5. Results

5.1. Collection and authentication of crude drugs

The drugs were collected from local market of Delhi, India and authenticate by Dr. H. B. Singh (Scientist G & Head, Raw Materials Herbarium & Museum) National Institute of Science Communication and Information Resources (NISCAIR), New Delhi. Voucher specimens were deposited at NISCAIR museum (NISCAIR/RHMD/Consult/-2009-10/1548/121).

Morphological and microscopically identification of all drugs were done and the results are shown in Table 2. There is no similarity between the all three drugs. The photographs of two drugs are shown in Fig. 13.

**Figure 13:** Photograph of *Rubia cordifolia* Linn. and *Saussurea costus* (Falc.) Lipsch. (Syn. *Saussurea lappa* (Decne.) C. B. Clarke).

*Rubia cordifolia*  
*Saussurea lappa*
Table 2: Data showing morphological features of *Achillea millefolium* flowers, *Rubia cordifolia* roots and *Saussurea lappa* roots.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Achillea millefolium flowers</th>
<th>Rubia cordifolia roots</th>
<th>Saussurea lappa roots</th>
</tr>
</thead>
<tbody>
<tr>
<td>Color</td>
<td>Pale yellow</td>
<td>Reddish brown</td>
<td>Brownish</td>
</tr>
<tr>
<td>Odour</td>
<td>Pleasant</td>
<td>Characteristic</td>
<td>Aromatic</td>
</tr>
<tr>
<td>Taste</td>
<td>Characteristic</td>
<td>Sweetish</td>
<td>Bitter Pungent</td>
</tr>
<tr>
<td>Form</td>
<td>Ray florets bracteate</td>
<td>Cylindrical</td>
<td>Conical</td>
</tr>
<tr>
<td>Shape &amp; extra features</td>
<td>Unisexual, 5 gamopetal, campanulate corolla, long style and bifid stigma, disc florets-bracteate</td>
<td>1.5 to 4 cm in diameter, 2 to 9 cm in length, 0.2 to 0.6 cm in width, surface smooth, rarely grooved, fracture short</td>
<td>15 cm long and 0.5 to 5 cm wide, surface straightly or spirally longitudinally wrinkled, fracture short pale brown</td>
</tr>
</tbody>
</table>

5.2. Proximate analysis

The moisture content, total ash, acid insoluble ash, ash, alcohol soluble extractive value, water-soluble extractive value and foreign matter of the two drugs are represented in Table 3.
Results

Table 3: Data showing proximate values of (moisture content, ash content, extractive value and foreign matter) Achillea millefolium, Rubia cordifolia & Saussurea lappa.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Sample</th>
<th>Moisture Content</th>
<th>Total Ash</th>
<th>Acid insoluble ash</th>
<th>Alcohol soluble extractive</th>
<th>Water soluble extractive</th>
<th>Foreign matter</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Achillea millefolium</em></td>
<td>3.01</td>
<td>7.2</td>
<td>2.18</td>
<td>8.53</td>
<td>11.89</td>
<td>0.87</td>
</tr>
<tr>
<td>2</td>
<td><em>Rubia cordifolia</em></td>
<td>5.87</td>
<td>6.8</td>
<td>0.66</td>
<td>25.2</td>
<td>49.8</td>
<td>0.74</td>
</tr>
<tr>
<td>3</td>
<td><em>Saussurea lappa</em></td>
<td>7.24</td>
<td>3.2</td>
<td>1.1</td>
<td>12.7</td>
<td>27.6</td>
<td>1.12</td>
</tr>
</tbody>
</table>

All the values are in %.

5.3. Chemical analysis

Chemical analysis of all three crude drugs was performed and results are tabulated in Table 4.

Table 4: Total alkaloid, starch and total polyphenol contents in Achillea millefolium, Rubia cordifolia & Saussurea lappa.

<table>
<thead>
<tr>
<th>Contents</th>
<th><em>Achillea millefolium</em> flowers</th>
<th><em>Rubia cordifolia</em> roots</th>
<th><em>Saussurea lappa</em> roots</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total alkaloid</td>
<td>0.94</td>
<td>0.17</td>
<td>1.48</td>
</tr>
<tr>
<td>Starch content</td>
<td>1.01</td>
<td>6.83</td>
<td>7.61</td>
</tr>
<tr>
<td>Total Polyphenol content</td>
<td>2.17</td>
<td>2.83</td>
<td>2.57</td>
</tr>
</tbody>
</table>

All the values are in % w/w.

5.4. Qualitative evaluation of extracts

Qualitative chemical tests were performed to analyse the individual crude drugs. The results are shown in Table 5.
The preliminary phytochemical screening of *A. millefolium* extract showed the presence of saponins, flavonoids, terpenoids, proteins and tannins. The *R. cordifolia* extract showed the presence of alkaloids, carbohydrates, anthraquinone glycoside, phytosterols, flavonoids and protein whereas *S. lappa* showed the presence of alkaloids, carbohydrates, glycosides, phytosterols, terpenoids and tannins. However, alkaloids, carbohydrates, glycosides, phytosterols, anthraquinones were absent in *A. mellifolium* flowers. Saponins, fats & oils and tannins showed negative results in *R. cordifolia* root. Along with saponins, flavonoids and anthraquinones showed negative results in *S. lappa*.

**Table 5:** Qualitative chemical tests of the extracts of *Achillea millefolium, Rubia cordifolia* & *Saussurea lappa*.

<table>
<thead>
<tr>
<th>Phytoconstituents</th>
<th>Tests</th>
<th>Achillea millefolium</th>
<th>Rubia cordifolia</th>
<th>Saussurea lappa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>Mayer’s test</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Dragendroff’s test</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Wagner’s test</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Hager’s test</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>Molisch’s test</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Benedict’s test</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Fehling’s test</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Anthraquinone</td>
<td>Bornträger’s test</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Glycosides</td>
<td>Legal’s test</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Cardiac Glycosides</td>
<td>Foam test</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Froth test</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Saponins</td>
<td>Liebermann Burchard’s</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>test</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phytosterols</td>
<td>Stain test</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Fats and oil</td>
<td>Lead acetate test</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
## Results

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Ninhydrin test</th>
<th>Biuret test</th>
<th>Tannins</th>
<th>Lead acetate test</th>
<th>Gelatine test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

5.5. **Extraction and isolation of bioactive compounds**

The percentage yield of isolated compound, Achillicin (blue crystals), Chamazulene (deep blue green crystals), Rubiadin (orange pale yellow crystals), Mollugin (reddish yellow crystals), Costunolide (white crystals), Dehydrocostus lactone (white crystals) and Cynaropicrin (white crystals) were found to be 6.01%, 5.5%, 0.018%, 0.036%, 0.04%, 0.03% and 0.02%, respectively.

5.6. **Characterization of isolated compounds**

The structures of isolated compounds were identified and confirmed by comparison of their earlier reported/published spectral data.

**Figure 14**: Chemical structure of achillicin, chamazulene, rubiadin-3-O-β-glucoside, mollugin, costunolide, dehydrocostus lactone and cynaropicrin.

