Studies on cytotoxic and phytotoxic potential of Mallotus tetracoccus (Roxb.) Kurz leaf extracts.

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Abstract: The present study was aimed to investigate the cytotoxicity and phytotoxicity of various organic extracts from the leaves of medicinal plant Mallotus tetracoccus (Roxb.) Kurz. (MT). The leaf powders of MT were extracted with different solvents such as petroleum ether (MT1), chloroform (MT2), ethyl acetate (MT3), acetone (MT4) and aqueous ethanol (MT5). The cytotoxicity of extracts was determined by using brine shrimp larvae. The phytotoxicity of plant extracts was assessed by using radish seeds (Raphanus sativus L.) by two methods: root, shoot length inhibition and seed germination studies. The ethanolic extract showed significant cytotoxicity value of 6.33 ± 0.8 µg/mL out of five extracts. The radish inhibition was observed in the order of MT3>MT2>MT4>MT5>MT1. There was considerable shoot length inhibition at 10,000 ppm for all MT extracts than root length. The radish seed germination rate for MT extracts was observed in the order of MT5>MT1>MT4>MT3>MT2. At 10,000 ppm, low germination index of 0.597 ± 0.089 and 0.769 ± 0.077 % were exhibited by ethyl acetate and chloroform extracts of MT. Thus the MT plant leaves in general was found to possess significant cytotoxic as well as allelopathic potential, which can be used as potential herbicide.

Key words: Mallotus tetracoccus, cytotoxicity, phytotoxicity, radish seed, Artemia salina.

Introduction

Medicinal plants are still used as radical scavengers to fight against many important diseases, such as cancer. The active phytochemical compounds present in the plants such as carotenoids, terpenoids, flavonoids, polyphenols, alkaloids, tannins, saponins possess various activities which

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help in scavenging the free radicals produced during initiation or progression of diseases [1]. Mallotus tetracoccus (Roxb.) Kurz. of family Euphorbiaceae, found in Western Ghats of India. The common names include Thavatta, Vatta, Vatta kumbil, Vetta kumbil (malayalam), Uppale mara (kannada) and “vatta kanni” in Tamil. Several species of the genus Mallotus are a rich source of biologically active compounds such as phloroglucinols, tannins, terpenoids, coumarins, benzopyrans and chalcones [2-5]. The reported bioactivities of the extracts or the individual chemical constituents isolated from this genus include antipyretic [6], anti-inflammatory, hepatoprotective [7], antioxidant and radical scavenging activities [8].

May and Ash has defined allelopathy as a mechanism in which allelochemicals produced by neighboring weeds for competition, may either have an inhibiting or stimulating effect on other plant growth [9], studied by observing the seed germination and plant growth. Weed species grow stronger, show competition on neighboring crops for light, water and nutrients, affecting the crop yields. In order to control weed growth application of chemical herbicides result in side effects affecting humans as well as causing environmental hazards, so herbicides from natural resources are preferred. Plants naturally produce a large amount of water-soluble toxins termed allelochemicals, released from leaves, flowers, seeds, stems and roots, to compete with other invaders present in their environment [10]. These chemical compounds are products of secondary metabolism of plants, such as alkaloids, phenols and terpenoids, affect the target plants seed germination, seedling growth, cell division and fungal activity [11]. Thus it is necessary to identify the compounds responsible for allelopathic effects which can be useful for developing green growth promoters [12].

The volatile compounds present in the MT leaf extract showed the presence of various chemical constituents such as bis (2-ethyl hexyl) phthalate (46.78 %), 3-methyl-2-(2-oxypropyl) furan (13.31 %), E-8-methyl-9-tetradecen-1-ol acetate (6.63 %), octadecanoic acid, 2-oxo (4.46 %) and longiborneol (2.39 %) analysed by GC-MS [13]. The bark extract of MT reported for higher phenolics, flavonoids and significant antioxidant potential [14]. Thus the objective was to study phytotoxicity using radish (Raphanus sativus L.) and cytotoxic studies using brine shrimps (Artemia salina) on different extracts of Mallotus tetracoccus leaves.

Materials and Methods

Collection of plant material
Healthy leaves of Mallotus tetracoccus were collected from Agasthiar Malai Reserve Forest, Western Ghats, South India, authenticated by the Director, Centre for Biodiversity and Forest Studies, Madurai Kamaraj University, and voucher specimens were deposited in the herbarium of Centre for Biodiversity and Forest Studies of our university (No.AM-03).

Extraction of plant material
The leaves were shade dried, powdered and packed in small packets and extracted successively with different solvents such as petroleum ether, chloroform, ethyl acetate, acetone and 70 % ethanol in the increasing order of polarity using soxhlet apparatus. The different solvent extracts
were filtered, concentrated, vacuum dried at 45°C for solvent removal and refrigerated in sterile bottles until use. The MT1, MT2, MT3, MT4 and MT5 indicate pet ether, chloroform, ethylacetate, acetone and aqueous ethanol extracts of MT respectively.

