*. This was followed by *B. cristata var. dichotoma* and *B. prionitis* with 0.70, 0.66 and 0.63 per cent respectively. In case of *B. cristata* (Blue flower and Pink variety) different compound peaks appeared at the same Rt.

5.3.3.2. Estimation of shanzhiside esters in different extracts of *B. prionitis* and *B. cristata var. dichotoma*

**a) Acetyl barlerin**
The maximum content of acetyl barlerin was found in ethyl acetate extract of *B. prionitis* followed by butanol and methanol/mother extract of the same plant and then of *B. cristata var. dichotoma* at 1.46, 0.43, 0.30 and 0.24 per cent respectively. The content in case of chloroform and hexane extract of *B. prionitis* was 0.08 and 0.07 per cent respectively. However acetyl barlerin was found to be absent in hexane, chloroform and ethyl acetate extract of *B. cristata* var. 365
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dichotoma. The content was found to be 0.07 per cent in case of methanol extract of B. cristata var. dichotoma.

b) Barlerin
The highest content of barlerin was found in butanol extract of B. prionitis at 1.48 per cent. This was followed by methanol and ethyl acetate extract of the same plant at 1.16 and 0.99 per cent content respectively. In the case of butanol and methanol extract of B. cristata var. dichotoma, content was found to be 0.97 and 0.21 per cent respectively. However it was absent in other three extracts viz., hexane, chloroform and ethyl acetate of B. cristata var. dichotoma. Barlerin per cent content in chloroform and hexane extract of B. prionitis was 0.08 and 0.02 respectively.

c) Shanzhiside methyl ester
The highest content of shanzhiside methyl ester was found in ethyl acetate extract of B. prionitis at 0.85 per cent whereas it was not detected in B. cristata var. dichotoma. This was followed by butanol extract of B. prionitis and methanol extract of B. cristata var. dichotoma at level of 0.69 and 0.66 per cent respectively. It was absent in hexane and chloroform extract of both the species. The per cent content in methanol extract of B. prionitis was found to be 0.63 and the minimum amount was found in butanol extract of B. cristata var. dichotoma at 0.38 per cent. Despite our efforts, the attempt was not successful in resolving the interfering compound in B. cristata var. dichotoma. As a result the content of shanzhiside methyl ester in mother/methanol extract was found to be on higher side as compared to the partitioned fractions. However, shanzhiside methyl ester was not detected in whole plant of B. cristata (Pink and Blue flower variety) and another compound was eluted at the same Rt.

5.3.4. Comparison of HPTLC, HPLC and UPLC Methods of Analysis of Shanzhiside Markers
Three different analytical techniques were used in the present study for quantitative analysis of shanzhiside esters. An attempt was made to develop simple, sensitive, selective, precise, robust, rapid and reliable method of analysis
for simultaneous determination of acetyl barlerin, barlerin and shanzhiside methyl ester (the major iridoids of Barleria). The validated method of analysis was developed for HPTLC/HPLC/UPLC as per ICH guidelines. However, the best results in terms of accuracy and reproducibility came from HPTLC. Although HPLC and UPLC are more sensitive techniques than HPTLC, but they could resolve the desired markers only in few samples and gave unexpectedly very high contents of acetyl barlerin, barlerin and shanzhiside methyl ester in other samples due to interference of other components eluting at same Rt but having different spectra. From these results it is concluded that for simultaneous determination of acetyl barlerin, barlerin and shanzhiside methyl ester, HPTLC is the best analytical method which otherwise also is the most simple and economical technique. However HPLC/UPLC can be effectively employed if determination of single iridoid is desired with good accuracy as both the techniques are extremely sensitive. Further, a critical analysis of observations and results also indicates that if some modification/improvements can be made in sample preparation, it is likely to get accurate, reliable and reproducible results with HPLC/UPLC as well.

5.4. BIOLOGICAL STUDIES
5.4.1. Toxicity Studies
5.4.1.1. Acute toxicity study (Up-and-Down procedure)
Toxicity studies were carried out as per OECD guidelines (2008). Acute toxicity was assessed in healthy adult female Sprague-Dawley rats by administering a single oral dose (limit dose) of the methanol extract of B. prionitis and observing the subjects for signs of toxicity over fourteen days period. Observations of interest included changes in behavioral, neurological, autonomic responses and body weight (table 92 & 93).

The methanolic extract suspended in 2 % v/v tween solution in normal saline was administered by gavage route to rats using a ball-tipped intubation needle (18 G). Five healthy female rats, acclimatized to laboratory conditions for five days prior to dosing, were used in this study. The rats were deprived of food for three to four hours before and after the administration of the test sample, but were given

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Continuous access to water during this period. A dose of 2000 mg/kg was selected as the test (limit) dose (figure 71). One animal was dosed at the test dose and as the animal survived, four additional animals were dosed sequentially so that a total of five animals are tested. Animals were observed individually at least once during the first 30 min after dosing, periodically during the first 24 h (with special attention given during the first 4 h), and daily thereafter, for a total of 14 days for changes in body weight (table 92, figure 106), skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behavioural pattern. Attention was directed to observations of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma (table 93). This study revealed non-significant (p < 0.05) signs of intoxication in the rats treated at the dose level of 2000 mg/kg when compared to control rats (table 93). All subjects survived the fourteen day study period. Gross as well as histopathological examination did not reveal any abnormalities in the test subject (table 93, figure 72 to 77). The AOT 425 statpgm test results and recommendations obtained from the acute oral toxicity (OECD test guidelines 425) are shown in figure 71.

Figure 106: Effect of limit dose (2000 mg/kg) of the methanol extract of B. prionitis on weight of rats on 14th day

The LD₅₀ of the methanolic extract was found to be greater than 2000 mg/kg (figure 71) and therefore it falls into Category 5 of the Globally Harmonised System (GHS) when administered orally in Sprague-Dawley rats. Category 5 includes those substances which are of relatively low acute toxicity and are anticipated to have oral or dermal LD₅₀ in the range of 2000-5000 mg/kg or equivalent doses for other routes.
5.4.2. Anti-inflammatory Studies Using Acute, Sub-acute and Topical Models of Inflammation

Barleria is a reputed plant of Ayurveda and enjoys high status for its versatile use in several ailments including inflammation. The genus is reported to contain iridoids, anthraquinones, flavonoids, sterols, and fatty acids.\(^{[101]}\) Iridoids constitute the major class of compounds isolated from Barleria and important bioactive iridoids are acetyl barlerin, barlerin and shanzhiside methyl ester.\(^{[102,103]}\) There is a continuing interest in iridoids as many of them have shown a host of biological and pharmacological activities. As Barleria is enriched in iridoids and reported to possess anti-inflammatory activity according to traditional claims, so the present study was planned to carry out the anti-inflammatory activity on various extracts of two most commonly grown Barleria species viz., B. cristata and B. prionitis. The biological evaluation of B. lupulina was not taken up because of very less quantity of the plant material and moreover its TLC profile was quite similar to B. prionitis. As B. cristata (Pink flower variety) and B. cristata (Blue flower variety) were found to be completely devoid of iridoid markers, so these two were also not proceeded for anti-inflammatory studies. Moreover, the studies done by previous workers had also shown poor biological activity in non-iridoid fractions of B. cristata and B. prionitis. Further to investigate the key molecules responsible for the activity, the three shanzhiside based iridoids (the major components of the plant) were also subjected to anti-inflammatory studies. It was also considered important to assess the anti-inflammatory activity in acute, sub-acute and topical models which in turn will enable us to assess the kind of inflammatory disorders against which the active extracts/compounds are likely to be effective.

5.4.2.1. Acute models of inflammation

5.4.2.1.1. Carrageenan induced paw oedema model

The anti-inflammatory activity of various extracts (viz., methanol, total aqueous, butanol, ethyl acetate, chloroform, hexane and left aqueous) of B. prionitis and B. cristata and isolated pure compounds were investigated in carrageenan induced paw edema model. The activity was evaluated at different dose levels of 100, 200 and 400 mg/kg for extracts and 0.1, 0.5, 1, 3 and 5 mg/kg for three pure isolates, using ibuprofen (50 mg/kg, p.o.) as the standard drug. Acute oedema was
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induced in the left hind paw of the rats by injecting freshly prepared carrageenan solution (1 % w/v) in normal saline which was measured using plethysmometer. The mean difference in paw volume was noted at 0, 1, 3, 6, 9, 24 h intervals and per cent protection was calculated to determine the level of activity of test drugs.

5.4.2.1.1. Anti-inflammatory activity of extracts of B. prionitis and B. cristata

B. prionitis: A definite and significant (p < 0.05) protection was observed with all active extracts except for left aqueous extract at 3, 6 and 9 h at 200 mg/kg of B. prionitis at different dose levels and various time intervals in carrageenan induced rat paw oedema model. The maximum activity during early phase of inflammation at 1h was shown by total aqueous extract of B. prionitis at 200 mg/kg dose with 72.97 per cent protection followed by ethyl acetate extract with 66.22 per cent protection at 400 mg/kg dose. Methanol, chloroform, butanol and left aqueous extracts of B. prionitis showed prominent response at 3 h. The best per cent protection of 73.83 was shown by 400 mg/kg dose of methanol extract, closely followed by butanol extract with 73.15 per cent reduction in oedema at 400 mg/kg dose. However, the maximum activity shown by chloroform extract was at 200 mg/kg with 64.43 per cent protection against carrageenan challenge and that of left aqueous extract at 400 mg/kg with 47.65 per cent inhibition of oedema. A gradual fall in protection of oedema was observed from 3 h onwards till 9 h. But interestingly, total aqueous, methanol and hexane extracts showed a reversal with increased activity at 100 mg/kg at 24 h with per cent protection of 39.33, 67.42 and 56.18 respectively (table 94 to 96, figure 107).
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Figure 107: Effect of various plant extracts of *B. prionitis* on carrageenan induced rat paw oedema

IBU = Ibuprofen, TAE = Total aqueous extract, ME = Methanol extract, HE = Hexane extract, CE = Chloroform extract, EAE = Ethyl acetate extract, BE = Butanol extract and LAE = Left aqueous extract. The % protection against paw oedema at all dose levels and time intervals is statistically significant at *p* < 0.05 except for LAE at 3, 6 and 9 h at 200 mg/kg.
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**B. cristata:** A definite and significant (p < 0.05) protection was observed with all active extracts except for butanol and left aqueous extract at 3, 6 and 9 h at 200 mg/kg of *B. cristata* at different dose levels and various time intervals in carrageenan induced rat paw oedema model. The maximum activity during the initial phase of inflammation was depicted by methanol extract at 400 mg/kg with 69.80 per cent protection. The two extracts viz., total aqueous and butanol exhibited nearly same per cent protection with significant (p < 0.05) anti-inflammatory activity. Total aqueous extract showed maximum activity at 6 h with 69.23 per cent protection at 100 mg/kg and butanol extract was found to be active at 400 mg/kg with 69.13 per cent inhibition of inflammation at 3 h. Further, both hexane and ethyl acetate extracts also showed promising results at 1 h with 62.16 and 59.45 per cent reduction in swelling, respectively, at 400 mg/kg dose. The chloroform and left aqueous extracts showed protection at 3 h with 55.70 (200 mg/kg) and 44.30 (400 mg/kg) per cent reduction in oedema at respective dose levels. Although both species of *Barleria* showed significant anti-inflammatory activity but the comparative overall activity profile of *B. cristata* was found to be less than *B. priotitis* (table 94 to 96, figure 108).