Acetic acid 6-hydroxy-3,6,9-trimethyl-2-oxo-2,3,3a,4,5,6,8,9b-octahydroazuleno[4,5-b]furan-4-yl ester
(Achillicin)
2-(3,8-Dimethyl-azulen-5-yl)-propionic acid  
(Chamazulene)

1,3-dihydroxy-2-methyl-9,10-anthraquinone 3-O-b-glucopyranoside  
(Rubiadin-3-O-b-glucoside)
6-Hydroxy-2,2-dimethyl-2H-benzo[h]chromene-5-carboxylic acid methyl ester
(Mollugin)

6,10-Dimethyl-3-methylene-3a,4,5,8,9,11a-hexahydro-3H-cyclodeca[b]furan-2-one
(Costunolide)
3,6,9-Trimethylene-decahydro-azuleno[4,5-b]furan-2-one
(Dehydrocostus lactone)

8-Hydroxy-4-(2-hydroxymethyl-acryloyl)-3,6,9-trimethylene-decahydro-
azuleno[4,5-b]furan-2-one
(Cynaropicrin)
Achillicin (8α-acetoxy-10-epi-artabsin): The achillicin was successfully isolated and identified by MS, IR and $^1$H and $^{13}$C NMR (Banh-Nhu et al., 1979). Blue crystals; MALDI-MS: m/z (% rel. int.): 306 (1) [M]$^+$; IR (KBr) v max cm$^{-1}$: 3453 (O-H str.), 1773 (C=O str. of γ-lactone), 1727 (C=O str.), 1613 (C=C str.) and 1153; $^1$H-NMR (CDCl$_3$; 300 MHz): δ 1.29 (CH$_3$(15) d, J = 7.2 Hz, 3H), 1.61 (CH$_3$(13), s, 3H), 1.65 (CH$_2$(9), d, J = 13.2 Hz, 2H), 1.72 (OH$_{(10)}$, br s, 1H), 2.08 (CH$_3$(17), s, 3H), 2.12 (CH$_7$(7), m, J = 10.2 Hz, 1H), 2.18 (CH$_3$(14), s, 3H), 2.61 (CH$_{11}$(11), m, J = 11.1 Hz, 1H), 2.89 (CH$_2$(3), d, J = 11.1, 1.5 Hz, 2H), 5.49 (CH$_{(6)}$, d, J = 1.5 Hz, 1H), 5.53 (CH$_{(8)}$, m, J = 13.2, 11.1, 4.2, 4.2 Hz, 1H). $^{13}$C-NMR (CDCl$_3$; 75 MHz): δ 178.20 (C-12), 169.96 (C-16), 149.84 (C-4), 137.85 (C-1), 134.04 (C-5), 124.54 (C-2), 78.24 (C-6), 73.46 (C-8), 69.40 (C-10), 55.94 (C-11), 46.50 (C-9), 45.58 (C-3), 40.50 (C-7), 30.38 (Me-15), 21.16 (Me-17), 15.50 (Me-13), 14.90 (Me-14).

The MS, IR, $^1$H and $^{13}$C NMR spectral data agree with a molecular formula of C$_{17}$H$_{22}$O$_3$ and literature (Banh-Nhu et al., 1979). The structure of achillicin is shown in Fig. 14.

Chamazulene ((S)-2-(r,3,8-Trimethylazulen-5-y)acetic acid): The isolated chamazulene was identified by MS, IR and $^1$H and $^{13}$C NMR (Ramadan et al., 2006). Deep blue green crystals; MALDI-MS: m/z (% rel. int.): 229 (22) [M]$^+$, 228 (100) [M]$^+$, 183 [M-CO$_2$H]$^+$ (99), 168 [M-CH$_3$CO$_2$H]$^+$ (60); IR (KBr) v max cm$^{-1}$: 3359 (O-H str.), 2975 (C-H str.), 2928, 2257, 1926, 1700 (C=O str.), 1457, 1381, 1330, 1275, 1090 and 1050; $^1$H-NMR (CDCl$_3$; 300 MHz): δ 1.63-1.59 (CH$_3$(13), d, J = 7.2 Hz, 3H), 2.65 (CH$_3$(15), s, 3H), 2.83 (CH$_3$(14), s, 3H), 3.89-3.85 (CH$_1$(11), q, J = 7.2 Hz, 1H), 7.01-6.98 (CH$_7$(7), d, J = 10.5 Hz, 1H), 7.27-7.26 (CH$_1$(1), d, J = 3.6 Hz, 1H), 7.45-7.44 (CH$_6$(6), dd, J = 10.5 Hz, J = 1.8 Hz, 1H), 7.65-7.63 (CH$_2$(2), d, J = 3.6 Hz, 1H), 8.22-8.21 (CH$_4$(4), d, J = 1.8, 1H), 11.96 (OH$_{(12)}$, br s, 1H); $^{13}$C-NMR (CDCl$_3$; 75 MHz): δ 179.21, 145.41, 137.34, 136.71, 136.07, 135.59, 133.74, 130.79, 126.85, 124.95, 114.11, 48.75, 20.07, 18.97, 12.87.

The MS, IR, $^1$H and $^{13}$C NMR spectral data agreed with a molecular formula of C$_{15}$H$_{16}$O$_3$ and literature (Ramadan et al., 2006). The structure of chamazulene is shown in Fig. 14.
Rubiadin (1,3-dihydroxy, 2-methyl-9,10-anthraquinone-3-O-β-glucopyranoside): The rubiadin was isolated and identified by MS, IR and $^1$H and $^{13}$C NMR (Yang et al., 1998; Rao et al., 2006). Orange yellow crystals; MALDI-MS: $m/z$ (% rel. int.): 254 (100) [M]$^+$ of aglycone [M$^+$]; IR (KBr) v max cm$^{-1}$: 3417.3 (OH str.), 2919.7 (alkyl neat CH str.), 2881.2, 1670 (unchelated C=O), 1631.5 (chelated C=O), 1589.1, 1577.5, 1330.7, 1286.3, 1230.4, 1195.7, 1157.1, 1076.1, 777.2, 756.0, 713.5 (4 adjacent ArH), 588.2; $^1$H-NMR (CDCl$_3$; 300 MHz): δ 2.18 (CH$_3$$_{(2)}$, s, 3H), 3.24-3.54 (Glc-CH$_3$$_{(3)}$, m, 5H), 3.69 (Glc-CH$_3$$_{(3)}$, d, $J$ = 11.7 Hz, 1H), 5.14 (Glc-H$_{(1)}$, d, $J$ = 5.7 Hz, 1H), 7.39 (CH$_3$$_{(4)}$, s, 1H), 7.92-7.93 (CH$_{(6&7)}$, d, $J$ = 4.2 Hz, 2H), 8.16-8.26 (CH$_{(5&8)}$, d, $J$ = 4.5 Hz, 2H), 12.98 (Ar-OH$_{(1)}$, br s, 1H); $^{13}$C-NMR (CDCl$_3$; 75 MHz): δ 188.6 (C-9), 181.5 (C-10), 161.3 (C-3), 160.2 (C-1), 134.7 (C-7), 134.5 (C-6), 132.6 (C-10a), 131.7 (C-8a), 131.6 (C-4a), 126.6 (C-8), 126.4 (C-5), 120.7 (C-2), 110.7 (C-9a), 105.6 (C-4), 100.1 (C-1’), 77.2 (C-5’), 76.1 (C-3’), 73.0 (C-2’), 69.2 (C-4’), 60.2 (C-6’), 8.3 (Me-2).

