Influence of 2,4-D and picloram on in vitro callus induction from Verbena bipinnatifida Nutt. and evaluation of in vivo anti-inflammatory activity of callus extract

Ezzat A. M. Genady

Department of Pharmacognosy, Faculty of Pharmacy, Al-Azhar University, Nasr City, Cairo,11651, Egypt.

Address For Correspondence: Ezzat A. M. Genady Department of Pharmacognosy, Faculty of Pharmacy, Al-Azhar University, Nasr City, Cairo,11651, Egypt.
E-mail: genady2004@yahoo.com

ABSTRACT

According to world health organization (WHO), infectious diseases are the first cause of death between population. Inflammation is a defense mechanism to minimize the damage by infection or irritation. Literature reports that medicinal plants are good way for preparation of modern drugs in pharmaceutical industry. The present study establish, for the first time, an efficient in vitro callus induction protocol from leaves explants of Verbena bipinnatifida Nutt. Family Verbenaceae by using different concentrations from 2,4-D (2,4-Dichlorophenoxy acetic acid) and picloram as well as in vivo anti-inflammatory activity of callus extract. The result showed that 2,4-D at concentration 4mg/L gave maximum degree of callus formation and callus induction percent (48.88 %) was recorded at 6mg/L, on other hand picloram at 2mg/L was the best concentration for degree of callus formation and callus induction percent (48.88 %). Furthermore, the anti-inflammatory effect experiment showed that 70% ethanol extract from callus at dose (50 mg/Kg, 100mg/Kg and 200/ Kg mg) exhibited dose dependent and significant anti-inflammatory activity in carrageenan induced hind paw edema.

INTRODUCTION

The wellbeing of human is directly proportional to the wellbeing of botanical around them (Okunlola, et al., 2016). The use of medicinal plants in health care is an old age practice and still an a part of medicine over all the world (Ghanbar, et al., 2016). The active constituents in medicinal plants are beneficial source for many drugs preparation due to the side effects and toxicity of synthetic drugs (Gaddaguti, et al., 2014). There are some difficulties in the propagation of many species of medicinal plants which lead to reduce the availability of several active constituents (Cherdshewasart, et al., 2007). Also, synthesis of bioactive substances in labs coast much many due to their structural complexity (Oksman, et al., 2004). Plant tissue culture is an alternative method for propagation of important medicinal plants especially which are sensitive to climatic changes (Rout, et al., 2000; Naika, et al., 2008). Callus induction is the unique technique in tissue culture for production of active constituents, many factors play important role in callus formation such as type of explants, composition of MS medium and environment (Renu, et al., 2011). Callus induction can be improved by employed of auxins and cytokinins, they enhance cell growth through stimulating cell division and elongation (coenen, et al., 1997). Verbena bipinnatifida Nutt. Family Verbenaceae cultivated in Egypt, as an ornamental plant (El-Hamouly, et al., 2000). Verbena bipinnatifida Nutt., contains important constituents such as flavonoids, iridoids and pentacyclic triterpene (Mohamed, et al., 2008), and has potential antimicrobial and hypotensive activity (El-Azizi, et al., 2000). This study described protocol for callus induction culture from Verbena bipinnatifida Nutt.
Family Verbenaceae cultivated in Egypt, using two growth regulators and as well as in vivo anti-inflammatory activity of callus extract. No published work on in vitro callus induction by using 2,4-D and picloram especially on this specie of Verbena which cultivated in Egypt.

MATERIALS AND METHODS

Plant materials:
Verbena bipinnatifida Nutt. fruits were collected from botanical garden of Medical Professions Syndicates Union Club, Nasr city, Cairo, Egypt. The plant was botanically confirmed by Mrs. Therese Labib consultant of Egyptian flora, Orman Garden, Giza, Egypt. Mature and healthy seeds were selected by physical appearance then kept at -20 ºC for two weeks before seedling.

Surface Sterilization:
Seeds sterilization is an essential step in tissue culture techniques to avoid contamination. The seeds were initially surface sterilized in 0.1% (w/v) fungicide for 10 min followed by soaking for 2 min in 70% (v/v) ethanol, then washed by sterile distilled water. Seeds were second surface sterilized with 30% commercial bleach (5.25% sodium hypochlorite) supplemented with few drops of Tween 20 for 20 min followed by rinsing for 3x5 min.,

Culture media for germination:
All components of MS medium (Murashige and Skoog, 1962) such as vitamins, macro and micronutrients, amino acids, carbon source etc. were freshly prepared before experiment and under aseptic conditions in laminar flow, the bench of the champer and all instruments were thoroughly cleaned before conduct experiment. After sterilization, fifteen seeds were cultured for each Petri dish on MS with 3.0 % sucrose and 0.8 % agar at 24± 2 ºC with 16/8-h (light/dark) photoperiod under 50 ± 5 % humidity in culture room for 15 days.