**Explanation:** The time course of the development of oedema after subplantar injection of carrageenan in the rat has been extensively studied and well reported.[133,134] A single sub-plantar injection of 1 % w/v carrageenan solution in normal saline produces a bi-phasic paw volume increase. The early phase I of inflammatory response plateauing at 0.5-1 h, early phase II at 3-6 h and late phase is 12 and 24 h. It has also been reported that significant oedema persisted 24 h after carrageenan administration and complete recovery is achieved in 7 days.[192] The prostaglandins (PGs) appear to play an important role in the development of carrageenan induced rat paw oedema. The activity of the prostaglandin E (PGE) type in oedema fluids squeezed out from the carrageenan inflamed rat paw has been demonstrated. The early expressed fluid contain histamine; later fluid show bradykinin activity but little PGE₂ (less than 5 ng/ml) is found until after 3 h. The concentration of PGE₂ rise to a plateau of around 80 ng/ml between 18 and 24 h. This late-phase rise of PGE₂ concentration is accompanied by a similar rise in histamine level, which reaches 1 μg/ml at 24 h.[192]
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Figure 108: Effect of various plant extracts of *B. cristata* on carrageenan induced rat paw oedema

IBU = Ibuprofen, TAE = Total aqueous extract, ME = Methanol extract, HE = Hexane extract, CE = Chloroform extract, EAE = Ethyl acetate extract, BE = Butanol extract and LAE = Left aqueous extract. The % protection against paw oedema at all dose levels and time intervals is statistically significant at p < 0.05 except for BE and LAE at 3, 6 and 9 h at 200 mg/kg.
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The carrageenan oedema can be inhibited by at least two mechanisms: inhibition of the primary mediators involved in the swelling or inhibition of the prostaglandin amplification mechanism. A test drug may exhibit one or the other inhibitory mechanisms or a combination of both. If the drug shows the first mechanism, its activity may or may not translate to man depending on the importance of the primary mediators in the human disease. Thus the finding of an activity profile in animals based on the first mechanism may be an additional reason (besides metabolism and species difference) for failure to find such activity in man. By contrast, the prostaglandin-dependent component may be surmised to be present in a number of human inflammatory diseases on the basis of the accuracy of the extrapolation of findings on this activity from the carrageenan oedema model to the clinic.\(^{[192]}\)

The activity profile generated by different extracts showed that majority of the test samples were active at 3 h and afterwards the activity either decreased or was almost the same except in few cases where reversal of fall in activity was observed. According to the general release mechanism of various mediators, it is known that the cellular fluid is enriched in histamine, bradykinin and less of prostaglandins during the early phase (till 6 h). This indicates their greater role in the inhibiting primary mediators and the inflammatory diseases associated with these mediators. However, the extracts showing enhanced protection during late phase showed inhibitory effect on prostaglandins indicating their likely role in arresting various inflammatory disorders of humans.

Thus from the generated anti-inflammatory pattern we can conclude that the different extracts of *Barleria* showing their maximum activity during the early or late phase may be of help to extrapolate the knowledge of various inflammatory disorders of humans and holds promise to be explored further. The findings clearly indicates that the extracts showing protection during both the phases have better promise to be investigated further.
5.4.2.1.1.2. Anti-inflammatory activity of pure shanzhiside esters of Barleria

The three shanzhiside iridoids namely acetyl barlerin, barlerin and shanzhiside methyl ester isolated from Barleria during present study were evaluated against carrageenan induced rat paw oedema at different dose levels. Acetyl barlerin and barlerin were tested at 0.1, 0.5 and 1 mg/kg, where as shanzhiside methyl ester was evaluated at 0.1, 0.5, 1, 3 and 5 mg/kg. Further, the data generated by the previous workers in our laboratory was of advantage where the evaluation was done at 1, 5 and 10 mg/kg dose levels and the best activity was shown by 1 mg/kg dose level. In order to confirm the established activity of 1 mg/kg, different dose levels at lower and upper side of this dose were selected in case of shanzhiside methyl ester. But in case of acetyl barlerin and barlerin, due to limited quantity, higher doses were not taken up.

Acetyl barlerin and barlerin showed significant dose dependent activity with maximum protection exhibited by 1 mg/kg of each iridoid at 1 h. Barlerin showed 84.38 per cent protection against phlogistic agent followed by acetyl barlerin with

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76.56 per cent reduction in inflammation. Shanzhiside methyl ester also showed dose dependent activity up to 1 mg/kg dose with maximum protection of 69 per cent at an early phase of inflammation. But at higher dose levels of 3 and 5 mg/kg, the activity got decreased. Interestingly, this iridoid showed reversal of activity at 24 h while other two isolates namely acetyl barlerin and barlerin did not follow the pattern shown by shanzhiside methyl ester (table 97, figure 109). The overall protection at all time intervals of 1, 3, 6, 9 and 24 h at various oral doses of each shanzhiside ester was significant (p < 0.05).

Explanation: The underlying bi-phasic mechanism of the paw oedema produced after sub-plantar injection of 1 % w/v carrageenan solution in normal saline has been already discussed under section 5.4.2.1.1.1. Further, the findings of the study done on pure compounds clearly indicates their role in decreasing the levels of mediators in the early as well as late phase. Acetyl barlerin and barlerin are effective in the early phase only whereas shanzhiside methyl ester was found to reduce inflammation during both early and late phases caused by various mediators like histamine, bradykinin and PGE₂. Thus the study may be helpful to correlate the therapeutic efficacy of these iridoids in various inflammatory disorders of humans.

As discussed under the mechanism of carrageenan action, it is well known that various mediators like histamine, bradykinin, prostaglandins etc are involved in the acute inflammation. Thus to confirm our findings, the response of pure iridoids against different mediators viz., histamine and dextran was also studied. The pattern of the inflammation was examined carefully and the anti-inflammatory data of different extracts and pure compounds was generated. Additionally a detailed study on different extracts and pure compounds was also taken up using sub-acute and topical models of inflammation. This was done with an aim to study their protective role in inflammatory disorder which are of sub-acute nature and takes longer period to develop than acute inflammatory disorders. Further, the role of test samples under study in inflammatory disorders which require topical application was also investigated.
5.4.2.1.2. Evaluation of anti-inflammatory activity with different standards
To understand the mechanism of action of the active extracts and pure compounds as anti-inflammatory agents, four different standard anti-inflammatory drugs were used in carrageenan induced paw oedema model and the results were compared with activity shown by extracts/pure compounds. To take account of all the possible mechanisms of action, different standards hence selected had different mechanism of anti-inflammatory action. These standards were ibuprofen (50 mg/kg), celecoxib (50 mg/kg), nimesulide (50 mg/kg) and dexamethasone (1 mg/kg) where, ibuprofen (a non-selective COX inhibitor), celecoxib (a selective COX-2 inhibitor) and nimesulide (preferential COX-2 inhibitor) were non-steroidal anti-inflammatory drugs and dexamethasone had a steroidal pathway to inhibit inflammation.

![Graph](image)

**Figure 110:** Effect of different standards at respective dose levels on carrageenan induced inflammation
All doses in mg/kg. **CELE** = Celecoxib, **DEXA** = Dexamethasone, **IBU** = Ibuprofen and **NIMU** = Nimesulide. The % protection against paw oedema of all standards at different time intervals is statistically significant at p < 0.05

**Ibuprofen**, showed a definite protection from 60 min to 24 h. The maximum protection of 63.76 per cent (table 96, figure 110) was shown at 3 h followed closely by 60 per cent protection at 6 h. The delayed protection at 24 h was comparatively lower (48.31 %). The overall protection at all time intervals of 1, 3, 6, 9 and 24 h at 50 mg/kg oral dose was significant (p < 0.05). The results are shown in table 98, figure 110.
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Ibuprofen is a nonselective COX inhibitor; that is, it inhibits two isoforms of cyclooxygenase, COX-1 and COX-2. The analgesic, antipyretic, and anti-inflammatory activity of this NSAID appears to be achieved mainly through inhibition of COX-2, whereas inhibition of COX-1 would be responsible for unwanted effects on platelet aggregation and the gastrointestinal tract. However, the role of the individual COX isoforms in the analgesic, anti-inflammatory, and gastric damage effects of NSAID is uncertain.

**Celecoxib**, a selective COX-2 inhibitor showed a very definite protection from 60 min to 24 h. Of all the standards, celecoxib exhibited the maximum protection of 89.93 per cent at 3 h. This was closely followed by 86.15 per cent protection at 6 h and a significant delayed protection of 83.14 per cent at 24 h. The overall protection at all the time intervals was highly significant (p < 0.05). The results are shown in table 98, figure 110.