The MS, IR, $^1$H and $^{13}$C NMR spectral data agree with a molecular formula of rubiadin (aglycone) $\text{C}_{15}\text{H}_{10}\text{O}_{4}$ and rubiadin-3-O-β-glucoside $\text{C}_{21}\text{H}_{20}\text{O}_{9}$ and literatures (Yang et al., 1998; Rao et al., 2006). The structure of rubiadin-3-O-β-glucoside is shown in Fig. 14.

Mollugin (6-hydroxy-2,2-dimethyl-2H-naphtho[1,2-b]pyran-5-carboxylic acid methyl ester): The mollugin was isolated and identified by MS, IR and $^1$H and $^{13}$C NMR (Schildknecht et al., 1976; Itokawa et al., 1983; Inoue et al., 1984; Gupta et al., 1999; Ozgen et al., 2006; Yanbin et al., 2007). Yellow flakes; MALDI-MS: $m/z$ (% rel. int.): 284.3 (100) [M]$^+$; IR (KBr) v max cm$^{-1}$: 3165 (O-H str.), 2972 (Neat C-H str.), 1645 (C=O), 1580, 1451, 1362, 1343, 1248, 1194, 1163, 1132, 1098, 1013, 957, 889, 806 and 770; $^1$H-NMR (CDCl$_3$; 300 MHz): δ 1.49 (2CH$_3$$_{(3)}$, s, 6H), 3.96 (OCH$_3$$_{(2)}$, s, 3H), 5.63 (CH$_2$$_{(2)}$, d, $J$ = 9.9 Hz, 1H), 7.05 (CH$_{(1)}$, d, $J$ = 9.9 Hz, 1H), 7.44-7.58 (CH$_{(6&7)}$, m, 2H), 8.16 (CH$_{(8)}$, dd, $J$ = 8.4 Hz, 1H), 8.34 (CH$_{(5)}$, dd, $J$ = 8.4 Hz, 1H), 12.13 (Ar-OH$_{(1)}$, br s, 1H); $^{13}$C-NMR (CDCl$_3$; 75 MHz): δ 172.6 (-C=O), 156.6 (C-1), 141.7 (C-4), 129.4 (C-6), 129.1 (C-4a), 122.4 (C-1’), 122.0 (C-8), 112.7 (C-3), 102.3 (C-2), 74.7 (C-3’), 52.3 (-OCH$_3$), 27.1 (C-4’), 27.0 (C-5’).

The MS, IR, $^1$H and $^{13}$C NMR spectral data agree with a molecular formula of $\text{C}_{17}\text{H}_{20}\text{O}_{4}$ and literatures (Schildknecht et al., 1976; Itokawa et al., 1983; Inoue et al.,
Costunolide ((3αS,6E,10E,11αR) -6,10- dimethyl -3- methylene -3,3α,4,5,8,9- hexahydrocyclodecane [b] furan-2 (11αH) -one): The costunolide was isolated and identified by MS, IR and $^1$H and $^{13}$C NMR (Gricco PA and Nishizaura M, 1976; Ahmed and Abdelgaleil, 2005; Vijayakannan et al., 2006). Colourless needles shaped crystals; MALDI-MS: m/z (% rel. int.): 232.15 (100) [M]+; IR (KBr) v max cm$^{-1}$: 2975, 2945 (C-H str.), 2870 (Methyl C-H str.), 1762 (C=O str.), 1670, 1459, 1442, 1410, 1390, 1315, 1295, 1252, 1213, 1185, 1145, 1090, 1060, 971, 950, 895, 878 and 840; $^1$H-NMR (CDCl$_3$; 300 MHz): δ 1.48 (CH$_2$(4), q, J = 1.2 Hz, 2H), 1.71 (2CH$_3$(13&14), s, 6H), 1.97 (CH$_2$(5), t, J = 1.2 Hz, 2H), 1.99 (CH$_2$(9), t, 2H), 2.02 (CH$_2$(8), q, 2H), 2.78 (CH$_3$(3a), q, J = 1.2 Hz, 1H), 4.59 (CH$_3$(11a), t, J = 8.7 Hz, 1H), 4.75 (CH$_3$(11), d, J = 9 Hz, 1H), 4.89 (CH$_7$(7), t, 1H), 5.58-6.05 (CH$_2$(12), s, 2H); $^{13}$C-NMR (CDCl$_3$; 75 MHz): δ 172.6 (-C=O), 156.6 (C-1), 141.7 (C-4), 129.4 (C-6), 129.1 (C-4a), 122.4 (C-1’), 122.0 (C-8), 112.7 (C-3), 102.3 (C-2), 74.7 (C-3’), 52.3 (-OCH$_3$), 27.1 (C-4’), 27.0 (C-5’).

The MS, IR, $^1$H and $^{13}$C NMR spectral data agree with a molecular formula of C$_{13}$H$_{20}$O$_2$ and literatures (Gricco PA and Nishizaura M, 1976; de Kraker et al., 2001; Ahmed and Abdelgaleil, 2005; Vijayakannan et al., 2006). The structure of costunolide is shown in Fig. 14.

Dehydrocostus lactone (6-hydroxyguaiia-4(15), 10(14), 11(13)-tri en-12-oic acid lactone): The dehydrocostus lactone was isolated and identified by MS, IR and $^1$H and $^{13}$C NMR (Hikino et al., 1964; Yuuya et al., 1999; de Kraker et al., 2001 & 2002; Hsu et al., 2009). Colourless crystals; MALDI-MS: m/z (% rel. int.): 230.13 (55) [M]$^+$; IR (KBr) v max cm$^{-1}$: 3100 (C=CH$_2$ str.), 1800 (C=O of γ lactone), 1762, 1644, 1641 (C=CH$_2$ str.) and 902 (C=CH$_2$ str.); $^1$H-NMR (CDCl$_3$; 300 MHz): δ 1.41 (CH$_2$(8), q, J = 13.2 Hz, 2H), 1.86 (CH$_2$(2), q, J = 13.2, 4.8, 2.7 Hz, 2H), 2.15 (CH$_2$(9), m, J = 12.3, 10.5, 5.7, 5.7 Hz, 2H), 2.52 (CH$_2$(3), t, J = 13.2, 7.5, 1.8 Hz, 2H), 2.86 (CH$_3$(5), s, J = 9.3, 3.0 Hz, 1H), 2.87 (CH$_3$(1), q, 1H), 2.94 (CH$_7$(7), m, 1H), 3.96 (CH$_6$(6), d, J = 9.3, 9.3 Hz, 1H), 4.81 (2CH$_2$(14&15), s, 4H), 5.48 (CH$_2$(12), s, J = 3.3 Hz, 2H); $^{13}$C-NMR (CDCl$_3$; 75 MHz): δ 169.27 (C-12), 165.27 (C-1’), 152.14 (C-4), 141.65 (C-2’), 139.11 (C-11), 137.20 (C-10), 126.80 (C-3’), 122.77 (C-13), 118.26 (C-14),
Results

113.64 (C-15), 78.42 (C-6), 74.29 (C-8), 73.74 (C-3), 62.30 (C-4’), 51.35 (C-5), 47.52 (C-7), 45.28 (C-1), 39.02 (C-2), 36.97 (C-9).