**Cytotoxicity bioassay using brine shrimps**

Brine shrimp cytotoxicity assay was performed according to the standard procedure described by McLaughlin, 1991 [15]. Brine shrimps (*Artemia salina*) were hatched using brine shrimp eggs in a conical flask (1L), filled with sterile artificial seawater (prepared using sea salt 38 g/L and adjusted to pH 8.5 using 1N NaOH) under constant aeration for 48 h. After hatching, active nauplii free from egg shells were collected from brighter portion of the hatching chamber and used for the assay. Twenty nauplii were drawn through a glass capillary and placed in each vial containing 4.5 ml of brine solution. In each experiment, 0.5 ml of the extract was added to 4.5 ml of brine solution and maintained at room temperature for 24 h under the light and surviving larvae were counted with a hand lens. Experiments were conducted along with control (vehicle treated), different concentrations (1-1000 µg/ml) of the test substances in triplicates.

**Phytotoxicity studies using radish seeds**

The phytotoxic properties of the various extracts of plant were evaluated using radish seed phytotoxicity assay [16, 17]. Two type of determination were done for this purpose:

**Root Length Determination**

Radish seeds were washed with distilled water and with 1 % mercuric chloride. Whatman #1 filter paper was kept on Petri dish and 5 ml of the plant extracts (100 ppm, 1000 ppm and 10,000 ppm) were added separately. Filter paper was dried at room temperature for reducing extra solvent. Five ml of double distilled water was added and then 20 radish seeds were placed on Petri dishes followed by tightly sealing and incubation at 23 ± 2°C. Root length was measured after 1, 3 and 5 days of interval. Only double distilled water containing Petri dish with seeds were used as control. Each assay was carried out in triplicates.

**Seed Germination Determination**

This part of the determination is similar to that of earlier determination except for the extract concentrations and number of seeds. Here two different concentrations (1000 ppm and 10,000 ppm) and 100 radish seeds were used. Germinated seeds were counted after every day up to 5 days. Each assay was carried out in triplicates.

**Data analysis**

The following parameters were adopted in this analysis to evaluate the conditions of seed germination: Relative germination rate and Germination Index. They were calculated based on the following equations according to previous reports [18]:

Relative germination rate = \([\text{Seeds germinated in test sample}/\text{Seeds germinated in control}] \times 100\)

Relative root elongation = \([\text{Mean root length in test sample}/\text{Mean root length in control}] \times 100\)
Germination Index = [Relative germination rate * Relative root elongation] / 100.

**Statistical analysis**

The values are presented as mean ± SD (standard deviation) of triplicate measurements. Multiple comparisons between more than two groups were performed by one way ANOVA supplemented with Duncan’s multiple range post hoc tests. Values at P < 0.05 were considered to indicate statistical significance.

**Results**

**Brine shrimp cytotoxicity bioassay**

Among the five extracts of *Mallotus tetracoccus*, all of them exhibited potent cytotoxicity against brine shrimp, where the LD₅₀ values where less than 100 µg/mL. LD₅₀ values for MT extracts were in the range of 6.33 ± 0.8 to 22.86 ± 1.658 µg/mL, where ethanolic extract showed noteworthy cytotoxicity value of 6.33 µg/mL (Table I). The MT ethyl acetate and acetone extracts showed almost similar LD₅₀ value of 12.36 ± 1.643 and 13.17 ± 1.88 µg/mL respectively. The MT pet ether and chloroform extracts showed LD₅₀ values of 22.86 ± 1.658 and 19.06 ± 1.259 µg/mL respectively. The results on brine shrimps assay indicate that the extracts had LC₅₀ value lesser than 20 µg/ml; the recommended cutoff point for detecting cytotoxic activity [19].

Table I: Percentage (%) Mortality of brine shrimps at different concentrations of MT extracts and their respective LD₅₀ value.

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Percentage (%) Mortality at different concentrations of the plant extracts</th>
<th>LD₅₀ (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 (µg/mL)</td>
<td>5 (µg/mL)</td>
</tr>
<tr>
<td>MT1</td>
<td>16.67</td>
<td>26.67</td>
</tr>
<tr>
<td>MT2</td>
<td>10</td>
<td>30</td>
</tr>
<tr>
<td>MT3</td>
<td>13.33</td>
<td>36.67</td>
</tr>
<tr>
<td>MT4</td>
<td>16.67</td>
<td>36.67</td>
</tr>
<tr>
<td>MT5</td>
<td>26.67</td>
<td>46.67</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SD of three determinations. Means marked with different letters, within each column, are significantly different (p < 0.05).