**Nimesulide**, a preferential COX-2 inhibitor also exhibited definite protection from 60 min to 24 h. The maximum protection by nimesulide at 3 h closely followed the level of celecoxib with a marginal increase at 6 h (81.87 and 86.15 per cent respectively). The protection remained static at 83.14 per cent during delayed period of 24 h. The overall protection at all the time intervals was highly significant (p < 0.05) as shown in table 98, figure 110.

**Dexamethasone**, an anti-inflammatory agent which acts by blocking the steroidal pathway again showed a definite protection from 60 min to 24 h. The maximum protection of 85.23 per cent shown at 3 h was very close to celecoxib and remained almost constant at 84.62 per cent level till 6 h and was down to 75.28 per cent at 24 h. The overall protection at 1, 3, 6, 9 and 24 h was highly significant (p < 0.05). The results are shown in table 98, figure 110.

**Explanation:** The critical insight view of the pattern followed by different standards *viz.*, ibuprofen, celecoxib, nimesulide and dexamethasone gave some indication regarding the close proximity of mechanism of anti-inflammatory action between test and standard samples. The pattern followed by extracts and pure compounds was more like Ibuprofen which clearly indicates that these test samples follow the
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path of non-selective COX inhibitors. But there is no reversal pattern followed by ibuprofen whereas reversal of fall in anti-inflammatory activity was seen in case of few extracts and shanzhisi methyl ester. The activity shown at 24 h cannot be explained by ibuprofen activity profile and this needs to be explored further.

5.4.2.1.3. Mediators (histamine, dextran) induced inflammation in rats

5.4.2.1.3.1. Histamine induced paw oedema model

The anti-inflammatory activity of extracts viz., total aqueous, methanol and butanol of B. cristata and B. prionitis showing maximum activity in carrageenan induced paw oedema model were further investigated in mediators (histamine, dextran) induced inflammation at three dose levels of 100, 200 and 400 mg/kg. However, iridoids were tested at a single dose level of 1 mg/kg as amongst the different tested doses of 0.1, 0.5, 1, 3 and 5 mg/kg evaluated in carrageenan induced paw oedema model, the 1 mg/kg dose had shown the best activity.

Acute oedema was induced in the left hind paw of the rats by injecting freshly prepared histamine solution (1 % w/v) in normal saline and was measured using plethysmometer. The mean difference in paw volume was noted at 0 and 1 h intervals as the effect of histamine lasts for 1-2 h. The per cent protection was calculated to determine the level of activity of test drug.

![Figure 111: Effect of different plant extracts of B. prionitis and B. cristata on histamine induced inflammation](image)

All doses in mg/kg. IBU = Ibuprofen, TAE = Total aqueous extract, ME = Methanol extract, HE = Hexane extract, CE = Chloroform extract, EAE = Ethyl acetate extract, BE = Butanol extract and LAE = Left aqueous extract. The % protection against paw oedema at all dose levels is statistically significant at p < 0.05
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Figure 112: Effect of shanzhiside esters on histamine induced inflammation

IBU = Ibuprofen, AB = Acetyl barlerin, B = Barlerin and SME = Shanzhiside methyl ester. The % protection against paw oedema is statistically significant at p < 0.05 at 1 mg/kg dose level

In histamine induced paw oedema, methanolic extract of *B. prionitis* produced 63.16 per cent inhibition at a dose of 200 mg/kg body weight which was very close to 65.79 per cent inhibition shown by standard drug ibuprofen. The ameliorating effect of *B. cristata* was found to be marginally lower than *B. prionitis* with 60.53 per cent protection (table 99, figure 111). In case of pure compounds, barlerin showed maximum protection of 67.20 per cent followed by acetyl barlerin and shanzhiside methyl ester with 63.49 and 60.32 per cent inhibition respectively (table 100, figure 112). The overall protection of extracts of both the species at 100, 200 and 400 mg/kg, and pure compounds at 1 mg/kg at time interval of 1 h was statistically significant at p < 0.05.

Explanation: The reduction in inflammation shown by extracts and pure compounds further substantiate our earlier findings and indicates promising activity against inflammations caused by various agents.

5.4.2.1.3.2. Dextran induced paw oedema model

Acute swelling was induced in the left hind paw of the rats by injecting freshly prepared dextran solution (6 % w/v) in normal saline which was measured using plethysmometer. The mean difference in paw volume was noted at 0 and 1 h intervals as the effect of dextran lasts for 1-2 h. The per cent protection was calculated to determine the level of activity of test drug.
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Figure 113: Effect of different plant extracts of *B. prionitis* and *B. cristata* on dextran induced inflammation

All doses in mg/kg. IBU = Ibuprofen, TAE = Total aqueous extract, ME = Methanol extract, HE = Hexane extract, CE = Chloroform extract, EAE = Ethyl acetate extract, BE = Butanol extract and LAE = Left aqueous extract. The % protection against paw oedema at all dose levels is statistically significant at p<0.05.

In case of dextran induced paw oedema, methanol extract of *B. prionitis* showed more promising results than *B. cristata* with 61.76 and 55.88 per cent inhibition respectively at a dose of 200 mg/kg body weight. The activity of methanol extract of *B. prionitis* was very close to standard drug ibuprofen (table 101, figure 113) which showed 63.24 per cent inhibition of oedema. The per cent inhibition of oedema of *B. cristata* was found to be significantly lower than the standard. In case of pure compounds, barlerin showed maximum protection of 65.41 per cent which was better than ibuprofen. It was followed by acetyl barlerin and shanzhiside methyl ester with 60.76 and 59.88 per cent inhibition of oedema respectively at...
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1 mg/kg dose. The study was made over a period of 1 h as the swelling gets drastically reduced afterwards (table 102, figure 114). The overall protection of extracts of both the species at 100, 200 and 400 mg/kg, and pure compounds at 1 mg/kg at time interval of 1 h was statistically significant at \( p < 0.05 \).

**Explanation:** The anti-inflammatory activity shown by extracts and pure compounds in dextran model further confirms their ameliorating effect in reducing acute inflammation.

**5.4.2.2. Sub-acute model of inflammation**

The anti-inflammatory activity of the extracts/pure compounds was next evaluated using sub-acute model of inflammation with an aim to further evaluate their activity against proliferative connective granuloma mass formed in sub-acute inflammation. The cotton pellet induced granuloma method is an established inflammatory model for this purpose.

**5.4.2.2.1. Cotton pellet induced granuloma model**

Foreign body granulomas were provoked in rats by subcutaneous implantation of compressed cotton pellets (20 ± 1 mg). The animals were treated with standard/test drug for 7 days orally. On 8th day, the animals were sacrificed, pellets removed and wet and dry weight of cotton pellet were determined as described under section 4.1.5.2.5.1.1. of materials and methods. The per cent change of granuloma weight relative to the control group was determined to evaluate the level of activity of the formulation.

In this model, the best activity was shown by methanol extract of both the species at 400 mg/kg with 42.04 and 37.90 per cent inhibition for *B. prionitis* and *B. cristata*, respectively, on wet basis as compared to ibuprofen which exhibited 57.92 per cent protection against granuloma formation. However, the total aqueous extract of both the species showed activity at lower dose of 100 mg/kg which was 40.06 (for *B. prionitis*) and 35.47 per cent (for *B. cristata*). This was followed by butanol (38.15 per cent at 200 mg/kg), ethyl acetate (35.31 per cent at 200 mg/kg), left aqueous (34.05 per cent at 200 mg/kg), hexane (21.35 per cent
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per cent at 400 mg/kg) and chloroform (20.17 per cent at 200 mg/kg) of B. prionitis. In case of B. cristata, the pattern of activity was same as B. prionitis with difference in their level of inhibition: butanol (34.09 per cent at 200 mg/kg) > ethyl acetate (21.63 per cent at 200 mg/kg) > left aqueous (20.25 per cent at 200 mg/kg) > hexane (19.07 per cent at 400 mg/kg) > chloroform (18.67 per cent at 200 mg/kg) (table 103, figure 115). The overall protection was found significant with p < 0.05 at 100, 200 and 400 mg/kg on wet basis.

Figure 115: Effect of various plant extracts of B. prionitis and B. cristata on pellet induced wet granuloma

All doses in mg/kg. IBU = Ibuprofen, TAE = Total aqueous extract, ME = Methanol extract, HE = Hexane extract, CE = Chloroform extract, EAE = Ethyl acetate extract, BE = Butanol extract and LAE = Lact solvent extract. The % protection against wet granuloma formation of all extracts at different dose levels is significant at p < 0.05

Figure 116: Effect of shanzhiside esters on cotton pellet induced wet granuloma

IBU = Ibuprofen, AB = Acetyl barlerin, B = Barlerin and SME = Shanzhiside methyl ester. The % protection against wet granuloma formation of pure compounds is statistically significant at p < 0.05 at 1 r level.
Results and Discussion

Pure compounds were also evaluated in cotton pellet induced granuloma model. Barlerin showed 34.04 per cent inhibition of the proliferative mass as compared to ibuprofen which showed 56.39 per cent protection. This was followed by acetyl barlerin and shanzhiside methyl ester with 30.10 and 23.19 per cent protection respectively against the formed giant mass respectively at a single dose level of 1 mg/kg on wet basis (table 104, figure 116). The anti-inflammatory activity was found to be significant at p < 0.05 on wet basis.