The MS, IR, \(^1^H\) and \(^{13}^C\) NMR spectral data agree with a molecular formula of C\(_{15}\)H\(_{18}\)O\(_2\) and literatures (Hikino et al., 1964; Yuuya et al., 1999; de Kraker et al., 2001 & 2002; Hsu et al., 2009). The structure of dehydrocostus lactone is shown in Fig. 14.

Cynaropicrin (8-Hydroxy-4- (2-hydroxymethyl-acryloyl)-3,6,9- trimethylene-decahydro-azuleno [4,5-b] furan-2-one): The cynaropicrin was isolated and identified MS, IR and \(^1^H\) and \(^{13}^C\) NMR (Yoshioka et al., 1971; Corbella et al., 1972; Cho et al., 1998; Yayli et al., 2006). Colourless crystals; MALDI-MS: \(m/z\) (% rel. int.): 330.15 (100) [M]+; IR (KBr) \(\nu_{\text{max}}\) cm\(^{-1}\): 3300 (O-H str.), 2945, 2870, 1762 (C=O str. of \(\gamma\)-lactone), 1670 (C=CH str.), 1459, 1442, 1410, 1390, 1315, 1295, 1252, 1213, 1185, 1145, 1090, 1060, 971, 950, 895, 878 and 840; \(^1^H\)-NMR (CDCl\(_3\); 300 MHz): \(\delta\) 1.71 (\(CH_2(2)\), t, \(J = 8.1\) Hz, 2H), 2.01 (O-H\((3&4')\) br s, 2H), 2.19 (\(CH_2(9)\), d, \(J = 12.0, 8.1\) Hz, 2H), 2.82 (\(CH_5\), d, 1H), 2.94 (\(CH_1\), t, \(J = 8.7\) Hz, 1H), 3.18 (\(CH_7\), d, 1H), 4.16 (\(CH_6\), t, \(J = 10.2\) Hz, 1H), 4.37 (\(CH_2(4')\) br s, 2H), 4.49 (\(CH_3\), t, \(J = 7.5\) Hz, 1H), 5.03 (\(CH_2(13)\), s, 2H), 5.12 (\(CH_8\), m, 1H), 5.38 (2\(CH_2(14&15)\), d, \(J = 1.8\) Hz, 4H), 5.98 (\(CH_2(3')\), s, 2H); \(^{13}^C\)-NMR (CDCl\(_3\); 75 MHz): \(\delta\) 169.27 (C-13), 165.27 (C-1’), 152.14 (C-4), 141.65 (C-2’), 139.11 (C-11), 137.20 (C-10), 126.80 (C-3’), 122.77 (C-12), 118.26 (C-14), 113.64 (C-15), 78.42 (C-6), 74.29 (C-8), 73.74 (C-3), 62.30 (C-4’), 51.35 (C-5), 47.52 (C-7), 45.28 (C-1), 39.02 (C-2), 36.97 (C-9).

The MS, IR, \(^1^H\) and \(^{13}^C\) NMR spectral data agree with a molecular formula of C\(_{19}\)H\(_{22}\)O\(_5\) and literatures (Yoshioka et al., 1971; Corbella et al., 1972; Cho et al., 1998; Yayli et al., 2006). The structure cynaropicrin is shown in Fig. 14.
5.7. Chromatographic profiling of isolated compounds by high performance thin-layer chromatography (HPTLC)

The identification of isolated compounds was confirmed by earlier reported $R_f$ values published in literatures (Table 6). The results of chromatographic profiling of individual compounds are tabulated as follows:

Table 6: TLC profile of isolated compounds.

<table>
<thead>
<tr>
<th>Name of compound</th>
<th>Mobile phase</th>
<th>Visualization agent</th>
<th>Observations and $R_f$ value</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Achillicin</td>
<td>Hexane/ ethyl acetate (EtOAc)/ chloroform (9:5:6)</td>
<td>EP reagent (0.2% w/v 4-dimethylaminobenzaldehyde and 3% v/v orthophosphoric acid in glacial acetic acid/water (50:50 v/v))</td>
<td>Blue spot 0.3</td>
<td>Banh-Nhu et al., 1979</td>
</tr>
<tr>
<td>Chamazulene carboxylic acid</td>
<td>$n$-hexane/ EtOAc/ MeOH (65:30:5)</td>
<td>UV irradiation</td>
<td>Dark blue spot 0.4</td>
<td>Ramadan et al., 2006</td>
</tr>
<tr>
<td>Rubiadin</td>
<td>Toluene/ ethyl acetate (85:15)</td>
<td>UV irradiation</td>
<td>Yellow orange spot 0.58</td>
<td>Rao et al., 2006</td>
</tr>
<tr>
<td>Mollugin</td>
<td>$n$-hexane/ ethyl acetate (95:5)</td>
<td>UV irradiation</td>
<td>Yellow spot 0.30</td>
<td>Gupta et al., 1999; Ho Li et al., 2001</td>
</tr>
<tr>
<td>Costunolide</td>
<td>Petroleum ether (60-80°C)/ acetone (9:1)</td>
<td>Anisaldehyde-sulphuric acid reagent</td>
<td>Greenish-blue spot 0.30</td>
<td>de Kraker et al., 2001; Vijayakannan et al., 2006</td>
</tr>
<tr>
<td>Dehydrocostus lactone</td>
<td>Petroleum ether (60-80°C)/ acetone (9:1)</td>
<td>Anisaldehyde-sulphuric acid reagent</td>
<td>Blue/ violet-coloured spot 0.35</td>
<td>de Kraker et al., 2001; Vijayakannan et al., 2006</td>
</tr>
<tr>
<td>Cynaropicrin</td>
<td>Chloroform/ acetone (60:20)</td>
<td>Anisaldehyde-sulphuric acid reagent</td>
<td>Violet colour 0.30</td>
<td>Corbella et al., 1972; Cho et al., 1998; Wagner and Bladt, 2009</td>
</tr>
</tbody>
</table>
**Figure 15:** Photograph of *Rubia cordifolia* Linn. and *Saussurea costus* (Falc.) Lipsch. (Syn. *Saussurea lappa* (Decne.) C. B. Clarke).
In this study TLC fingerprinting of individual crude drug extracts and isolated compounds revealed several bands, showed in Fig. 15. The $R_f$ values of isolated compounds were identified by comparing the individual plant extracts.
5.8. Animal study

5.8.1. *In-vitro* study (Spasmolytic activity in isolated tracheal strip)

Cumulative concentration-response curves of contraction and relaxation were expressed as a percentage of the maximal response for each substance. The degree of contraction or relaxation in tracheal strips was determined from the tension applied. Tested compounds and isoprenaline caused relaxation of carbachol contracted rat trachea in a concentration dependent manner, their pD₂ values are tabulated in Table 7. All test compounds and isoprenaline were able to ameliorate contraction induced by 30 μM carbachol.

The CCRC of carbachol revealed sealing effect on 100 μM concentration where 100 % contraction was achieved therefore, 30 μM carbachol was selected to attain sub-maximal response with the IC₅₀ 2.24 μM and pD₂ 5.65±0.63 (Fig. 16).

**Figure 16:** Cumulative concentration-response curves fitted by non-linear iterative regression analysis of carbachol. Responses are expressed as % contraction of the tension produced by the spasmogen. Vertical bars shows S.E. values, n = 6.