Results for percentage mortality of brine shrimp indicates that highest percentage was observed at 500 µg/mL by most of the extracts tested. At a concentration of 10 µg/mL, only ethanolic and...
ethyl acetate extracts of MT presented significant mortality values of 53.33 %; 60 % respectively (table I).

**Radish Seed Phytotoxicity Assay**
For radish (*Raphanus sativus L.*). seed germination, root, shoot length and percent of seed germination were determined. From the root length inhibition and seed germination percentage, germination index were calculated.

**Radish seedling root length inhibition**
The effect of three different concentrations (10,000, 1000 and 100 ppm) of the plant extracts were studied on root growth inhibition or stimulation of radish seedling. The radish root and shoot length at fifth day of study were observed.

All extracts of MT inhibited root growth at 10,000 ppm. The radish root length for MT extracts were observed in range of 22.85 ± 0.427 to 32.8 ± 0.681 mm for 100 ppm, 10.1 ± 0.486 to 28.7 ± 1.021 mm for 1000 ppm and 3.02 ± 0.176 to 9.9 ± 0.651 mm for 10,000 ppm when compared with control length of 60.07 ± 1.706 mm (Figure I). At 100, 1000 ppm concentrations, all MT extracts exhibited inhibition in the range of 38.04 ± 0.711 to 54.55 ± 1.133 % and 16.79 ± 0.808 to 47.76 ± 1.7 % respectively. At 10,000 ppm concentration of plant extracts, the ethyl acetate and chloroform extracts showed root inhibition of 5.02 ± 0.292 and 5.66 ± 0.44 % respectively. Thus the radish inhibition were observed in the order of MT3>MT2>MT4>MT5>MT1 (Table II).

Figure I: Effect of different concentrations (100, 1000 and 10,000 ppm) of MT extracts on radish root length at fifth day. Values with similar letters don’t show significant difference (p>0.05).
Table II: Percentage of radish root growth inhibition/stimulation at different concentrations of plant extracts.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Root growth inhibition/stimulation at concentration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100 ppm</td>
</tr>
<tr>
<td>MT1</td>
<td>50.25±1.369&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>MT2</td>
<td>38.04±0.711&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>MT3</td>
<td>42.98±0.958&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>MT4</td>
<td>45.28±1.04&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>MT5</td>
<td>54.55±1.133&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SD of three determinations. Means marked with different letters, within each column, are significantly different (p < 0.05).

Similarly the shoot length of radish seeds after five days were recorded. The radish shoot length for MT extracts were observed in range of 11.2 ± 0.643 to 20.3 ± 1.130 mm for 100 ppm, 8.1 ± 0.293 to 16.8 ± 0.765 mm for 1000 ppm and 1 ± 0.379 to 7.6 ± 0.493 mm for 10,000 ppm when compared with control length of 21.94 ± 0.487 mm. Thus there was high shoot length inhibition at 10,000 ppm for all MT extracts (Figure II).

Radish seed germination

The radish seed germination percentage of all the extracts were studied at two different concentrations 1000 and 10,000 ppm (Figure IIIa and IIIb). For 1000 ppm concentration of MT extracts tested, germination rate observed were in the range of 41.46 ± 0.604 to 72.47 ± 0.604 %.
At 10,000 ppm, lowest rates of 11.85 ± 1.207 and 13.59 ± 1.045 % were observed by ethyl acetate and chloroform extracts of MT. High germination was observed in MT ethanolic and petroleum ether extracts (62.02 ± 0.604 and 61.67 ± 1.045 % respectively). Thus the radish seed germination rate for MT extracts were observed in the order of MT5>MT1>MT4>MT>MT2 (Table III). At 1000 ppm, lowest and highest germination index of 6.96 ± 0.430 and 32.94 ± 0.810 % were exhibited by chloroform and ethanolic extracts of MT. At 10,000 ppm, low germination index of 0.597 ± 0.089 and 0.769 ± 0.077 % were exhibited by ethyl acetate and chloroform extracts of MT. Thus the germination index in the range of 0.597 ± 0.089 to 10.13 ± 0.768 % shows highest level of inhibition by the extracts at higher concentrations (Table III).