Figure 117: Effect of various plant extracts of B. prionitis and B. cristata on cotton pellet induced dry granuloma

All doses in mg/kg. IBU = Ibuprofen, TAE = Total aqueous extract, ME = Methanol extract, HE = Hexane extract, CE = Chloroform extract, EAE = Ethyl acetate extract, BE = Butanol extract and LAE = Left aqueous extract. The % protection against dry granuloma formation of various extracts at different dose levels is statistically significant at p < 0.05 except for HE at 100, 200 mg/kg for BP and BC, TAE at 200, 400 mg/kg for BC, 400 mg/kg for BP and LAE at 100 and 200 mg/kg

On dry weight basis, the maximum activity was exhibited by methanol extract of both the species at 400 mg/kg having 40.52 and 36.68 per cent inhibition for B. prionitis and B. cristata respectively as compared to ibuprofen which depicted 40.70 per cent protection against proliferative mass. The high difference in value of % decrease in wet weight of granuloma from 57.92 to 40.70 % decrease in dry weight of granuloma of ibuprofen was observed whereas it was not found in other test samples and no reasons of such drop in value has been found in literature. However, the total aqueous extract of both the species showed their potential at lower dose of 100 mg/kg with 33.92 (for B. prionitis) and 30.91 per cent (for B. cristata). This was followed by butanol (31.90 per cent at 200 mg/kg), ethyl
acetate (31.66 per cent at 200 mg/kg), left aqueous (28.89 per cent at 200 mg/kg)
chloroform (17.58 per cent at 200 mg/kg) and hexane extract (16.58 per cent at 200 mg/kg) for B. prionitis. In case of B. cristata, the sequence of extracts showing their inhibition potential was as follows: butanol (30.39 per cent at 200 mg/kg) ethyl acetate (19.60 per cent at 200 mg/kg) > left aqueous (16.33 per cent at 40 mg/kg) > chloroform (15.57 per cent at 200 mg/kg) > hexane extract (14.82 per cent at 400 mg/kg) (table 103, figure 117).

Figure 118: Effect of shanzhiside esters on cotton pellet induced dry granuloma

Amongst pure compounds, barlerin showed its best activity with 29.09 per cent protection against the granuloma formation on dry weight basis as compared to ibuprofen which showed 38.93 per cent protection. This was followed by acetyl barlerin and shanzhiside methyl ester with 25.99 and 19.13 per cent protection respectively at a single tested dose level of 1 mg/kg (table 104, figure 118). The activity was found to be significant at $p < 0.05$ on dry basis.

Figure 119: Effect of different extracts and shanzhiside esters on serum nitrite/nitrate level

Amongst pure compounds, barlerin showed its best activity with 29.09 per cent protection against the granuloma formation on dry weight basis as compared to ibuprofen which showed 38.93 per cent protection. This was followed by acetyl barlerin and shanzhiside methyl ester with 25.99 and 19.13 per cent protection respectively at a single tested dose level of 1 mg/kg (table 104, figure 118). The activity was found to be significant at $p < 0.05$ on dry basis.
Results and Discussion

After sub-acute induction of cotton pellet induced granuloma, a marked increase in serum nitrate/nitrite level was noted in control. The rise in level is an indicator of the enhanced activity of iNOS enzyme. This further leads to release of inflammatory mediator NO which raises nitrate/nitrite level. In our study, methanol extract significantly (p<0.05) decreased nitrite/nitrate level to 2.03 μM in comparison to ibuprofen which decreased it up to 1.65 μM. This was followed by significant (p<0.05) activity of butanol extract (2.19 μM) and barlerin (2.25 μM). Acetyl barlerin (2.46 μM), total aqueous extract (2.67 μM) and shanzhiside methyl ester (2.79 μM) however, exhibited non significant activity (table 105, figure 119).

Explanation: The cotton pellet induced granuloma has been widely used to evaluate the transudative, exudative and proliferative components of inflammation. The wet and dried weight of the pellets correlates well with the amount of fluid adsorbed and granulomatous tissue formed respectively. In this model, inflammation is induced due to implantation of the foreign body. Sub-acute inflammation is a reaction arising when acute response is insufficient to eliminate pro-inflammatory agents. It induces a proliferation of fibroblasts and the infiltration of neutrophils and exudation and occurs by the development of proliferative cells. The inflammatory cascade further involves the induction of iNOS which leads to formation of NO and raised levels of nitrate/nitrite. This causes vasodilation, increased vascular permeability and oedema formation. It is proposed that the active extracts and pure isolated compounds may have caused a decrease in granuloma tissue formation by inhibiting proliferation of fibroblasts and granulocyte infiltration possibly by attenuating iNOS activity.[179,180]

5.4.2.3. Topical model of inflammation

The topical model of inflammation gives an indication of the myeloperoxidase (MPO) enzyme activity which is the marker of inflammatory reaction. This model is used to find the antiphlogistic activity of the substance. Further, the significant topical anti-inflammatory activity shown by the substance can be explored to formulate topical preparation with low toxicity and better therapeutic index. With this viewpoint, the present study was planned to assess the topical anti-
Results and Discussion

Inflammatory activity of pure compounds/extracts of both the species using croton oil ear oedema model.

5.4.2.3.1. Croton oil induced ear oedema model
The croton oil solution, standard drug solution and test sample solutions were prepared as described under section 4.1.5.2.6. A 40 μl of croton oil solution was applied to right ear of each animal of control group. Similarly, the same volume of standard drug solution and test drug (extracts/pure compounds) solution was applied to the respective groups. For effective test drugs comparison, the solution of ibuprofen was prepared by suspending 0.3 g of powdered ibuprofen in the working croton oil solution, at a concentration of 300 mg/kg. The working solution was then made for application to the right ear (12 mg/40 μl per ear). The left ear of each animal remained untreated. The solution was applied under ethane anaesthesia. Six hours after the application the animals were sacrificed. Both ears were excised with the help of sharp scissors and weighed separately on an analytical balance. The weight difference between the treated and untreated ears hence recorded indicated the degree of inflammatory oedema. Per cent increase in ear weight of each animal in a group was calculated. In a similar way per cent decrease in ear oedema was calculated due to topical application of test standard samples as compared to control group.

![Figure 120: Effect of the different extracts of B. prionitis and B. cristata on croton oil induced ear edema](image)

All doses in mg/ml. TAE = Total aqueous extract, ME = Methanol extract, HE = Hexane extract, CE = Chloroform extract, EAE = Ethyl acetate extract, BE = Butanol extract and IBU = Ibuprofen. The inhibition of ear oedema of different extracts at different dose levels is statistically significant at p < 0.05.
Results and Discussion

All the tested extracts of both the species showed significant (p < 0.05) ear oedema reduction when compared to ibuprofen which exhibited 62.95 per cent protection. The chloroform extract of *B. prionitis* at a dose of 200 mg/ml showed highly significant (p < 0.05) inhibition of ear oedema at 88.31 per cent followed closely by same extract of *B. cristata* which exhibited 80.24 per cent protection at 200 mg/ml dose. A very close level of activity was shown by butanol extract of both the species at 79.92 and 79.35 per cent for *B. prionitis* and *B. cristata* respectively followed by methanol extract of *B. prionitis* (78.35 per cent). Total aqueous and hexane extract of *B. prionitis* showed protection of 75.79 and 75.30 per cent respectively whereas ethyl acetate extract was found to be active against the phlogistic agent with 69.83 per cent protection. In case of *B. cristata*, total aqueous extract exhibited 73.44 per cent inhibition followed by ethyl acetate and hexane extracts with 66.67 and 63.30 per cent inhibition respectively (table 106, figure 120).

![Figure 121: Effect of shanzhiside esters on croton oil induced ear edema](image)

The pure compounds, barlerin and acetyl barlerin also remarkably attenuated the ear oedema with 67.44 and 65.49 per cent which was in close proximity to the standard value. However, shanzhiside methyl ester was found to possess lower value of 57.11 per cent. All the compounds were tested at a single active dose of 1 mg/kg and were found to be statistically significant (p<0.05) when compared to control (table 107, figure 121).
Results and Discussion

Explanation: The absorption through skin is highly affected by the presence of intercellular stacked lipid bilayer. These hydrophobic layers provides hindrance to the compounds of high polarity and this supports our finding as chloroform extract showed more per cent decrease in ear oedema by disrupting the skin structure as compared to methanolic extracts and thus showed enhanced penetration. The chloroform extract mainly consists of steroids and triterpenoidal type of compounds and methanol extract is enriched in iridoids. The more activity exhibited by the chloroform extract might be due to the presence of steroidal components like β-sitosterol. Similarly, barlerin and acetyl barlerin showed more per cent protection against oedema as compared to the polar shanzhiside methyl ester which consists of more polar hydroxyl moieties.

Croton oil is a phlogistic agent extracted from Croton tiglium L., Euphorbiaceae, and it has an irritant and vesiculant effect on the skin. Croton oil contains phorbol esters, being the 12-O-tetradecanoylphorbol-13-acetate (TPA) the predominant phorbol ester. Topical application of croton oil or TPA promotes an acute inflammatory reaction triggered by protein kinase C (PKC) activation, which promotes an increase in the activity of phospholipase A2 (PLA2), prostaglandins, leukotrienes and several immune mediators such as cytokines and chemokines. Cellular infiltration represents an important feature in skin inflammation, and neutrophils are the predominant type of cells that infiltrate the area and causes an increase in expression of cyclooxygenase-2. MPO is an enzyme known to be a marker of neutrophil infiltration. Thus, inhibition of MPO activity can be used to indicate the presence of an anti-inflammatory reaction.\textsuperscript{1193,1941} Topical treatments with the different extracts or pure compounds showed remarkable anti-inflammatory activity, indicating that these compounds may influence cell migration during the inflammatory process. However, it is too early to propose a detailed mechanism through which the extract/compounds exert their topical anti-inflammatory activity. Barleria extracts and three isolated shanzhiside esters could be influencing one or more steps of the croton oil-induced inflammatory cascade, such as protein kinase C and phospholipase A2 activation, cyclooxygenase-2 induction, and cytokine production and release.
Results and Discussion

Conclusion: The anti-inflammatory data generated in the present study using various models/inflammogens/standard drugs clearly indicate a significant role of Barleria and shanzhiside esters in curing inflammations of various types. The iridoids of the plant have a promising potential to be developed as anti-inflammatory drugs after detailed investigations at molecular level.

5.4.3. Evaluation of Analgesic Activity Using Different Models

Inflammation in many disorders is associated with pain and anti-inflammatory drugs are often associated with either mild, moderate or strong analgesic activity. As marked anti-inflammatory activity has been shown by different extracts and isolated pure compounds of Barleria in various inflammatory models, so the next study was planned to evaluate their pain reducing potential. Hence, the analgesic activity of different extracts and pure compounds was determined using central (tail flick model) and peripheral (acetic acid induced writhing) model. It was evaluated in mice at three dose levels of 100, 200 and 400 mg/ml of extracts and 1 mg/ml of pure compounds.