The CCRC of isoprenaline hydrochloride showed contraction induced by carbachol was completely diminished at 100 nM concentration of isoprenaline with the EC₅₀ 71.42 nM and pD₂ 7.2±0. 82 (Fig. 17).
**Figure 17:** Cumulative concentration-response curves fitted by non-linear iterative regression analysis of isoprenaline. The preparations were pre-contracted by carbachol. Responses are expressed as % relaxation of the tension produced by the spasmogen. Vertical bars shows S.E. values, n = 6.

The CCRC of achillicin revealed contraction induced by carbachol was completely diminished at 1 mM concentration of achillicin with the EC$_{50}$ 35.48 µM and pD$_2$ 4.45±1.16 (Fig. 18).

**Figure 18:** Cumulative concentration-response curves fitted by non-linear iterative regression analysis of achillicin. The preparations were pre-contracted by carbachol. Responses are expressed as % relaxation of the tension produced by the spasmogen. Vertical bars shows S.E. values, n = 6.
The CCRC of chamazulene carboxylic acid revealed contraction induced by carbachol was completely diminished at 100 µM concentration of chamazulene with the EC$_{50}$ 3.98 µM and pD$_2$ 5.41±2.07 (Fig. 19).

**Figure 19:** Cumulative concentration-response curves fitted by non-linear iterative regression analysis of chamazulene carboxylic acid. The preparations were pre-contracted by carbachol. Responses are expressed as % relaxation of the tension produced by the spasmogen. Vertical bars shows S.E. values, n = 6.

![Cumulative concentration response curve (CCRC)](image)

The CCRC of rubiadin revealed contraction induced by carbachol was completely diminished at 100 µM concentration of rubiadin with the EC$_{50}$ 5.62 µM and pD$_2$ 5.25±1.11 (Fig. 20).

**Figure 20:** Cumulative concentration-response curves fitted by non-linear iterative regression analysis of rubiadin. The preparations were pre-contracted by carbachol.
Responses are expressed as % relaxation of the tension produced by the spasmogen. Vertical bars shows S.E. values, n = 6.

The CCRC of mollugin revealed contraction induced by carbachol was completely diminished at 100 µM concentration of mollugin with the EC$_{50}$ 1.26 µM and pD$_2$ 5.90±2.02 (Fig. 21).

Figure 21: Cumulative concentration-response curves fitted by non-linear iterative regression analysis of mollugin. The preparations were pre-contracted by carbachol. Responses are expressed as % relaxation of the tension produced by the spasmogen. Vertical bars shows S.E. values, n = 6.
The CCRC of costunolide revealed contraction induced by carbachol was completely diminished at 100 µM concentration of costunolide with the EC\textsubscript{50} 1.26 µM and pD\textsubscript{2} 5.90±2.16 (Fig. 22).

**Figure 22:** Cumulative concentration-response curves fitted by non-linear iterative regression analysis of costunolide. The preparations were pre-contracted by carbachol. Responses are expressed as % relaxation of the tension produced by the spasmogen. Vertical bars shows S.E. values, n = 6.

<table>
<thead>
<tr>
<th>% Relaxation</th>
<th>-4</th>
<th>-2</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>log_{10} [costunolide] nM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The CCRC of dehydrocostus lactone revealed contraction induced by carbachol was completely diminished at 300 µM concentration of dehydrocostus lactone with the EC\textsubscript{50} 12.59 µM and pD\textsubscript{2} 4.91±1.04 (Fig. 23).
Figure 23: Cumulative concentration-response curves fitted by non-linear iterative regression analysis of dehydrocostus lactone. The preparations were pre-contracted by carbachol. Responses are expressed as % relaxation of the tension produced by the spasmogen. Vertical bars shows S.E. values, n = 6.

The CCRC of cynaropicrin revealed contraction induced by carbachol was completely diminished at 300 μM concentration of cynaropicrin with the EC\textsubscript{50} 7.94 μM and pD\textsubscript{2} 5.11±1.16 (Fig. 24).

Figure 24: Cumulative concentration-response curves fitted by non-linear iterative regression analysis of cynaropicrin. The preparations were pre-contracted by carbachol. Responses are expressed as % relaxation of the tension produced by the spasmogen. Vertical bars shows S.E. values, n = 6.

Judging from the CCRC (Fig. 17), exposure of tracheal strips to 3 X 10\textsuperscript{-6} M isoprenaline completely restored the spasm induced by submaximal concentration (30 μM) of carbachol. Among all test compounds, the most potent spasmolytic compound
was mollugin and costunolide, which was found to be equipotent while, chamazulene was also able to ameliorate the spasm induced by carbachol in tracheal smooth muscles in a significant manner. On the other hand, achiillicin was found to be least potent when compared to isoprenaline. The potency of isolated compounds was found in the order; costunolide ≥ mollugin > chamazulene > rubiadin > cynaropicrin > dehydrocostus lactone > achiillicin. We extended these preliminary in-vitro findings to the in-vivo level to further explore the possible mechanism of action responsible for spasmolytic activity of the most potent compounds on the basis of EC$_{50}$ values i.e. costunolide, mollugin and chamazulene, among all seven isolated compounds.

**Table 7:** Table showing different concentration ranges of individual reference standards & isolated compounds with pD$_2$ values.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Starting concentration in moles</th>
<th>Ending concentration in moles</th>
<th>n</th>
<th>EC$_{50}$ values</th>
<th>pD$_2$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbachol</td>
<td>1 X 10$^{-9}$</td>
<td>3 X 10$^{-4}$</td>
<td>6</td>
<td>2.24 µM</td>
<td>5.65±0.63</td>
</tr>
<tr>
<td>Isoprenaline</td>
<td>1 X 10$^{-12}$</td>
<td>3 X 10$^{-6}$</td>
<td>6</td>
<td>71.42 nM</td>
<td>7.20±0.82</td>
</tr>
<tr>
<td>Achiillicin</td>
<td>1 X 10$^{-12}$</td>
<td>3 X 10$^{-3}$</td>
<td>6</td>
<td>35.48 µM</td>
<td>4.45±1.16***</td>
</tr>
<tr>
<td>Chamazulene carboxylic acid</td>
<td>1 X 10$^{-12}$</td>
<td>3 X 10$^{-4}$</td>
<td>6</td>
<td>3.98 µM</td>
<td>5.41±2.07**</td>
</tr>
<tr>
<td>Rubiadin</td>
<td>1 X 10$^{-12}$</td>
<td>3 X 10$^{-4}$</td>
<td>6</td>
<td>5.62 µM</td>
<td>5.25±1.11**</td>
</tr>
<tr>
<td>Mollugin</td>
<td>1 X 10$^{-12}$</td>
<td>3 X 10$^{-4}$</td>
<td>6</td>
<td>1.26 µM</td>
<td>5.90±2.02*</td>
</tr>
<tr>
<td>Costunolide</td>
<td>1 X 10$^{-12}$</td>
<td>3 X 10$^{-4}$</td>
<td>6</td>
<td>1.26 µM</td>
<td>5.90±2.16*</td>
</tr>
<tr>
<td>Dehydrocostus lactone</td>
<td>1 X 10$^{-12}$</td>
<td>3 X 10$^{-4}$</td>
<td>6</td>
<td>12.59 µM</td>
<td>4.91±1.04***</td>
</tr>
<tr>
<td>Cynaropicrin</td>
<td>1 X 10$^{-12}$</td>
<td>3 X 10$^{-4}$</td>
<td>6</td>
<td>7.94 µM</td>
<td>5.11±1.16***</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SEM; n: number of rat tracheal strips; pD$_2$: negative log of IC$_{50}$. *P < 0.05; **P < 0.01; ***P < 0.001 as compared to isoprenaline.

