Larvicidal activity of Artemisia annua L. callus culture against Anopheles stephensi larvae

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Abstract: The emergence of resistance by both Plasmodium falciparum and Anopheles stephensi made the search for an alternative environmentally safe plant based insecticide inevitable. Artemisia annua is a well known antimalarial. Present study is an attempt to induce callus production from young leaves of Artemisia annua plant and study its larvicidal activity against larvae of Anopheles stephensi. Callus was initiated by using different concentrations of auxins and cytokinins. A suitable culture media was standardized for optimal growth of callus. Healthy callus cultures were obtained in the slightly modified Murashige and Skoog’s medium + NAA and BAP (0.03 and 0.2 mg l⁻¹ respectively) + Sucrose 20 gm l⁻¹ + Agar 6 gm l⁻¹ within 28 days of inoculation. Callus was successively extracted in order of increasing polarity of solvents. Larvicidal activity, in terms of lethal concentration (LC₅₀) of the callus extract in chloroform was calculated to be 18.45 ± 0.75 ppm after 72 hr against third instar larvae of A. stephensi.

Key words: Artemisia annua, Callus cultures, Larvicidal component

Introduction

The world is loosefight against malaria. That is the grim picture painted by a series of reports on malaria (Phillips, 2004). Malaria in humans is caused by the parasitic Plasmodium falciparum. Plasmodium falciparum accounts for the majority of infections and the vector Anopheles stephensi is responsible for the transmission of disease. Today the mosquito can breed anywhere from a rice field to a cupful of water and they are killed by spraying insecticides in ditches, and ponds (Bernard, 2006; Bansal and Singh, 2007; Shanmugasundaram et al., 2008). Development of resistance to various organic insecticides poses a serious threat to the conventional control measures against malaria vector (Bansal and Singh, 2006). This has necessitated the need for search and development of environmentally safe, biodegradable, low cost, indigenous methods for vector control, which can be used with minimum care by individual and communities in specific situations (Mittal and Subbarao, 2003; Setia et al., 2007).

Plants are an inexhaustible source of a diverse array of chemicals such as flavors, fragrances, natural pigments, pesticides and pharmaceuticals. This seemingly unrelated collection of chemicals could be grouped together under a broad category of plant secondary metabolites (Bhoywani et al., 2002). The continuous production of the desired bioactive compounds for pharmaceuticals industries requires sustained input of plant materials from nature. So, it is necessary to focus attention towards plant cell and organ cultures for the large scale production of bioactive compounds as an alternative to traditional agricultural based production (Fulzele, 2005). In callus culture, the culture conditions and variables can be more easily optimized and it also offers better selectivity and yield of the desired bioactive compound (Prakash et al., 2002). With the callus culture method production can be more controllably ensured in terms of the product quality and quantity, independent of geographical and climatic barriers. Many researches focused on the production of artemisinin, an antimalarial drug by callus cultures of A. annua (Martinez and Staba, 1988; Woerdenbag et al., 1993). One study reported the larvicidal activity of leaves of Artemisia annua (Tonk et al., 2006). But no study is reported about the larvicidal activity of A. annua callus. Present study focuses on the production of callus of A. annua and study of its larvicidal activity against larvae of Anopheles stephensi.

Materials and Methods

Plant material: Leaves of Artemisia annua were collected from botanic garden of Dayalbagh Educational Institute, Dayalbagh, Agra.

Production of callus culture of A. annua: Unorganized callus tissue of Artemisia annua was raised from the leaf segments. Young leaves were excised to 4-8 mm segments and washed under tap water for 15 min, followed by sterilization for 15 min with 1% NaOCl (sodium hypochlorite) solution by constant stirring. After that, leaves were soaked in 70% ethanol for 20-30 and finally washed 3-4 times with autoclaved distilled water and cultured in Murashige and Skoog’s (MS) medium (Murashige and Skoog, 1962). All experiments were done under aseptic conditions. MS media with different alterations in concentration of growth hormones i.e. auxin NAA and cytokinin BAP were tried. pH of the media was adjusted to 5.8 using dilute solution of NaOH and HCl (0.1 N). The medium (15-20 ml) was dispensed in test tubes and autoclaved at 15 psi (123°C for 15 min) after plugging with non-absorbent cotton wrapped in cheese-cloth. The tissue thus established was maintained on standardized medium by subculturings after four weeks in the test tubes containing approximately 20 ml fresh growth medium. Cultures were grown and maintained at 27± 2°C in dark. Each treatment had 24 replicates of culture and experiments were repeated twice.