5.4.3.1. Tail flick model (Central model of analgesic activity)

The tip of the mice was subjected to radiant source and the flicking time was recorded. The reaction time was noted at 5, 15, 30, 60 and 120 min after the drug administration as discussed in section 4.1.5.3.1. The percentage increase in reaction time (index of analgesia) was calculated at each time interval.
Results and Discussion

**Figure 122: Effect of extracts of *B. prionitis* on pain induced by heat in tail flick model**

PENTZ = Pentazocin, TAE = Total aqueous extract, ME = Methanol extract, BE = Butanol extract, EAE = Ethyl acetate extract, CE = Chloroform extract, HE = Hexane extract and LAE = Left aqueous extract. Data showing index of analgesia is statistically significant at \( p < 0.05 \) for:
- PENTZ at 30', 45', 60' and 120';
- TAE, ME and EAE at 30', 45', 60' and 120' at 100 mg/kg; TAE and ME at 30', TAE, ME and EA at 45', PENTZ, TAE, ME, BE, and EAE at 60' and 120' and HE at 120' at 200 mg/kg; BE at 45', ME, TAE, BE and EAE at 60' and ME and BE at 120' at 400 mg/kg.
Results and Discussion

Figure 123: Effect of extracts of *B. cristata* on pain induced by heat in tail flick model

PENTZ = Pentazocin, TAE = Total aqueous extract, ME = Methanol extract, BE = Butanol extract, EAE = Ethyl acetate extract, CE = Chloroform extract, HE = Hexane extract and LAE = Left aqueous extract.

Data showing index of analgesia is statistically significant at p < 0.05 for: PENTZ at 30', 45', 60' and 120'; TAE, ME and EAE at 30', 45', 60' and 120' and HE at 60' at 100 mg/kg; ME and EA at 45'; TAE, ME, BE, and EAE at 60' and 120' and HE at 120' at 200 mg/kg; ME, BE at 45'; TAE, BE and EAE at 60' and BE at 120' at 400 mg/kg.
Results and Discussion

The analgesic effect of both the species was nearly same. The methanolic extract of *B. prionitis* and *B. cristata* at a dose of 100 mg/kg showed 69.05 and 68.80 per cent protection in pain respectively after 1 h of oral administration. The total aqueous and ethyl acetate extracts of both the species also showed good protection. The per cent reduction in pain of total aqueous and ethyl acetate extracts for *B. prionitis* was 66.95 and 65.38 per cent and for *B. cristata*, it was 59.73 and 61.60 per cent respectively. However, extracts viz., butanol, hexane, chloroform and left aqueous of both the species showed almost same percentage reduction in pain. The order of activity of extracts for *B. prionitis* was as follows: butanol (54.40 per cent at 400 mg/kg after 2 h) > hexane (30.95 per cent at 200 mg/kg after 1 h) > chloroform (33.33 per cent at 400 mg/kg after 1 h) > left aqueous (18.18 per cent at 200 mg/kg after 2h). The pattern of activity depicted by extracts of *B. cristata* was as butanol (53.49 per cent at 400 mg/kg after 2 h) > hexane (31.06 per cent at 200 mg/kg after 1 h) > chloroform (30.40 per cent at 400 mg/kg after 1 h) > left aqueous (17.81 per cent at 200 mg/kg after 2 h) (table 108 to 110, figure 122 & 123). Data showing index of analgesia of extracts is statistically significant at p < 0.05 for: pentazocin at 30’, 45’, 60’ and 120’; total aqueous, methanol and ethyl acetate of both *B. prionitis* and *B. cristata* at 30’, 45’, 60’ and 120’ and hexane of BC at 60’ at 100 mg/kg; total aqueous and methanol of *B. prionitis* at 30’; total aqueous (*B. prionitis*), methanol (*B. prionitis* and *B. cristata*) and ethyl acetate (*B. prionitis* and *B. cristata*) at 45’; total aqueous, methanol, butanol, and ethyl acetate of both *B. prionitis* and *B. cristata* at 60’ and 120’ and hexane (*B. prionitis* and *B. cristata*) at 120’ at 200 mg/kg; methanol (*B. cristata*), butanol (*B. prionitis* and *B. cristata*) at 45’; methanol, total aqueous, butanol and ethyl acetate of both *B. prionitis* and *B. cristata* at 60’ and methanol (*B. prionitis*) and butanol (*B. prionitis* and *B. cristata*) at 120’ at 400 mg/kg.
Results and Discussion

Amongst the shanzhiside iridoids, shanzhiside methyl ester showed the peak protection of 45.78 per cent against pain, followed by the close values of barlerin and acetyl barlerin at 43.11 and 42.42 per cent respectively. All the three compounds at a single selected dose of 1 mg/kg (which had shown maximum anti-inflammatory activity) showed protection at 60 mins of their administration beyond which the activity diminished. The activity profile was profoundly low as compared to standard which showed 76.65 per cent reduction in pain after 1 h of administration, indicating that these compounds possess mild analgesic activity (table 111, figure 124). The activity was found to be statistically significant at p < 0.05 for pentazocin at 30’, 45’, 60’ and 120’; acetyl barlerin, barlerin and shanzhiside methyl ester at 60’ and acetyl barlerin and shanzhiside methyl ester at 120’.

Explanation: The various endogenous opioid peptide precursor molecules are present in the brain sites that have been implicated in the pain modulation and are activated during stress such as that produced by pain or the anticipation of pain. The antinociceptive action occurs when mu (μ), kappa (κ) and delta (δ) receptors located on primary afferent neurons get activated. This reduces intracellular cAMP formation and opens K⁺ channels (mainly through mu and delta receptors) or suppresses voltage gated N type Ca²⁺ channels (mainly kappa receptors). These
actions result in neuronal hyperpolarization and reduced availability of intracellular Ca\(^{2+}\), which further leads to decreased neurotransmitter release by CNS and myenteric neurons. All these receptors are G protein coupled.\(^{195,196}\) The antinociceptive activity shown by various extracts and pure compounds might be the result of activation of any of these G protein coupled receptors.

5.4.3.2. Acetic acid induced writhing model (Peripheral model of analgesic activity)

The writhing in animals was induced by injecting 0.1 ml of 1% acetic acid in normal saline. After 30 minutes of drug administration, acetic acid solution was administered intraperitoneally and the inhibition of pain response (antinociceptive activity) was noted for a period of 20 mins (Section 4.1.5.3.2).

Figure 125: Effect of extracts of B. prionitis and B. cristata in acetic acid induced writhing model

All doses in mg/kg. **ASP** = Aspirin, **TAE** = Total aqueous extract, **ME** = Methanol extract, **BE** = Butanol extract, **EAE** = Ethyl acetate extract, **CE** = Chloroform extract, **HE** = Hexane extract, **LAE** = Left aqueous extract, **BP** = B. prionitis and B. cristata. Data showing % inhibition of wriths of different extracts is statistically significant at p<0.05 at all dose levels except for LAE (BC) at 400 mg/kg

Figure 126: Effect of shanzhiside esters in acetic acid induced writhing model

**ASP** = Aspirin, **AB** = Acetyl barlerin, **B** = Barlerin and **SME** = Shanzhiside methyl ester. Data showing % inhibition of wriths of pure compounds is statistically significant at p < 0.05 at 1 mg/kg dose level
The overall analgesic activity of extracts of both species was found to be statistically significant (p < 0.05) at all dose levels except for left aqueous extract of *B. cristata* at 400 mg/kg. The 400 mg/kg dose of butanol extract of *B. prionitis* showed promising results with 72.00 per cent reduction in wriths as compared to 85.14 per cent of standard while a lower dose of 100 mg/kg of the methanol extract was found to be effective with 68.57 per cent inhibition of wriths. The antinociceptive potential of the other extracts of *B. prionitis* was in the order: chloroform (63.43 at 400 mg/kg) > ethyl acetate (63.14 at 100 mg/kg) > hexane (59.43 at 200 mg/kg) > total aqueous (57.86 at 100 mg/kg) > left aqueous (42.57 at 100 mg/kg). In case of *B. cristata*, the activity profile was found to be slightly lower than *B. prionitis* with butanol extract exhibiting 67.14 per cent decrease in wriths. The analgesic activity in terms of per cent of other extracts of *B. cristata* was in order: chloroform (62.57 at 400 mg/kg) > methanol (62.00 at 100 mg/kg) > ethyl acetate (58.00 at 100 mg/kg) > hexane (55.43 at 200 mg/kg) > total aqueous (52.57 at 100 mg/kg) > left aqueous (29.71 at 100 mg/kg) (table 112, figure 125).

Pure compounds when evaluated at a single selected dose of 1 mg/kg showed less significant activity as compared to aspirin which exhibited 80.66 per cent protection against pain. Shanzhiside methyl ester was found to be most effective amongst all the three compounds with 43.87 per cent inhibition of wriths. Acetyl barlerin also showed mild antinociceptive activity with 37.74 per cent reduction in wriths and barlerin attenuated the wriths by only 35.85 per cent (table 113, figure 126). The activity shown by pure compounds was significant (p < 0.05) when compared to control.

**Explanation:** Prostaglandins especially E₂ and I₂ induce hyperalgesia by affecting the transductive property of free afferent nerve endings either through chemical or mechanical stimuli. They irritate the mucous membrane and produces the long lasting pain. The analgesic activity is mainly due to obtunding of peripheral pain receptors and prevention of PG mediated sensitization of nerve endings. A central subcortical action raising threshold to pain perception also contributes to the analgesic activity.$^{[97]}$ Thus the activity shown by extracts/pure compounds...
possibly could be due to the inhibition of prostaglandins or the rise in threshold value to the pain.