Culture media for callus induction:
MS medium contained 30% (w/v) sucrose supplemented with different concentrations of 2,4-D (0, 2, 4, 6 and 8 mg/L) and picloram (0, 1, 2, 3 and 4 mg/ L) as sole of growth regulators. The pH of the medium was adjusted to 5.7± 0.1 using 0.1 M NaOH or 0.1 M HCl and solidified with 0.8% (w/v) agar. The media were then autoclaved at 121 ºC for 20 min. explants from in vitro grown seedlings were used. Young leaves were cut in portion of about 0.25 cm² and the adaxial face was placed on the above MS medium. Cultures were then incubated in dark at 24±2 ºC for two weeks. Fifteen explants were cultured for each Petri dish and triplicate for each treatment. Analysis of the percentage of callus induction, morphology and color of the callus and intensity of callus growth were observed every five days.

Anti-inflammatory activity:
Experimental Animal:
Adult healthy female Wister albino rats weighting between 120-140gm were used for the study.

In vivo anti-inflammatory activity:
5gm from well dried hydro alcohol callus extract dissolve in 50 ml distilled water. The initial paw volume of each rat was noted by the usage of caliper. Thirty animals were used in this study and divided into 5 groups (six animals per each). Group-1 was served for carrageenan injection in the right hind paw at dose 0.1 ml. Group-2 was received diclofenac sodium at a dose of 6.75 mg/kg body weight, whereas group 3-5 received callus extract at three different doses. After thirty minutes from intraperitoneal injection and one hour from the administration of callus extract orally at a dose of (50) mg/kg, (100) mg/kg, and (200) mg/kg, followed by 1% w/v from Carrageenan solution (0.1 ml/paw) was injected subcutaneously into the plantar surface of the right hind paw of the rat. The paw volume of the left legs that considered as control for each animal in carrageenan, standard and tested doses of callus extract were measured with the help of official caliper during the time intervals of 1, 2, 3, 6, 12 and 24h after carrageenan injection (Macedo, et al., 2016; Sepideh and Sadegh, 2016; Speroni, et al., 2007).

RESULTS AND DISCUSSION

Callus induction:
Tissue culture methods have been employed as an important aid to conventional methods of plant improvement (Kaladhar, 2012). Callus induction is the best technique for improvement of plant through somaclonal variation (Hidayat Ullah, et al., 2007). Different active constituents in medicinal plants have been found in tissue culture especially callus culture (Ana Hortencia, et al., 2016). Plant cells in callus has advantages
for producing the active constituents by defined system of production and also it take short time, this technique provide us with continuous supply of such important secondary metabolites, furthermore callus cells are free from diseases, not affected by climate changes and sometimes give us new active constituents which not found in original plant (Loredo, 2013). The best auxin for callus induction is 2,4-D which produce sustained and continuous growth of callus (Renu, et al., 2011). In this study callus induced as shown in (Figure 1) by two auxins (2,4-D and picloram). Both auxins were incorporated separately on MS medium at different concentration. 2,4-D at concentration (2, 4, 6 & 8 mg/L) produced callus with white color and variable growth; and picloram at concentration (1, 2, 3 & 4 mg/L) gives callus with white and whitish brown color and variable growth (Figure 1). Results in callus induction (Table 1) showed that the maximum degree of callus formation in 2,4-D at concentration 4 mg/L, while the best callus induction percent (73 %) at 6 mg/L. From the same table we noted that the best concentration from picloram were 2 mg/L for callus induction percent (48.88 %) and callus formation.

