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A Comparative Study of Anti-*Candida* Activity and Phenolic Contents of the Calluses from *Lythrum salicaria* L. in Different Treatments

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Abstract In the study, anti-*Candida* activity and phenol contents of *Lythrum salicaria* L. calli and wild species have been evaluated. The seeds of *L. salicaria* (Lythraceae), collected from Lahidjan City in the north of Iran, were cultured in Murashige and Skoog medium (MSM) with a supplement, gibberellin, to germinate. Callus inductions were performed from segments of seedling on MSM containing different concentrations of plant growth regulators, 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzylaminopurine (BAP). The activity of calluses extracts, wild plant, gallic acid, and 3,3′,4′-tri-O-methylellagic acid-4-O-β-D-glucopyranoside (TMEG) as the main phenolic compounds against *Candida albicans* was

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assessed using cup plate diffusion method. The total phenols contents of calli and wild plant extracts were analyzed using Folin–Ciocalteu reagent. The callus formation in MSM supplemented with various concentrations of 2,4-D and BAP were 0–100 %. Anti-*Candida* activity of callus extract which obtained from MSM supplemented with 2,4-D and BAP (1 mg dm^{-3}) was similar to the wild plant extract. Minimum inhibitory concentration values of gallic acid and TMEG were obtained as 0.312 and 2.5 mg cm^{-3}, respectively. Gallic acid equivalent values in all treatments were from 0 to 288 μg GAE mg^{-1}. Phenolic contents of plant aerial parts (331±3.7 μg GAE mg^{-1}) and the callus, which developed in MSM including 1 mg dm^{-3} of both 2,4-D and BAP, showed the same phenolic value and exhibited anti-*Candida* extract activity.

Keywords BAP · 2,4-D · Lythraceae · Fungicidal activity · 3,3′,4′-tri-O-methylellagic acid-4-O-β-D-glucopyranoside

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
BAP	6-Benzylaminopurine
MSM	Murashige and Skoog Medium
GAE	Gallic acid equivalent
TMEG	3,3′,4′-tri-O-methylellagic acid-4-O-β-D-glucopyranoside
DMSO	Dimethyl sulfoxide
DW	Distilled water
TMS	Trimethylsilane

Introduction

Plant cell culture is viewed as a potential means of producing useful metabolite [1]. Several strategies such as screening and selection of growth and production media have been followed to improve yields of secondary metabolites in plant cell cultures [2]. Since each plant species requires different kinds and levels of phytohormones, for this reason it is important to select the most appropriate growth regulators and their optimal concentrations [1]. Polyphenols are one of the most considerable and ubiquitous classes of plant metabolites, which provide the various beneficial effect on human health [3]. In Flora Iranica, *Lythrum* (Lythraceae) is almost represented by 30 species; seven species are widely growing in Iran [4]. It is known as Turbinkwash and Surmankhal in Persian [5]. Flowering aerial parts have been used for the treatment of diarrhea, chronic intestinal catarrh, hemorrhoid, eczema, varicose veins, bleeding of the gums, and vaginitis [6, 7]. Phytochemical investigation on this plant revealed some phenolic components such as tannins, anthocyanins, and flavonoids [7]. The extract of the plant contained antifungal and antibacterial components and exhibited antioxidant activity similar to reference substance [8–11]. Previous study revealed fungicide activity of the plant against *Candida albicans* [7, 8, 12, 13], the second the cause of vaginitis in the USA and leading cause in Europe [14]. Phenolic compounds display potent antifungal activity against *C. albicans* [15]. There is a report about an efficient plant regeneration system for *Lythrum salicaria* on medium containing different concentrations of various plant growth regulators [16]. In the present study, the appropriate medium containing different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzylaminopurine (BAP) were investigated for callus formation of *L. salicaria*. Furthermore, callus extracts were evaluated against *C. albicans* and total phenol contents. Percentage of callus forming, weight, density, and color of calli has been analyzed. The obtained results were compared

with the plant extract fungicidal activity and its phenolic contents. Gallic acid and 3,3′,4′-tri-O-methylellagic acid-4-O-β-D-glucopyranoside (TMEG) as phenolic compounds which the latter one purified from *L. salicaria* were examined for antifungal activity.

Materials and Methods

Culture Materials

Murashige and Skoog medium and the plant regulators (2,4-D and BAP) were purchased from Duchefa (Haarlem, The Netherlands). All other chemicals with high purity were obtained from Merck (Darmstadt, Germany).