5.4.4. Evaluation of Antiulcer Activity

Anti-inflammatory activity of drugs is often associated with the high level of prostaglandin, although prostaglandins play a key role in maintaining the mucosal lining. One of these is the stomach lining, where prostaglandins serve a protective role, preventing the stomach mucosa from being eroded by its own acid. The prostaglandins are produced by mast cells, macrophages, endothelial cells and many other cell types by the action of two cyclooxygenases, the constitutively expressed COX-1 and the inducible enzyme COX-2 and are involved in the vascular and systemic reactions of inflammation. COX-2 is an enzyme facultatively expressed in inflammation, and it is the inhibition of COX-2 that produces the desirable effects of NSAIDs. When nonselective COX-1/COX-2 inhibitors (such as aspirin, ibuprofen, and naproxen) are used, they lower stomach prostaglandin levels. As a result, ulcers of the stomach or duodenum internal bleeding, nausea, vomiting etc can result. The pattern of anti-inflammatory activity followed by extracts and pure compounds in this study was same as that of Ibuprofen which clearly indicated that these test samples follow the path of non selective COX inhibitors. Hence, it was planned to also investigate the antiulcer potential of active extracts/pure compounds of Barleria.

5.4.4.1. Pyloric ligation induced gastric ulcer model

The animals were fasted (with free access to water) for a period of 24 h so as to ensure complete gastric emptying and a steady state gastric acid secretion. The 24 h fasted animals were administered with the test drugs/vehicle on the morning of the experiment. The procedure details of the experiment are given in section 4.1.5.4.1.1. The various parameters studied in the collected gastric contents were gastric fluid volume, pH, ulcer index and total & free acidity.
Results and Discussion

Figure 127: Effect of different extracts of *B. prionitis* and *B. cristata* on gastric fluid volume

RANT = Ranitidine, CTRL = Control, TAE = Total aqueous extract, ME = Methanol extract, BE = Butanol extract, BP = *B. prionitis* and BC = *B. cristata*. The values showing reduction in gastric fluid volume of extracts are statistically significant at p < 0.05 at all dose levels.

Figure 128: Effect of shanzhiside esters on gastric fluid volume

RANT = Ranitidine, CTRL = Control, AB = Acetyl barlerin, B = Barlerin and SME = Shanzhiside methyl ester. The values showing reduction in gastric fluid volume of pure compounds are statistically significant at p < 0.05 at 1 mg/kg dose level.

All the extracts produced a dose-dependent and significant (p < 0.05) reduction in gastric fluid volume. Methanol extract showed maximum effect in both the species at a dose of 400 mg/kg (table 114, figure 127). Pure compounds were also significantly (p < 0.05) effective in reducing the gastric fluid quantity with shanzhiside methyl ester showing maximum activity followed by acetyl barlerin and barlerin (table 114, figure 128).
Results and Discussion

Figure 129: Effect of different extracts of *B. prionitis* and *B. cristata* on gastric pH

RANT = Ranitidine, CTRL = Control, TAE = Total aqueous extract, ME = Methanol extract, BE = Butanol extract, BP = *B. prionitis* and BC = *B. cristata*. The values showing rise in pH of extracts are statistically significant at p < 0.05 at all dose levels except for TAE (BC) at 100 mg/kg.

Figure 130: Effect of shanzhisisde esters on gastric pH

RANT = Ranitidine, CTRL = Control, AB = Acetyl barlerin, B = Barlerin and SME = Shanzhisisde methyl ester. The values showing rise in pH of pure compounds are statistically significant at p<0.05 at 1 mg/kg dose level.

All the extracts and pure compounds produced a significant (p < 0.05) rise in gastric pH as compared to control, with maximum increase being produced by standard drug ranitidine indicating its protective role for the gastric mucosa (table 114, figure 129 & 130).
Results and Discussion

Figure 131: Effect of different extracts of *B. prionitis* and *B. cristata* on ulcer index

RANT = Ranitidine, CTRL = Control, TAE = Total aqueous extract, ME = Methanol extract, BE = Butanol extract, BP = *B. prionitis* and BC = *B. cristata*. The values showing ulcer index of extracts are statistically significant at p < 0.05 at all dose levels.

Maximum reduction in ulcer index was seen in the ranitidine treated group with a percentage protection of 86.73 against ulcer. All the tested doses of extracts and pure compounds also produced significant (p < 0.05) reduction of ulcer index as compared to the control. The maximum per cent protection against ulcer formation was shown by methanol extract with per cent value of 80.97 for *B. prionitis* and...
Results and Discussion

76.11 per cent for *B. cristata*. However, ulcer protective activity of shanzhiside methyl ester was found to be 72.57 per cent followed by 61.06 and 59.73 of acetyl barlerin and barlerin respectively (table 114, figure 131 & 132).

![Figure 133: Effect of different extracts of *B. prionitis* and *B. cristata* on total acidity](image)

*RANT* = Ranitidine, *CTRL* = Control, *TAE* = Total aqueous extract, *ME* = Methanol extract, *BE* = Butanol extract, *BP* = *B. prionitis* and *BC* = *B. cristata*. The values showing total acidity of extracts are statistically significant at *p* < 0.05 at all dose levels.

![Figure 134: Effect of different extracts of *B. prionitis* and *B. cristata* on free acidity](image)

*RANT* = Ranitidine, *CTRL* = Control, *TAE* = Total aqueous extract, *ME* = Methanol extract, *BE* = Butanol extract, *BP* = *B. prionitis* and *BC* = *B. cristata*. The values showing free acidity of extracts are statistically significant at *p* < 0.05 at all dose levels.
Results and Discussion

Figure 135: Effect of shanzhiside esters on total and free acidity

RANT = Ranitidine, CTRL = Control, AB = Acetyl barlerin, B = Barlerin and SME = Shanzhiside methyl ester. The values showing total and free of pure compounds are statistically significant at p < 0.05 at 1 mg/kg dose level

The volume of total and free acidity (table 114, figure 133 to 135) was significantly (p < 0.05) decreased in all drug treated groups as compared to control.

Explanation: The etiology of peptic ulcers is not clearly known but it results probably due to an imbalance between the aggressive (acid, pepsin and H. pylori) and the defensive (gastric mucus and bicarbonate secretion, prostaglandins, innate resistance of the mucosal cells) factors which causes an activation of cAMP mediated proton pump through enzyme H⁺K⁺ATPase which further causes mobilization of intracellular Ca²⁺ ions. Diffusion rate across the cell membrane is directly proportional to the gradient but it also depends on the molecule's lipid solubility, size, degree of ionization, and the area of absorptive surface. Because the cell membrane is lipoid, lipid-soluble drugs diffuse most rapidly. Another concept is that small molecules tend to penetrate membranes more rapidly than larger ones. On the contrary, if the drug is absorbed at a fast rate, the desired level of plasma concentration will be achieved but cannot be maintained with absolute consistency because of metabolic breakdown and excretory clearance. In the present study, the exact pattern of the antiulcer activity of different extracts and pure compounds cannot be well defined but it may be said that the key factor behind the activity is the molecular size. Shanzhise methyl ester and methanol possess lesser size than the other test substances and thus, the penetration of these molecules is more, thus showing enhanced activity. Regarding pure compounds, another factor could be the stearic hindrance of acetyl moieties which are more in number in case of acetyl barlerin and barlerin.
5.4.5. Chronic Model of Inflammation
5.4.5.1. Evaluation of antiarthritic activity in adjuvant induced ar
model
Adjuvant arthritis, a chronic inflammatory disease in the rat, is conside
pose some but not all the features of human rheumatoid arthritis. It is th
widely used chronic model for studying the anti-inflammatory and ir
suppressive properties of drugs.

The literature reports indicate that adjuvant arthritis arises from an al
immune response primarily involving cell-mediated hypersensitivity. Sub
administration of Complete Freund's Adjuvant (CFA) in rat paw res
significant production of inflammation which can be steadily maintained
days. The various parameters recorded during the present study viz., body \n\n\n\npaw volume changes, haematological parameters, arthritic score, radiologic
histopathological examination and estimation of serum TNF-α and IL-1β
were studied as per the recommended procedure.\[186-188,189\]

5.4.5.1.1. Body weight
Changes in body weight were noted to assess the course of the disease a
response to therapy of anti-inflammatory drugs. As the incidence and sev\narthritis increased, the changes in the body weights of the rats also oc
during the course of the experimental period.

Figure 136: Effect of active extracts and shanzhiside esters on body
changes
CFA = Complete freud's adjuvant, LP = Liquid paraffin, IND = Indomethacin, ME = Methanol extract, BE = Butan
AB = Acetyl barlerin, B = Barlerin and SME = Shanzhiside methyl ester. The increase in body weight was s
significant (p < 0.05) from day 9 to 21 for all test samples

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Results and Discussion

There was significant decrease during 21 day study in the body weight of control rats (with CFA treatment) from 168 to 140 g as compared to the normal rats (without CFA treatment) after subplantar administration of CFA due to the generation of immune response. However, treatment with active extracts and pure shanzhiside iridoids showed significant ($p < 0.05$) increase in body weight form day 9 to day 21 as compared to control rats (table 116). Rats treated with indomethacin (3 mg/kg) also significantly ($p < 0.05$) attenuated this decrease in body weight (140 to 174) from day 9 to 21 as compared to control rats (table 116, figure 136).

Explanation: Arthritis is associated with bone loss or loss in bone density. As a result the weight is reduced. In the present findings, the active extracts and shanzhiside iridoids are effective in reducing bone erosion which is shown by the significant effects on increase in rat body weights.

5.4.5.1.2. Paw volume

The arthritic rats showed a soft tissue swelling that was noticeable around the ankle joints during the acute phase of arthritis and was due to the oedema of periarticular tissues such as ligaments and joint capsules. The swelling was found to be increasing in the initial phase of inflammation and then it became nearly constant in the second week. After the 12-15 days of CFA injection immune response was visible with induced secondary arthritis.

Figure 137: Effect of active extracts and shanzhiside esters on hind paw oedema

CFA = Complete freud’s adjuvant, LP = Liquid paraffin, IND = Indomethacin, ME = Methanol extract, BE = Butanol extract, AB = Acetyl barlerin, B = Barlerin and SME = Shanzhiside methyl ester. The decrease in paw volume was statistically significant ($p < 0.05$) from day 7 to 21 for all test samples except standard which showed statistically significant ($p < 0.05$) effects from day 3.
Results and Discussion

When compared with normal rats, the control rats showed significant increase in the paw volume after subplantar CFA administration. In the primary phase of the arthritis i.e. from day 4 to 6, a non-significant decrease in the paw volume was observed in the drug (extracts/pure compounds) treated rats, while animals of the standard group showed a significant decrease in the paw volume. Rats treated with different test samples showed significant (p < 0.05) attenuation in paw volume from day 7 onward whereas indomethacin (3 mg/kg) treated rats had significantly (p < 0.05) decreased paw volume from day 3 to 21 as compared to control rats (table 117, figure 137).