Extraction of callus: Calli obtained were collected, air dried and weighed. The calli were successively extracted in petroleum ether,
Table - 1: The response of callus production from leaf segment to various modifications of MS medium

<table>
<thead>
<tr>
<th>Medium</th>
<th>Growth NAA (mg l⁻¹)</th>
<th>Regulators</th>
<th>Callus development (days)</th>
<th>Type of response</th>
<th>Survival of cultures (%)</th>
<th>Fresh / dry weight per culture tube (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS basal medium</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>No callus formation</td>
<td>30.0</td>
<td>-</td>
</tr>
<tr>
<td>50% concentration of MS salts*</td>
<td>0.05</td>
<td>0.2</td>
<td>40</td>
<td>Slow growth</td>
<td>16.6</td>
<td>11.2+3-20 / 4.40+2.1</td>
</tr>
<tr>
<td>MS basal medium</td>
<td>2.0</td>
<td>0.2</td>
<td>35</td>
<td>Callus with roots differentiation</td>
<td>35.0</td>
<td>23.1+4.1 / 10.1+4.3</td>
</tr>
<tr>
<td>MS basal medium</td>
<td>0.2</td>
<td>2.0</td>
<td>28</td>
<td>Callus with shoots differentiation</td>
<td>41.6</td>
<td>29.4+1.6 / 12.1+5.5</td>
</tr>
<tr>
<td>MS medium + 1 mg l⁻¹ Thiamine HCl</td>
<td>0.03</td>
<td>0.2</td>
<td>28</td>
<td>Healthy golden yellow unorganised callus</td>
<td>87.5</td>
<td>541.2+3.6/35.5+2.5</td>
</tr>
</tbody>
</table>

Each treatment has 24 replicates of culture, MS = Murashige skoog’s

Table - 2: Bioefficacy (lethal concentration i.e. LC₅₀) of extracts of A. annua callus against Anopheles stephensi

<table>
<thead>
<tr>
<th>Phyto-extract</th>
<th>Exposure period (hr)</th>
<th>LC₅₀ (mg l⁻¹)</th>
<th>Fiducial limit</th>
<th>Regression equation</th>
<th>Regression coefficient (R²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Upper</td>
<td>Lower</td>
<td></td>
</tr>
<tr>
<td>Chloroform</td>
<td>24</td>
<td>66.4</td>
<td>67.09</td>
<td>65.70</td>
<td>y=1.2946x+2.6411</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>25.22</td>
<td>25.87</td>
<td>24.56</td>
<td>y=0.9562x+3.6604</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>18.45</td>
<td>19.20</td>
<td>17.70</td>
<td>y=2.1889x+2.2321</td>
</tr>
</tbody>
</table>

For the callus extract stock solution of 500 ppm was prepared by dissolving 0.05 g of crude in 0.5 ml of acetone and volume raised to 100 ml with distilled water. From this, different dilutions of 10, 20, 30, 40, 60 and 80 ppm were prepared in 200 ml distilled water and 20 third instar larvae were released in it.

Percent mortality observed for each concentration (10 – 100 mg l⁻¹) of most effective crude i.e. chloroform extract obtained from callus was converted into probit by using probit table (Finney, 1971). By using these values regression equations along with regression co-efficient were obtained LC₅₀ value was calculated from these equations.

Bioassay: Anopheles stephensi third instar larvae used, were collected from various places with stagnant water in Dayalbagh, Agra. They were cleaned several times with distilled water. Bioefficacy of all the crude extracts were conducted according to WHO (1963) Technical Report Series. Values for the 50% lethal concentration (LC₅₀) of callus extract were calculated. Bioefficacy was also tested using acetone in blank. The acetone amount in blank was varied between 0.1 to 1.0% and tested against third instar larvae of Anopheles stephensi. The results showed no effect of acetone up to 0.5%.