**5.8.2. In-vivo study (Bronchospasmolytic activity in Wistar albino rat)**
To assess the efficacy of costunolide, mollugin and chamazulene in asthma, they were subject to pulmonary vascular permeability characteristic, haematological parameter and biochemical analysis. Apart from this these compounds were also subjected to change in airway immunohistochemistry.

5.8.2.1. Wet-to-dry (W/D) lung weight ratio

Wet/dry weight ratio was higher in the snc group (4.49±0.21) when compared with the unsn group (2.92±0.11). Pretreatment with 20 mg/kg b.w. of cham (3.63±0.18), moll (3.24±0.16), cost (3.13±0.16) significantly reduced (P<0.01) the wet/dry weight ratio which was increased during the OVA induced asthma (Fig. 25). However, comb group (2.96±0.15) was more significantly (P<0.001) able to decrease the W/D ratio when compared to other groups.

Figure 25: Pulmonary vascular permeability characteristics of Wistar albino rats: Wet/dry weight ratios were significantly altered by test compounds (Cham, Moll, Cos and comb), although wet/dry weight was highly significant in Comb group and less significant in Moll group when compared to Dexamethasone. Unsn, Unsensitized; Sn, Sensitized; SnC, Sensitized+Challange; Dexam, Dexamethasone; Cham, Chamazulene; Moll, Mollugin; Cost, Costunolide; Comb, Combination. Results are expressed as mean±SEM. *P<0.05, **P<0.01, ***P<0.001 compared to veh/PBS, #P<0.05, ##P<0.01, ###P<0.001 compared to OVA/OVA, and $P<0.05, $$$P<0.01, $$$$P<0.001 compared to Dexam.
5.8.2.2. Total leucocyte count & differential cell count in blood and BALF

The total leucocyte (11.8±0.59), eosinophil (12.1±0.60), neutrophil (31.7±1.58), monocyte (11±0.55), macrophage (11±0.55) and basophil (0.9±0.04) count was significantly (P<0.001) increased in the snc group when compared with unsn group (Fig. 26b and 26c) whereas lymphocyte (44.3±2.21) count was decreased significantly. Dexamethasone (1 mg/kg, i.p.) showed significant (P<0.001) suppressive effect on the total leucocyte, eosinophil, neutrophil, monocyte, macrophage and basophil count in the blood and BALF as compared to the snc group.

**Figure 26:** Effect of cham, moll, cost and comb on the recruitment of inflammatory cells in blood and BALF obtained from ovalbumin-induced asthma model in rat. Inflammatory cells were significantly altered in test groups, although highly significant results were observed in comb group and less significant in cham group when compared to dexa group. Unsn, Unsensitized; Sn, Sensitized; SnC, Sensitized+Challange; Dexta, Dexamethasone; Cham, Chamazulene; Moll, Mollugin; Cost, Costunolide; Comb, Combination. Results are expressed as mean±SEM. *P<0.05, **P<0.01, ***P<0.001 compared to veh/PBS, #P<0.05, ##P<0.01, ###P<0.001 compared to OVA/OVA, and $P<0.05, $P<0.01, $$$P<0.001 compared to Dexta.

**Figure 26a:** Total cell count (10^3/mm^3) infiltration in blood and BALF at the site of inflammation.

[Graph showing cell count in blood and BALF for different groups]

Comb and cost group more significantly inhibited (P<0.001) the total leukocytes (6.6±0.33; 7.1±0.35), eosinophil (4.5±0.22; 5.1±0.25), neutrophils
Results

(17.1±0.85; 18.1±0.90), monocytes (3.4±0.17; 4.2±0.21), macrophages (4.1±0.20; 4.5±0.21) and basophil (0.1±0.004; 0.1±0.005) in the both blood and BALF at the dose of 20 mg/kg b.w. p.o. (Fig. 26b and 26c), whereas it significantly restored (P<0.001) the lymphocyte count (74.9±3.74; 72.5±3.62), while cham and moll group also showed significant inhibition of total leukocytes (8.5±0.42; 7.5±0.37), eosinophil (8.6±0.43; 6.7±0.33), neutrophils (24.7±1.23; 20.8±1.04), monocytes (6.3±0.31; 5.63±0.28), macrophages (9.0±0.45; 7.0±0.35) and basophil (0.5±0.02; 0.37±0.01) in the both blood and BALF and restored the lymphocyte count (59.9±2.99; 66.5±3.32). Moll group showed equivalent effect as compared to dexe group. Cham group was not able to produce the promising results as compared to dexe group.

**Figure 26b:** The effect on differential cell count: lymphocytes, eosinophils, monocyte, basophil and neutrophils in the blood 24 h after challenge. Unsn, Unsensitized; Sn, Sensitized; SnC, Sensitized+Challange; Dexa, Dexamethasone; Cham, Chamazulene; Moll, Mollugin; Cost, Costunolide; Comb, Combination. Results are expressed as mean±SEM. *P < 0.05, **P < 0.01, ***P<0.001 compared to veh/PBS, #P < 0.05, ##P < 0.01, ###P<0.001 compared to OVA/OVA, and $P < 0.05, $$P < 0.01, $$$P<0.001 compared to Dexe.

**Figure 26c:** The percentage of alveolar macrophage (AM), lymphocytes, eosinophils and neutrophils in the lung lavage fluid 24 h after challenge. Unsn, Unsensitized; Sn, Sensitized; SnC, Sensitized+Challange; Dexa, Dexamethasone; Cham, Chamazulene; Moll, Mollugin; Cost, Costunolide; Comb, Combination. Results are expressed as
Results

mean±SEM. *P < 0.05, **P < 0.01, ***P<0.001 compared to veh/PBS, #P < 0.05, ##P < 0.01, ###P<0.001 compared to OVA/OVA, and $P < 0.05, $$P < 0.01, $$$P<0.001 compared to Dexa.

5.8.2.3. Biochemical parameter analysis in lung tissue homogenate and BALF

5.8.2.3.1. Platelet activating factor (PAF) estimation

PAF was measured in lung tissue homogenate and BALF of rats. Level of PAF significantly (P<0.001) elevated in snc group (1.98±0.091; 0.824±0.042) as compared to unsn (0.12±0.011; 0.019±0.001) and sn group (0.37±0.021; 0.053±0.002) in tissue as well as in BALF. This was the result of hyperresponsiveness to cholinergic stimulation through IgE-mediated mast cell degranulation. The increased levels of PAF level was significantly (P<0.001) shattered in pre-treated groups of isolated compounds. Out of all isolated compounds their combination (0.53±0.003; 0.109±0.005) and costunolide (0.72±0.036; 0.297±0.015) was found to be highest potent as compared to dexta group (0.91±0.045; 0.403±0.020). The test drugs reduced the PAF level in the order cost > moll > cham. Cham group (1.12±0.056; 0.514±0.026) were not able to produce significant inhibition of PAF level as compared to dexta (Fig.27).
**Figure 27:** Effect of cham, moll, cost and comb on the elevated levels of PAF in ovalbumin induced asthmatic rat blood and BALF. PAF levels were significantly restored in test groups, although highly significant results were observed in comb group and less significant in cham group when compared to dexa group. Unsn, Unsensitized; Sn, Sensitized; SnC, Sensitized+Challange; Dexa, Dexamethasone; Cham, Chamazulene; Moll, Mollugin; Cost, Costunolide; Comb, Combination. Results are expressed as mean±SEM. *P<0.05, **P<0.01, ***P<0.001 compared to veh/PBS, #P<0.05, ##P<0.01, ###P<0.001 compared to OVA/OVA, and $P<0.05, $$$P<0.01, $$$$P<0.001 compared to Dexa.