Explanation: This oedema formation can be related to PGE$_2$ which enhances the effects of chemotactic factor mediators, such as leukotriene B and C5a. One of the potentiating effects of PGs include oedema formation. Interference by test samples (extracts and pure compounds) with the synthesis of PGs, which are reported to stimulate bone resorption and collagenase secretion from macrophages and to diminish proteoglycan synthesis in cartilage, can be claimed to be effective in preventing cartilage and bone pathology in adjuvant induced arthritis in rats.

5.4.5.1.3. Arthritic score

The progression of the rheumatoid arthritis in form of redness, swelling and bending of claws can be assessed using score known as arthritis score. The high arthritic score signifies the severity of chronic inflammation.

![Figure 138: Effect of active extracts and shanzhiside esters on arthritic score](image)

CFA = Complete freud's adjuvant, STD = Standard, ME = Methanol extract, BE = Butanol extract, AB = Acetyl barlerin, B = Barlerin and SME = Shanzhiside methyl ester. The values showing arthritic score of extracts (200 mg/kg) and shanzhiside esters (1 mg/kg) are statistically significant at p < 0.05 from day 7 and of standard from day 5 onwards.

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Results and Discussion

The subplantar administration of CFA results in significant increase in arthritic score in all CFA treated rats as compared to normal rats and this had shown a biphasic response (first phase is from day 1 to day 5 or 7 and second phase is from day 5 or 7 to 21). There was a decrease in the arthritic score from day 9 to 13. The arthritic score was significantly increased from day 15 in control rats which remained significantly increased till the end of the study i.e. up to 21st day as compared to normal rats. Rats treated with active extracts and pure compounds showed significant (p < 0.05) decrease in arthritic score from day 7 onward till the end of the study as compared to control rats. A significant decrease in arthritic score was also observed from day 5 in the indomethacin treated rats (table 118, figure 138). A decrease in arthritic score in methanol and butanol extract and shanzhisdie iridoids treated groups was clearly observed which indicated lessening in the severity of arthritis.

Explanation: The arthritic score was found to be reduced in various drug treated groups as there was minimal redness, swelling and blending of claws thereby indicating a significant control on chronicity of inflammation.

5.4.5.1.4. Haematological parameters: The systemic manifestation of the inflammatory response to the inciting agent, Complete Freund’s Adjuvant, used in animal models of arthritis is reflected in several peripheral blood-based parameters which are discussed below:

Erythrocyte sedimentation rate (ESR): ESR is an estimate of the suspension stability of RBC’s in plasma. It is related to the number and size of the red cells and to the relative concentration of plasma proteins, especially fibrinogen and β globulins. Increase in the rate is an indication of active but obscure disease processes.\(^\text{(12001)}\)
Results and Discussion

Figure 139: Effect of active extracts and shanzhiside esters on ESR levels

ME = Methanol extract, BE = Butanol extract, AB = Acetyl barlerin, B = Barlerin and SME = Shanzhisi methyl ester, LP = Liquid paraffin, IND = Indomethacin, CFA = Complete freud’s adjuvant. The values showing ESR of extracts (200 mg/kg) and shanzhiside esters (1 mg/kg) are statistically significant at p < 0.05.

The ESR count, which drastically increased in arthritic control group, was remarkably counteracted by the standard drug, extracts and pure compounds Barleria back to normal (table 119, figure 139), thus justifying its major role protecting arthritic conditions. The reduction in ESR count by extracts/pure compounds at their respective doses was found to be statistically significant (p < 0.05).

Total leukocyte count (TLC): In arthritis condition, there is a mild to moderate rise in WBC count due to induction of IL-1β mediated inflammatory response. IL-1β increases the production of both granulocyte and macrophage color stimulating factor.

Figure 140: Effect of active extracts and shanzhiside esters on total leukocyte count levels

ME = Methanol extract, BE = Butanol extract, AB = Acetyl barlerin, B = Barlerin and SME = Shanzhisi methyl ester, LP = Liquid paraffin, IND = Indomethacin, CFA = Complete freud’s adjuvant. The values showing rise in TLC cells level of extracts (200 mg/kg) and pure compounds (1 mg/kg) are statistically significant at p < 0.05 for ME.
Results and Discussion

In the present investigation, the migration of leucocytes into the inflammed area was non-significantly suppressed by the standard drug, pure compounds and test extracts except for methanol extract which showed significant reduction in TLC level (table 119, figure 140).

Haemoglobin: It has been reported that in the arthritic condition the haemoglobin levels get decreased which has been associated with anaemia. Anaemia may be due to reduced erythrocyte deformability. The reduced deformability leads to a shortened life span of erythrocytes, which results in depression of RBC's, a marker of rheumatoid disease.[200,201]

There was significant decrease in haemoglobin level in control rats as compared to normal rats. Rats treated with active extracts and pure compounds showed significant (p < 0.05) increase in haemoglobin level when compared to control (table 119, figure 141).

Figure 141: Effect of active extracts and shanzhiside esters on haemoglobin levels

ME = Methanol extract, BE = Butanol extract, AB = Acetyl barlerin, B = Barlerin and SME = Shanzhiside methyl ester, LP = Liquid paraffin, IND = Indomethacin, CFA = Complete freud's adjuvant. The values showing Hb level of extracts (200 mg/kg) and shanzhiside esters (1 mg/kg) are statistically significant at p < 0.05

Packed cell volume (PCV): PCV is defined as the volume of the blood cells in a sample of blood after it has been centrifuged in the hematocrit and normally, it amounts to 45% of the blood sample.
Results and Discussion

Figure 142: Effect of active extracts and shanzhiside esters on packed cell volume

ME = Methanol extract, BE = Butanol extract, AB = Acetyl barlerin, B = Barlerin and SME = Shanzhiside methyl ester, LP = Liquid paraffin, IND = Indomethacin, CFA = Complete freud’s adjuvant. The values showing PCV level of extracts (200 mg/kg) and shanzhiside esters (1 mg/kg) are statistically significant at p < 0.05 except for BE and IND.

A significant (p < 0.05) increase in packed cell volume was observed in case of rats treated with methanol extract and pure compounds prophylactically. However, there was a non-significant increase in packed cell volume in butanol extract and standard - indomethacin treated rats (table 119, figure 142).

5.4.5.1.5. Radiological and histopathological examination

The potent antiarthritic effect of active extracts viz., methanol and butanol and pure compounds was further confirmed by radiological studies (figure 104). The X-ray appearance, commonly referred to as diminished joint space is the hallmark of arthritis. In positive control rats challenged with CFA, metacarpophalangeal area depicted radiographical changes that included periosteal reaction along the metacarpals and at locations bone loss was also evident. Most of metacarpophalangeal joints showed reduced joint spaces. Moderate to severe soft tissue swelling was also present which was clearly visible in the lateral view. However, in the negative control rats challenged with liquid paraffin, no periosteal reaction and tissue swelling was seen and metacarpophalangeal joints were having normal joint spaces.

The following observations were made in the groups treated with test samples (extracts/pure compounds/standard):
Results and Discussion

a) The rats of the standard group (indomethacin treated) exhibited soft tissue swelling of hind paws but with respect to metacarpophalangeal joints spacing, results were near normal with mild periosteal reaction (figure 104).

b) The methanol extract treated rats showed response which was almost comparable to standard group with respect to joint space and periosteal reaction. However, the hind paws showed more soft tissue swelling but with respect to metacarpophalangeal joints spacing, results were near normal with mild periosteal reaction and joint spacing was also evident (figure 104).

c) The rats treated with butanol extract also showed results almost comparable to standard. The hind paws showed decreased soft tissue swelling but with respect to metacarpophalangeal joints spacing, results were near normal with mild periosteal reaction (figure 104).

d) In the shanzhiside methyl ester treated rats, minimal bone changes were observed showing mild periosteal reaction with reduced soft tissue swelling. Metacarpophalangeal joint space was also evident (figure 104).

e) In the acetyl barlerin treated rats, mild periosteal reaction was observed with moderate soft tissue swelling. Metacarpophalanges showed better uniform bone density. Joint space was also clearly visible (figure 104).

f) In the rats treated with pure compound barlerin, mild periosteal reaction was observed with moderate soft tissue swelling. Metacarpophalanges showed better uniform bone density when compared to acetyl barlerin treated rats. Joint space was also clearly visible (figure 104).

The radiological observations were further supported by histopathological examination (figure 105) and following conclusions were drawn:

a) The animals treated with liquid paraffin showed normal joint space around bone while those treated with CFA showed soft tissue inflammation with decreased joint space (figure 105).

b) The standard drug, indomethacin, exhibited a clear joint space (figure 105).
c) The rats treated prophylactically with methanol extract of *B. prionitis* demonstrated normal joint space with normal synovial membrane although mild soft tissue and synovial swelling around the ankle heel bone was present (figure 105).

d) The butanol extract treated group of rats showed clear joint space with no inflammation, although, hyperplasia and lobular thickening of synovial membrane was observed (figure 105).

e) In the rats treated with pure compound acetyl barlerin, joint space showed the swelling. Pannus formation (new vascularization of the synovium) along the edges of the joint space was also observed with villous (finger like) formation (figure 105).

f) The rats to which pure compound barlerin was given, the results were most significant as compared to other shanzhiside esters. Joint space was clear and the synovial membrane along the edge was mildly inflamed and having hyperplastic surface. However, mild soft tissue swelling was there (figure 105).

g) In the shanzhiside methyl ester treated rats, joint space around the tip of a tendon showed severe inflammatory hyperplastic synovial membrane with mild synovial reaction (figure 105).