Figure III: Effect of different concentrations (a. 1000 ppm and b. 10,000 ppm) on seed germination of MT extracts at fifth day. Values with similar letters don’t show significant difference (p>0.05).
Table III: Effect of plant extracts on seed germination rate and germination index.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Relative germination rate (%)</th>
<th>Germination index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT1</td>
<td>72.47±0.604a</td>
<td>28.96±0.943a</td>
</tr>
<tr>
<td></td>
<td>61.67±1.045f</td>
<td>10.13±0.768f</td>
</tr>
<tr>
<td>MT2</td>
<td>41.46±0.604b</td>
<td>6.96±0.430b</td>
</tr>
<tr>
<td></td>
<td>13.59±1.045g</td>
<td>0.769±0.077g</td>
</tr>
<tr>
<td>MT3</td>
<td>43.90±1.045c</td>
<td>10.22±0.521c</td>
</tr>
<tr>
<td></td>
<td>11.85±1.207g</td>
<td>0.60±0.089g</td>
</tr>
<tr>
<td>MT4</td>
<td>58.89±0.604d</td>
<td>20.93±0.769d</td>
</tr>
<tr>
<td></td>
<td>44.60±1.597h</td>
<td>6.19±0.410h</td>
</tr>
<tr>
<td>MT5</td>
<td>68.99±1.045e</td>
<td>32.94±0.810f</td>
</tr>
<tr>
<td></td>
<td>62.02±0.604f</td>
<td>9.48±0.077f</td>
</tr>
</tbody>
</table>

Discussion

**Brine shrimp cytotoxicity bioassay**

Brine shrimp lethality bioassay (BSLT) is the general bioassay for detecting broad spectrum of bioactivity present in plant crude extracts [20]. The following study results on the extracts of *Mallotus tetracoccus* leaves showed that the ethanolic and ethyl acetate extracts revealed higher level of cytotoxicity when compared to other extracts. Similar study report by researchers also show that the polar fractions exhibited high cytotoxic activity than others. The methanolic extracts of *Physalis minima* and *Argemone Mexicana* showed LC₅₀ values of 36.67 and 54.42 µg/mL [21]. The stem bark, leaf and root extracts of *Combretum adenogonium* showed LC₅₀ values of 65.77, 76.97 and 110.04 µg/mL respectively [22]. The leaf extract of *Mesua ferrea* exhibited cytotoxicity value at doses of 500 ppm [23]. Five Bangladesh medicinal plants were studied for cytotoxicity, of which highest activity were observed only in *Curcuma longa* (26.63 µg/mL), whereas other plants *Curcuma zedoaria*, *Streblus asper*, *Enhydra fluctunas* and *Scoparia dulcis* exhibited activity at dose of >150 µg/mL [24]. Forty five Medicinal plants of Kenya possessing antimalarial activity were studied for cytotoxicity of which only 23 (51 %) plants showed activity at concentrations of 100 µg/mL [25]. Thirty one medicinal plants of eastern Nicaragua studied for cytotoxicity showed that only 4 (13%) plants exhibited activity at dose of <1000 µg/mL, whereas other 27 plants showed activity >1000 µg/mL [26]. On comparison of our study results with other research work, our extracts possessed significant cytotoxic activity at concentrations of <50 µg/mL.

**Radish Seed Phytotoxicity Assay**

Phytotoxicity is said to be an important attribute in determination of allelopathic potential of a plant species [27]. The phytotoxicity study on MT extracts showed that activity is found at higher concentrations. Our study reports are similar to study reports by other researchers too. The aqueous extracts of *Nicotiana glauca* Graham (stems, roots and fruits) were evaluated for phytotoxicity on two crops (lettuce and radish), where seed germination and root length inhibition was between 15 and 100 %, due to the presence of phenolics. Root length inhibition was more obvious than shoot length, which is an indicator of phytotoxicity [28].
Studies on cytotoxic and phytotoxic potential of Mallotus………

The essential oils of Salvia hierosolymitana Boiss. and Salvia multicaulis Vahl. var. simplicifolia Boiss. studied for phytotoxic effects, showed that extracts inhibited and promoted radish seed germination at dose of 0.625 μg/mL and 0.24 μg/mL. Radical elongations of radish were inhibited significantly in response to 0.125 μg/mL and 1.25 μg/mL of Salvia multicaulis var. simplicifolia. Radical elongation seemed to be more affected than germination [29].

Thus the overall cytotoxic and phytotoxic activity of Mallotus tetracoccus extracts might be due to the presence of volatile terpenoid compounds such as longiborneol (2.39 %); p-menth-8(10)-en-9-ol (a terpene alcohol, 1.49 %) and fatty acid esters (48.11 %) such as bis (2-ethyl hexyl) phthalate (46.78 %); di-n-octyl phthalate (1.33 %) analysed by GC-MS [13]. The qualitative study on MT extract illustrated the presence of sugars, tannins, alkaloid, flavonoid, steroids, terpenoids and phenolic acids (unreported results). Recently the cytotoxic studies on nanodrug from MT leaf extract showed cytotoxicity upto 76.8 to 84.9 % at 20-100μL concentrations [30].

Conclusion

The different extracts of Mallotus tetracoccus leaves studied for cytotoxicity and phytotoxicity studies confirmed that ethanolic extract revealed cytotoxic activity and ethyl acetate extract demonstrated high phytotoxicity on radish seeds. Thus isolation of pure compounds from the ethanolic and ethyl acetate extracts can be studied for in-vitro and in-vivo studies for further application as herbicide.

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