For the callus extract stock solution of 500 ppm was prepared by dissolving 0.05 g of crude in 0.5 ml of acetone and volume raised to 100 ml with distilled water. From this, different dilutions of 10, 20, 30, 40, 60 and 80 ppm were prepared in 200 ml distilled water and 20 third instar larvae were released in it.

Results and Discussion

Young leaves were used to initiate callus culture in Murashige and Skoog’s medium. In order to check the best medium for callus production, cultures were grown in basal media (MS medium), and media supplemented with different concentrations of growth hormones i.e. auxin and cytokinin. The response of callus production to various modifications of MS medium has been shown in Table 1. Out of several media used, in MS medium supplemented with low concentrations of both the growth hormones i.e. NAA 0.03 mg l⁻¹ and BAP 0.2 mg l⁻¹ and adding 1 mg l⁻¹ Thiamine HCl, leaf responded within a week after showing an initial sign of curling. Callus formation occurred all over the surface after 15-20 days of culture. The callus was golden yellow, unorganized and appeared healthier in comparison with callus grown in other media, which were tried. Good growth was observed in 87.5% of cultures after 4 weeks (Fig. 1). The fresh weight of callus per culture tube also increased i.e. 80-1000 mg. All the cultures were kept in BOD incubator at 27+2°C in dark. These calli were maintained by subculturing after four weeks in same media. But after two weeks of subculturing, browning and differentiation of callus was observed. The browning of callus was probably due to oxidation of phenolic compounds produced by plant cells. Callus was extracted prior to browning and differentiation of callus to avoid loss of secondary compounds.

Yields obtained: Successive extraction of callus of Artemisia annua in petroleum ether, chloroform and methanol gave the following yields:
Percent mortality observed for each concentration of most effective crude i.e. chloroform extracts obtained from callus was converted into probit by using probit table (Finney, 1971). By using these values regression equations along with regression coefficient were obtained. \( LC_{50} \) value was calculated from these equations. \( LC_{50} \) values along with fiducial limits, regression equations and coefficient are presented in Table 2.

In earlier studies extracts of Artemisia annua cultures have been assessed for in vitro activity against the malarial parasite, Plasmodium falciparum both in n-hexane extract of the plant cell culture medium and in the chloroform extract of the cells. Trace amounts of the antimalarial sesquiterpene lactone artemisinin may account for the activity of the n-hexane fraction but only the methoxylated flavonoids artemetin, chrysoplenetin, chrysoplisol-D and cirsilineol can account for the activity of the chloroform extract. (Tawfiq et al., 1989; Liu et al., 1992). Scopoletin, a coumarin isolated from A. annua callus has been also reported to possess anti-inflammatory activity.

Larvicidal property, in terms of concentration \( (LC_{50} - 19.9 \text{ ppm after 72 hr}) \) in the leaves of A. annua has been reported by Tonk et al. (2006). The yield of active crude against Anopheles stephensi obtained from direct extraction of leaves in petroleum ether was too low i.e. 2%, while in the present study the yield obtained from chloroform extract of callus is 11.76%. The callus is further maintained by subculturing on fresh nutrient medium which results in greatly increased yield. So, by tissue culture technique, active crude can be extracted in high amount and it is also totally independent of seasonal variability and inconsistencies in the quality and quantity of active component. The present study shows the larvicidal activity of callus extract of A. annua. Larval mortality of callus extract was scored at different concentrations after 24, 48 and 72 hr in all the extracts. Petroleum ether and methanol extracts did not show larvicidal activity even at high concentrations (100 mg l\(^{-1}\)). In chloroform extract, larval mortality ranged from 18.3 - 63.3% at 24 hr as compared to 0% in control. At 48 hr it ranged from 36.6-73.3% as compared to 1.6% in control and 41.6 - 100% at 72 hr as compared to 3.6% in control. There was a progressive increase in larval mortality with the increase in concentration. The \( LC_{50} \) value of active principle from callus was calculated as 18.45 ppm after 72 hr.

An increment in the yield as well as the activity of the crude extract against Anopheles stephensi obtained from callus of Artemisia annua in comparison to the crude obtained from direct extraction of leaves, shows that the tissue culture technique is a promising alternative for the production of active principle against larvae of Anopheles stephensi.

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References