Localised bone loss in the form of bone erosions and peri-articular osteopenia constitutes important signs for the diagnosis of rheumatois arthritis. This is accompanied by soft tissue inflammation and vascularization of the synovium. In the present study, the above mentioned findings clearly indicates the anti-arthritic activity of extracts and pure compounds, which acts by modulating bone erosion.

5.4.5.1.6. Estimation of serum TNF-α and IL-1β levels

It has been speculated that rheumatoid arthritis (RA) could be triggered by a T-cell response to infectious agents. Through cell-cell contact and different cytokines, these stimulated T cells activate monocytes, macrophages, and synovial fibroblasts. The latter then overproduce pro-inflammatory cytokines, mainly TNF-α, IL-1β, and IL-6.[202] These soluble molecules, once engaged to their receptors, trigger various signal transduction cascades that lead to the activation of
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transcription factors and the subsequent induction of genes whose products such as matrix metalloproteinases (MMPs) mediate tissue degradation.\textsuperscript{[201,202]}

In addition, TNF-\(\alpha\) and IL-1 also induce receptor activator of nuclear factor-\(\kappa\)B (RANK) on macrophages which differentiate into osteoclasts that resorb and destroy bone. Keeping in mind the above mentioned activation cascade, levels of different cytokines- TNF-\(\alpha\) and IL-1\(\beta\) were determined in the blood serum in different groups of rats. Consistent with the joint swelling and ESR, the serum levels of TNF-\(\alpha\) and IL-1\(\beta\) in the diseased control group (vehicle treated) were significantly high while prophylactic treatment with active extracts and pure compounds, significantly (\(p < 0.05\)) reduced the elevated levels of cytokines (table 120 & 121, figure 143 & 144). This reduction in the serum cytokines level by the active extracts and shanzhiside iridoids clearly indicates their protective role. Further the positive effect of the test samples in controlling the various parameters associated with the progression of arthritis showed their significant antiarthritic property.

![Graph](image-url)

Figure 143: Effect of active extracts and shanzhiside iridoids on serum IL-1\(\beta\)

\textbf{ME} = Methanol extract, \textbf{BE} = Butanol extract, \textbf{AB} = Acetyl barlerin, \textbf{B} = Barlerin and \textbf{SME} = Shanzhiside methyl ester, \textbf{LP} = Liquid paraffin, \textbf{CFA} = Complete freud’s adjuvant, \textbf{IND} = Indomethacin. The values showing serum IL-1\(\beta\) level of extracts (200 mg/kg) and shanzhiside iridoids (1 mg/kg) are statistically significant at \(p < 0.05\).
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Treatment groups

Figure 144: Effect of active extracts and shanzhiside iridoids on serum TNF-\(\alpha\)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Serum TNF-(\alpha) (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ME</td>
<td>160</td>
</tr>
<tr>
<td>BE</td>
<td>140</td>
</tr>
<tr>
<td>AB</td>
<td>120</td>
</tr>
<tr>
<td>B</td>
<td>100</td>
</tr>
<tr>
<td>SME</td>
<td>80</td>
</tr>
<tr>
<td>IND</td>
<td>60</td>
</tr>
<tr>
<td>LP</td>
<td>40</td>
</tr>
<tr>
<td>CFA</td>
<td>20</td>
</tr>
</tbody>
</table>

From the results observed in the current investigation, it may be concluded that *Barleria* and isolated shanzhiside esters from the same plant can be strongly categorized under potential antiarthritic drugs since both were active in all the inflammatory models including adjuvant induced arthritic model. The crude extracts and shanzhiside esters possess significant antiulcer activity while the pure compounds in addition also showed mild analgesic activity. From the investigations carried out on the comparative activity profile of two species of *Barleria*, *B. prionitis* definitely has more potential to curb the underlying causes involved in various inflammatory disorders with better gastric protection and needs to be explored further. Shanzhiside iridoids, with barlerin in particular are potential lead molecules and might prove to be very promising in various drug discovery programmes. Hence we can say that *Barleria* can be used safely in chronic diseases like arthritis as it has shown gastro protective potential which is the major problem associated with modern drug therapy like NSAIDs. Further, the pain relieving ability of this plant is an added advantage which takes care of the pain allied to many inflammatory disorders. Additionally, the selected plant *Barleria* as well as the pure shanzhiside esters are expected to be very effective if used in topical formulation for alleviating inflammation and pain associated with diseases like psoriasis, rheumatoid arthritis and others.
5.5. PROPOSED MECHANISM OF ACTION OF EXTRACTS/PURE COMPOUNDS

(i) Anti-inflammatory activity:

**Acute study:** A careful analysis of extracts/pure compounds of *Barleria* in acute, sub-chronic and topical models of inflammation was made. A good anti-inflammatory response was observed in carrageenan induced paw oedema. It is well known that a single sub-plantar injection of 1 % w/v carrageenan solution in normal saline produces a bi-phasic paw volume increase. The early phase I of inflammatory response plateauing at 0.5-1 h, early phase II at 3-6 h and late phase is 12 and 24 h. The prostaglandins appear to play a significant role in the development of carrageenan induced rat paw oedema. The activity of the prostaglandin E type in oedema fluids squeezed out from the carrageenan inflammed rat paw has been well demonstrated in literature. The early expressed fluid contains histamine; later fluid show bradykinin activity but little PGE₂ is found until after 3 hours. The concentration of PGE₂ rose between 18 and 24 h.

The extracts and compounds of *Barleria* showed significant prevention of paw oedema from 60 min to 24 h. Both early and late phases were significantly suppressed by pretreatment with the test samples. The carrageenan oedema can be inhibited by at least two mechanisms: inhibition of the primary mediators involved in the swelling or inhibition of the prostaglandin amplification mechanism. The significant protection shown by the extracts/pure compounds in the early phase indicates that these might be acting by inhibiting the primary mediators. However in some extracts, reversal of activity at 24 h was observed showing their protective role in the late phase, by inhibiting prostaglandin-dependent component of carrageenan which induces oedema.

**Different mediators:** Histamine is a low-molecular weight compound stored as complex with heparin in mast cells and basophils. It is responsible for producing arteriolar dilation and is the principal mediator of the immediate phase of increased vascular permeability, inducing venular endothelial contraction and interendothelial gaps. Significant protection was visible in histamine induced paw oedema thereby showing the inhibitory effect of extracts/pure compounds on acute and transient inflammation produced by histamine.
Dextran causes the degranulation of mast cells and release of several inflammation mediators such as histamine and serotonin. The anti-inflammatory activity shown by the extracts and pure compounds of Barleria are the result of partial inhibition of release of inflammatory mediators.

Sub-acute study: The cotton pellet induced granuloma has been widely used to evaluate the transudative and proliferative components of inflammation and the wet and dried weight of the pellets correlates well with the amount of granulomatous tissue formed. In this model, inflammation is induced due to implantation of the foreign body. Sub-acute inflammation is a reaction arising when acute response is insufficient to eliminate pro-inflammatory agents. Sub-acute inflammation induces a proliferation of fibroblasts with the infiltration of neutrophils & exudation and mast cell proliferation also takes place. It is proposed that extracts/pure compounds may have caused a decrease in granuloma tissue formation by inhibiting proliferation of fibroblasts and granulocyte infiltration.

Topical study: Studies have reported that the acute inflammation induced by topical administration of croton oil results from increased vascular permeability and vasodilatation, stimulating the migration of polymorphonuclear leukocytes (principally neutrophils), the liberation of histamine and serotonin, and the moderate synthesis of eicosanoids. The significant inhibition of ear oedema by extracts/pure compounds depicts its promising role in the inhibition of inflammatory cascade produced by croton oil.

(ii) Analgesic activity:
The generated data reveals that the analgesic activity (both central and peripheral) are exhibited by the Barleria extracts. In the central analgesic activity, pain induced by heat causes the activation of G protein coupled receptors viz., mu (\(\mu\)), kappa (\(\kappa\)) and delta (\(\delta\)) which reduces intracellular Ca\(^{2+}\) and this further leads to decreased neurotransmitter release by neurons. The central antinociceptive activity shown by various extracts and pure compounds might be the result of activation of any of these G protein coupled receptors which exercise inhibitory modulation by decreasing the release of the junctional transmitters. The peripheral antinociceptive activity of the extracts is mainly due to obtunding of peripheral pain receptors and prevention of PG especially E\(_2\) and I\(_2\) mediated sensitization of
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nerve endings. The activity shown by extracts was found to be more when compared to the activity of pure compounds of *Barleria*. This might be the result of synergistic activity of various compounds present in extracts. However, less activity shown by the pure compounds indicates that they are mild analgesics.

(iii) Antiulcer activity:
Accumulation of acid and pepsin in pyloric ligation induced gastric ulcer model leads to auto digestion of gastric mucosa and ulceration. Gastric mucosal damage can be attributed to the decrease in mucosal defence due to starvation, increase in vagal discharge resulting in degranulation of mast cells and depletion of histamine in gastric tissue. Vagally induced gastric secretion in this model has been shown to be mediated through histamine and significant protection shown by the extracts/pure compounds indicates its possible role in inhibition of this primary mediator of inflammation.

(iv) Antiarthritic activity:
It was observed that extracts/pure compounds could significantly inhibit the disease progression of AIA rats and markedly protect the affected joints against bone erosion in rats, presumably by suppressing the abundant production of pro-inflammatory cytokines TNF-α and IL-1β in the blood serum. Also, oedema formation due to PGE₂ mediated enhancement of the effects of chemotactic factor mediators was significantly inhibited by the active extracts/pure compounds of *Barleria*. It seems probable that the effect of active extracts/pure compounds in adjuvant induced arthritis come from two major actions: inhibition of swelling of soft tissues via elimination of the potentiating effects of PGEs on oedema formation and elimination of the effects of PGEs on bone remodelling.

Hence from the various studies conducted, it was found that the genus *Barleria* possess very good anti-inflammatory activity with good gastric protection. Further it is also useful in treating various problems associated with arthritis. Interestingly, it was found to be a mild analgesic. The various findings were further confirmed by performing the same set of studies on the isolated pure shanzhiside iridoids. Thus the traditional use of genus *Barleria* is fully justified by the generated bioactivity profiles.