Plant Materials and Callus Culture

The aerial parts and seeds of *L. salicaria* were collected from Lahidjan City (Gilan Province, the north of Iran), in June and September 2010, respectively. A voucher specimen was deposited at Central Herbarium of Medicinal Plants, Karaj, Iran (Ajani 313). Since the wild plant had a lot of microbial contamination, it was considered to employ sterile seedlings as an explant. The seed sterilization process was optimized. They were washed with fungicide, benomyl (saturated solution), for 15 min and dipped in 70 % ethanol for 2.5 min, rinsed with deionized water and then sodium hypochlorite (2.5 %) was applied to surface sterilization for 20 min, and eventually washed with deionized water three times. Sterilized seeds were germinated in glass bottles containing 50 cm^3 Murashige and Skoog medium (MSM) supplemented with 0.55 % plant agar (pH5.7, autoclaved for 20 min at 121 °C and 105 kPa). Gibberellin (GA_3) was added (0.5 $mg\,dm^{-3}$) after filtration through a 0.22 μm membrane filter. After 2 weeks incubation at 25 °C on this medium, seedling segments were transferred to bottles containing MSM supplemented with 0.55 % plant agar, 30 $g\,dm^{-3}$ sucrose, and different combinations and concentrations of plant regulators 2,4-D (0.1, 1, 2.5, or 5 $mg\,dm^{-3}$) and BAP (0, 0.5, 1, 2 $mg\,dm^{-3}$), followed by incubating these media in the darkness for 3 weeks. Calluses were extracted with methanol three times; the extracts were evaporated to concentrate in a rotary evaporator and dried by freeze dryer, then stored at 4 °C for measuring antifungal activity and total phenolic assay.

Anti-*Candida* Evaluation

Antifungal activity of the crude extract was determined using cup plate diffusion method [17]. The *C. albicans* (ATCC 10231) inocula were prepared by suspending colonies overnight from 48- and 72-h-old Sabouraud dextrose (SD) agar cultures in 0.9 % saline, respectively. The inocula were adjusted photometrically at 600 nm to a cell density equivalent to 0.5 McFarland standard ($1.5\times10^8\,CFU\,cm^{-3}$). SD agar plates (100 mm diameter) were seeded individually with fungal suspensions using a sterile cotton swab. Wells were done by punching a stainless steel cylinder onto the agar plates and removing the agar to form a well. Extracts and components were dissolved in DMSO/DW (50/50) at a concentration of 20 $mg\,cm^{-3}$and diluted in a twofold manner to make the concentrations of 10, 5, 2.5, 1.25, 0.625, 0.312, and 0.156 $mg\,cm^{-3}$. In three wells, 100 μdm^3 of each dilute was placed individually. The plates were incubated at 20–25 °C for 48 h. The solvent DMSO/DW (50/50) was used as negative control for all the experiments. After incubation,

the mean inhibition zone diameter for each concentration was determined and the lowest one considered as the minimum inhibitory concentrations (MICs) which presented in Table 2.

Isolation of Constituent

Aerial parts of the plant (180 g) were crushed and extracted three times with methanol. The methanolic extract of the plant (33 g) was subjected to column chromatography on silica gel (70–230 mesh) eluted with chloroform/methanol (49:1, 0:1) afforded A (8 g) and B (23 g) fractions. Fraction B was loaded on C18 reverse phase column eluted with methanol/water (7:3, 10:0) to give five fractions (B1–B5). Fraction B3 (1.2 g) that was subjected on silica gel (70–230 mesh) and eluted with chloroform/methanol (19:1, 0:1) provided nine fractions (B31–B39). Fraction B34 (24 mg) was subjected on sephadex LH20 and eluted with methanol yielding pure compound (10 mg) (Fig. 1). Gallic acid has been purified from this plant as a major component in the previous study [18]. The peaks obtained are shown below: ^{13}C-NMR (125 MHz, DMSO-d6): 112.94 (C-1), 141.2 (C-2), 140.0 (C-3), 154 (C-4), 108 (C-5), 112.94 (C-6), 158.23 (C-7), 113.73 (C-1′), 141.2 (C-2′), 140.0 (C-3′), 151.92 (C-4′), 112 (C-5′),111.95 (C-6′),157 (C-7′),61.72 (OMe-3), 61.38(OMe-3′), 56.82(OMe-4′),101 (C-1″), 73.34 (C-2″), 76.48 (C-3″), 69.48 (C-4″), 77.29 (C-5″), and 60.53 (C-6"). ^{1}H-NMR (500 MHz, DMSO-*d6*); 7.67 (H-5, s,1H), 7.85 (H-5′, s, 1H), 4.05 (OMe-3, s, 3H), 4.02 (OMe-3′, s, 3H), 4.1(OMe-4′, s, 3H), 5.18 (H-1″, d, 1H), 3.45 (H-2″, 1H), 3.44 (H-3″, 1H), 3.17 (H-4″, 1H), 3.47 (H-5″, 1H), 3.7 (Ha-6″, 1H), and 3.5 (Hb-6″, 1H).

NMR Instrument

NMR spectra were obtained at ambient temperature on Bruker Avance DPX-500 spectrometer operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C. Sample was dissolved in DMSO-d_6 and chemical shifts (δ, in parts per million) were relative to internal TMS.