**Table 1:** Effect of different concentrations of 2,4-D and picloram on callus induction from leaf explant of Verbena bipinnatifida Nutt. after 4 weeks culture:

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Concentration (mg/L)</th>
<th>Degree of callus formation</th>
<th>Morphology of callus</th>
<th>Colour of callus</th>
<th>Callus induction %</th>
</tr>
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<tbody>
<tr>
<td>2,4-D</td>
<td>0</td>
<td>-</td>
<td>Friable</td>
<td>W</td>
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<tr>
<td></td>
<td>2</td>
<td>++</td>
<td>Friable</td>
<td>W</td>
<td>46.66</td>
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<tr>
<td></td>
<td>4</td>
<td>+++</td>
<td>Friable</td>
<td>W</td>
<td>73.33</td>
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<tr>
<td></td>
<td>6</td>
<td>+</td>
<td>Friable</td>
<td>W</td>
<td>57.77</td>
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<tr>
<td></td>
<td>8</td>
<td>+</td>
<td>Friable</td>
<td>W</td>
<td>44.44</td>
</tr>
<tr>
<td>Picloram</td>
<td>0</td>
<td>-</td>
<td>-</td>
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<td></td>
<td>1</td>
<td>++</td>
<td>Compact</td>
<td>WB</td>
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<tr>
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<td>2</td>
<td>+++</td>
<td>Friable</td>
<td>W</td>
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<td></td>
<td>4</td>
<td>+</td>
<td>Friable</td>
<td>W</td>
<td>42.22</td>
</tr>
</tbody>
</table>


A, Callus after 1 week; B, Callus after 2 weeks; C, Callus after 3 weeks & D, Callus after 4 weeks.

**Fig. 1:** Stages of callus induction from leaves explants of Verbena bipinnatifida Nutt.

The anti-inflammatory activity of diclofenac sodium and tested callus extract in carrageenan injected rats:

The decrease in paw swelling volume is a good index in determining the protective effect of anti-inflammatory agents (Waseem, et al., 2017). Data in Table (2) show that injection of 0.1 ml carrageenan in right hind paw (S.C) in a dose of 1% w/v caused a significant increase of inflammation in right paw volume to about (46%, 58%, 94%, 119%, 102% and 66% after 1, 2, 3, 6, 12 and 24 h respectively when compared to mean volume of left leg, while pre-treatment with diclofenac sodium (i.p.) in a dose of 6.75 mg/kg caused a significant reduction of inflammation in right paw volume to (19%, 12%, 11% 15 %, 10% and 12%), when compared to carrageenan injected group at 1, 2, 3, 6, 12 and 24 h respectively. The callus extract solution at a dose of (50 mg/kg orally) shows a significant reduction of inflammation in right hind paw volume to (35%, 58%, 94%, 74%, 28% and 24%) after 1, 2, 3, 6, 12 and 24 h respectively, when compared to carrageenan injected group, taken into consideration that callus extract had little activity than diclofenac. The use of The callus extract at (100 mg/kg orally) had intermediate reduction in right paw volume to (31%, 43%, 54%, 42%, 28% and 14%) after 1, 2, 3, 6, 12 and 24 h respectively, when compared to carrageenan injected group. The use of The callus extract at (200 mg/kg orally) had very high activity when compared to standard drug and also nearly the same volume of left hind paw by reduction edema to (26%, 30%, 24%, 15%, 6% and 6%) after 1, 2, 3, 6, 12 and 24 h respectively.
The present study showed that all the selected doses from callus extract were evaluated for their in vivo anti-inflammatory activity and compared to diclofenac sodium as a reference was measured before and after 1, 2, 3, 6, 12, and 24 hours from carageenan injection. Mean percent of the edema was calculated as a regard to carrageenan control group and potency was calculated as a regard to the percentage of the change of the diclofenac and tested doses of callus extract, it was observed that the low dose of callus extract (50 mg/kg) had shown low anti-inflammatory activity at the end of experiment (24 %) and the intermediate dose (100 mg/kg) had shown moderate activity (14 %) after 24 hours, while, the highest dose (200 mg/kg) had shown highest activity (6 %) which more than diclofenac sodium (10 %) after 12 hours.

**Conclusion:**

From the above data, it was concluded that picloram is more effective in degree of callus formation than 2,4-D, while 2,4-D is more effective than picloram in callus induction percent on this specie of Verbena. This is the first time to study the effect of two auxins (2, 4-D and picloram) in different concentrations on callus induction of *Verbena bipinnatifida* Nutt. Family Verbenaceae cultivated in Egypt. Anti-inflammatory effect of hydro alcohol extract of callus showed reduction of inflammation in all rats at all doses and the highest dose (200mg/kg) produce a good activity than Stander.

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