5.8.2.3.2. Interleukin-6 (IL-6) estimation

As shown in Fig. 28, the recruitment of the cells results significantly increase (P<0.001) in IL-6 levels of lung tissue homogenate and BALF fluids in snC group (221.83±11.09; 182.33±9.12) as compared with unsn (112.17±5.60; 89.5±4.47) and sn (126.33±6.32; 94.83±4.74) control animals. The level of IL-6 tended to be significantly (P<0.001) decreased by the systemic treatments with dexamethasone (154.33±7.72; 129.5±6.47) and cham (177.33±8.87; 138.67±6.93), moll (148.5±7.42; 122.17±6.11), cost (139.5±6.97; 113.83±5.69) & comb (130±6.50; 100.5±5.02) in the antigen-challenged rats. Moreover, comb group represents significantly restored the elevated levels of IL-6 as compared to dexa group. No significant difference was observed between the unsn & sn and dexa & moll groups in measured IL-6 levels. Moll group found to be equipotent whereas cham group was not able to significantly restore the elevated IL-6 levels as compared to dexa group.
**Figure 28**: Effects of systemic pre-treatments with cham, moll, cost and comb on increased IL-6 levels in lung tissue homogenate and in bronchoalveolar lavage fluid (BALF) in ovalbumin induced rat asthma model. Each bar represents the mean ±SE from 6 different animals. Unsn, Unsensitized; Sn, Sensitized; SnC, Sensitized+Challange; Dexa, Dexamethasone; Cham, Chamazulene; Moll, Mollugin; Cost, Costunolide; Comb, Combination. Results are expressed as mean±SEM. *P<0.05, **P<0.01, ***P<0.001 compared to veh/PBS, #P<0.05, ##P<0.01, ###P<0.001 compared to OVA/OVA, and $P<0.05, $$P<0.01, $$$P<0.001 compared to Dexa.

![Graph](image)

**5.8.2.3.3. Interleukin-8 (IL-8) estimation**

Analysis of the concentration of IL-8 in the lung tissue homogenate and BALF indicated that significantly higher (P<0.001) levels of IL-8 was detected in both from the asthmatic rats (72.67±3.63; 42.50±2.12), as compared with that in the unsensitized (46.33±2.31; 20.17±1.00) and sensitized (48.0±2.40; 24.33±1.22) groups of rats (**Fig. 29**). Furthermore, the levels of IL-8 in lung tissue homogenate and BALF from the asthmatic rats that had been treated group with dexamethasone (55.67±2.78; 31.0±1.55), chamazulene (61.33±3.07; 34.0±1.70), mollugin (54.17±2.70; 28.50±1.42), costunolide (50.0±2.50; 25.17±1.26) and combination (47.5±2.37; 22.83±1.41) were significantly (P<0.001) lower than that in the asthmatic rats with 0.2 % CMC (drug vehicle) treatment. Among all tested compound, costunolide and combination found to be highly potential (P<0.001) whereas mollugin was found to be equivalent action when compared to dexamethasone group. Chamazulene was not
able to produce significant inhibition of IL-8 when compared to standard group. Apparently, treatment with all tested compounds inhibited the ovalbumin-induced pro-inflammatory cytokine production in the lungs.

**Figure 29:** Pre-treatment with cham, moll, cost and comb decreases the level of IL-8 in the lungs of rats. The levels of IL-8 in lung tissue homogenate and BALF of individual rats were analysed by ELISA. Data shown are mean values for each group of rats (n = 6). Unsn, Unsensitized; Sn, Sensitized; SnC, Sensitized+Challange; Dexa, Dexamethasone; Cham, Chamazulene; Moll, Mollugin; Cost, Costunolide; Comb, Combination. Results are expressed as mean±SEM. *P<0.05, **P<0.01, ***P<0.001 compared to veh/PBS, #P<0.05, ##P<0.01, ###P<0.001 compared to OVA/OVA, and $P<0.05, $$P<0.01, $$$P<0.001 compared to Dexa.

![Graph showing IL-8 levels in lungs and BALF](image)

5.8.2.3.4. Eosinophil peroxidase (EPO) assay

Ovalbumin induced asthmatic (1.953±0.098; 0.689±0.034) animals were found to produce high level (P<0.001) of EPO, an eosinophil marker, which was highly expressed after 24 hrs on instillation of ova aerosol challenge (**Fig. 30**) when compared to unsensitized animals (0.172±0.008; 0.061±0.003) instilled with normal saline & PBS and sensitized animals (0.692±0.035; 0.092±0.005). Depletion of the complement system by pre-treatment with dexa (0.558±0.028; 0.117±0.006), cham (0.602±0.030; 0.123±0.006), moll (0.587±0.029; 0.119±0.006), cost (0.547±0.027; 0.103±0.005) and comb (0.522±0.026; 0.099±0.005) had a significant (P<0.001)
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deterioration in the EPO levels of lung tissue homogenate and BALF. Comb was able to indicated maximum (P<0.001) inhibition whereas cost and moll groups showed equivalent inhibition of EPO when compared to dexe group. Cham pre-treatment did not significantly affect the ovalbumin-induced increase in EPO activity in the lung.

Figure 30: Effect of cham, moll, cost and comb pre-treatment on EPO activities recovered in the lung tissue homogenate and BALF after 24 hr of ova aerosol challenge. Data shown are optical density (OD) at 492 nm for each group of rats (n = 6). Unsn, Unsensitized; Sn, Sensitized; SnC, Sensitized+Challange; Dexe, Dexamethasone; Cham, Chamazulene; Moll, Mollugin; Cost, Costunolide; Comb, Combination. Results are expressed as mean±SEM. *P<0.05, **P<0.01, ***P<0.001 compared to veh/PBS, #P<0.05, ##P<0.01, ###P<0.001 compared to OVA/OVA, and $P<0.05, $$$P<0.01, $$$$P<0.001 compared to Dexe.

5.8.2.3.5. Superoxide dismutase (SOD) estimation

Ovalbumin significantly (P<0.001) decreased the level of SOD in the OVA sensitized asthmatic rats (53.33±2.67; 34.33±1.72) when compared with un-sensitized (102.17±5.11; 86.33±4.32) and sensitized (99.17±4.96; 74.83±3.74) group (Fig. 31). Treatment with Dexe (86.50±4.32; 68.17±3.41), cham (74.66±3.73; 51.83±2.59), moll (91.17±4.56; 65.17±3.26), cost (98.66±4.93; 71.33±3.57) and comb (101.66±5.08; 76.50±3.82) groups significantly restored (P<0.001) the level of SOD. The potency of isolated compounds in SOD activity was fond to be in the order Comb > Cost > Moll > Cham.
Figure 31: Effect of cham, moll, cost and comb on superoxide dismutase (SOD) content in ovalbumin induced asthmatic rats. Data shown are mean values for each group of rats (n = 6). Unsn, Unsensitized; Sn, Sensitized; SnC, Sensitized+Challenge; Daxa, Dexamethasone; Cham, Chamazulene; Moll, Mollugin; Cost, Costunolide; Comb, Combination. Results are expressed as mean±SEM. *P<0.05, **P<0.01, ***P<0.001 compared to veh/PBS, #P<0.05, ##P<0.01, ###P<0.001 compared to OVA/OVA, and $P<0.05, $$P<0.01, $$$P<0.001 compared to Dexa.

5.8.2.3.6. Tumor necrosis factor alpha (TNF-α) estimation

The amount of TNF-α liberated into lung tissue homogenate and BALF during the asthmatic condition (268.50±13.42; 119.66±5.98), unsensitized (89.33±4.47; 13.83±0.69) & sensitized (93.17±4.66; 20.50±1.02) control, dexamethasone (119.66±5.98; 31.83±1.59), chamazulene (126.83±6.34; 39.66±1.98), mollugin (118.33±5.92; 32.17±1.61), costunolide (107.17±5.36; 24.33±1.22) and combination (92.0±4.60; 18.50±0.92) pre-treated group rats was measured by ELISA as shown in Fig. 32. Marked levels of TNF-α was overproduced in serum of immunized rats which was significantly (P<0.001) inhibited in the dexa and test compounds treated groups with comb and cost showing the maximum protection followed by moll and cham treatment.

Figure 32: Effect of chamazulene, mollugin, costunolide and combination on TNF-α content in ovalbumin induced asthmatic rats. Data shown are mean values for each
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group of rats (n = 6). Unsn, Unsensitized; Sn, Sensitized; SnC, Sensitized+Challenge; D exa, Dexamethasone; Cham, Chamazulene; Moll, Mollugin; Cost, Costunolide; Comb, Combination. Results are expressed as mean±SEM. *P<0.05, **P<0.01, ***P<0.001 compared to veh/PBS, †P<0.05, ††P<0.01, †††P<0.001 compared to OVA/OVA, and $P<0.05, $$P<0.01, $$$P<0.001 compared to Dexa.

5.8.2.3.7. Nitric oxide (NO) estimation

NO levels were found to be significantly increased (P<0.001) in case of snC group (52.17±2.61; 38.50±1.92) compared to unsn (23.83±1.19; 15.83±0.79) and sn (25.66±1.28; 19.83±0.99) controls. This is indicative that sensitising the animals twice with BSA followed by challenge with OVA has increased in IgE levels, resulting in hypersensitive reactions. The increased levels of NO correlates with increased IgE levels. Furthermore in the treatment combination of isolated compounds was found to significantly (P<0.001) reduce the elevated levels of NO in comparison to other treated group in both lung homogenates and BALF. The other isolated compounds significantly reduced (P<0.001) the elevated levels of NO in the order cost > moll > cham. When compared to standard dexa (32.66±1.63; 23.17±1.16) moll (31.00±1.55; 22.33±1.12), cost (28.17±1.41; 19.17±0.96) and comb (24.33±1.22; 16.66±0.83) groups were found to be significantly more effective whereas cham (36.16±1.81; 28.00±1.40) was less effective than standard dexa group (Fig. 33).
**Figure 33:** Effect of different treatments on lung tissue homogenate and BALF nitric oxide concentration content in ovalbumin induced asthmatic rats. Data shown are mean values for each group of rats ($n = 6$). Unsn, Unsensitized; Sn, Sensitized; SnC, Sensitized+Challenge; Dexa, Dexamethasone; Cham, Chamazulene; Moll, Mollugin; Cost, Costunolide; Comb, Combination. Results are expressed as mean±SEM. *P<0.05, **P<0.01, ***P<0.001 compared to veh/PBS, #P<0.05, ##P<0.01, ###P<0.001 compared to OVA/OVA, and $^\|$P<0.05, $$^\|\|P<0.01, $$$^\|\|\|P<0.001$ compared to Dexa.

5.8.2.3.8. Lung lavage protein concentration

Due to the immunomodulation by ovalbumin induced asthma, the level of total protein in asthmatic rats (4.48±0.22) significantly (P<0.001) elevated as compared to unsensitized (0.99±0.05) and sensitized (1.51±0.07) control. Dexamethasone (2.17±0.10), chamazulene (2.93±0.15), mollugin (1.89±0.09), costunolide (1.75±0.09) and combination (1.14±0.06) pre-treated groups were significantly restored the uplifted protein levels. **Fig. 34** represents the effect of isolated compounds on total proteins levels in BALF. There was a marked increase in total protein snc rats. These alterations were reversed back to near normal in all treatment groups. All drug treatment found to show significant (P<0.001) action as compare to dexa group.

**Figure 34:** Effect of cham, moll, cost and comb on BALF total protein concentration content in ovalbumin induced asthmatic rats. Data shown are mean values for each group of rats ($n = 6$). Unsn, Unsensitized; Sn, Sensitized; SnC, Sensitized+Challenge; Dexa, Dexamethasone; Cham, Chamazulene; Moll, Mollugin; Cost, Costunolide;
Comb, Combination. Results are expressed as mean±SEM. *P<0.05, **P<0.01, ***P<0.001 compared to veh/PBS, #P<0.05, ##P<0.01, ###P<0.001 compared to OVA/OVA, and $P<0.05, $$P<0.01, $$$P<0.001 compared to Dexa.

5.8.2.4. Airway immunohistochemistry

Hematoxylin and eosin (H&E) staining were performed on the lung tissues to analyse the effects of isolated compounds on the histological feature of asthma. Histological sections of lung tissue from rat exposed to OVA exhibited airway inflammation and were found to have serious pathological changes such as swollen walls and extensive infiltration of eosinophils & mononuclear cells in the peribronchial regions of the lung, around pulmonary blood vessels and airways in the lung interstitium (Fig. 35). Conversely, airway inflammation was decreased in sections of lung tissue obtained from rats that were treated with dexamethasone, chamazulene, mollugin, costunolide and combination. These findings demonstrate that all isolated compounds have the potential to counteract allergic asthma-associated airway remodelling.

Figure 35: Effects of isolated compounds on airway inflammation. Representative haematoxylin-eosin stained sections of lung from: (A) Normal saline sensitized and PBS-challenged rats; (B) Bovine albumin sensitized and PBS-challenged rats; (C) Bovine albumin sensitized and OVA-challenged rats; (D) Bovine albumin sensitized and OVA-challenged mice treated with dexamethasone; (E) Bovine albumin
sensitized and OVA-challenged mice treated with chamazulene; (F) Bovine albumin sensitized and OVA-challenged mice treated with mollugin; (G) Bovine albumin sensitized and OVA-challenged mice treated with costunolide; (H) Bovine albumin sensitized and OVA-challenged mice treated with combination. The left panel is magnified × 100, scale bars = 100 μm. The right panel is magnified × 400, scale bars = 50 μm.