Total Phenols Assay

Total phenolic contents were examined as GAE, expressed as microgram GAE per milligram extract [19]. The callus and the wild plant extracts were transferred to glass tubes, to which 5 cm^3 Folin–Ciocalteu reagent (diluted 1:10) was added subsequently and incubated at room temperature. After 10 min, 4 cm^{3}sodium bicarbonate (75 mg cm^{-3}) was added to the mixture up to 10 cm^3 using distilled water, then incubated for 30 min at room temperature, and the absorbance was measured at 765 nm compared to gallic acid calibration curve. All determinations were carried out in triplicate and the mean values±standard errors were calculated.

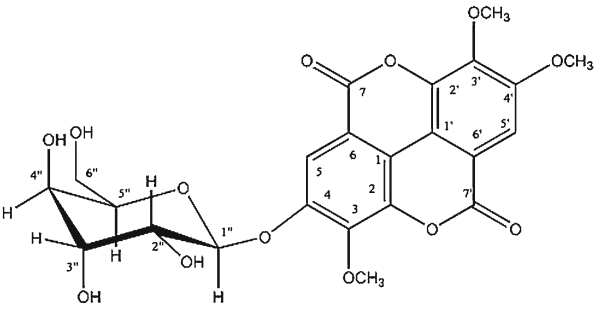

Fig. 1 3,3′,4′-tri-O-methylellagic acid-4-O-β-D-glucopyranoside molecular formula

Statistical Analysis

Data are presented as mean values±standard error. All statistical analyses were carried out using SPSS 11.5. (SPSS Inc., Chicago, IL). Analysis of variance was performed by ANOVA procedures. Significant differences between means were determined by Tukey's pairwise comparison test at a level of $p<0.05$.

Results

Callus Formation

The results presented here demonstrate a practical process by which suitable callus formation with anti-*Candida* activity and high amount of total phenolic contents can be achieved; in addition, wild plant extract and two pure components were analyzed for antifungal activity. Explants from germinated seedling were used to examine the effects of different combinations of 2,4-D and BAP at varying concentrations. Total phenol contents, percentage of callus formation, (wet–dry) weights, densities, and colors in different media were reported in Table 1. Results revealed that treatments nos. 5, 6, and 7 exhibited 100 % callus. Other media with different combinations of plant growth regulators stimulated callus formation about 70–80 %. In the medium supplemented by 2,4-D, callus formation was about 0–70 %. The callus generation in treatment nos. 12 and 13 were about 30 and 40 %, respectively. Combination of 2,4-D (0.1, 1, and 2.5 mgdm^{-3}) with BAP (0.5, 1, and 2 mg dm^{-3}) appeared to have promoted callus development in the seedling. Depending on the concentration of cytokinin and auxin, the responses to callus properties such as (wet–dry) weights, densities, and colors were varied among treatments.

Anti-*Candida* Activity

The results indicated that among all callus extract tested which was obtained from MSM supplemented with 2,4-D and BAP (1 mgdm^{-3}) recorded fungicidal property toward *C. albicans* as well as the plant extract (Table 2).

Identification of Constituent

On the basis of detailed study of spectroscopic data from ^{1}H NMR and ^{13}C NMR, the purified compound was identified as TMEG [20–23]. Due to our knowledge, this ellagic acid derivative was reported for the first time from *L. salicaria*. Plant extract, TMEG, and gallic acid MICs values were assessed as 20, 2.5, and 0.312 mgcm^{-3}, respectively (Table 2).

Total Phenol Assay

Efficiency of phenolic compounds in the callus from different treatments were indicated that diverse concentrations of plant growth regulators were significantly affected production of plant secondary metabolites in wide range (0–288±13.7 μg GAE mg^{-1}). Content of phenolics in aerial parts of the wild plant was determined as 331±3.7 μg GAE mg^{-1}. Treatment nos.10, 14, and 11presented the higher phenolic contents among other treatments. This three media with high production of total phenols have no significant differences. Other phenol contents were reported in Table 1.

Table 1 Callus generation and total phenolic contents of the callus extracts from explants cultured on MSM medium containing dual combinations of 2,4-D and BAP

No.	Treatments ($mgdm^{-3}$)	Eq. μg Gallic in 1 mg callus ext.	Callus forming (%)	(Wet–dry) weight (g)	Density (L, D)	Color
1	0.1 2,4-D + 0 BAP	72.14±3.7abc	70	0.14	L	Brown
2	1 2,4-D + 0 BAP	0	30	0.17	L	Brown
3	2.5 2,4-D + 0 BAP	0	36	0.19	L	Brown
4	5 2,4-D + 0 BAP	–	0	–	–	–
5	0.1 2,4-D + 0.5 BAP	180.61±5.5fg	100	0.21	D	Orange–white
6	1 2,4-D + 0.5 BAP	140.16±10.8ef	100	0.23	L	Orange–brown
7	2.5 2,4-D + 0.5 BAP	65.02±0.68abc	100	0.39	L	Brown
8	5 2,4-D + 0.5 BAP	184.08±0.94fg	70	0.21	D	Orange–brown
9	0.1 2,4-D + 1 BAP	123±8.7de	80	0.36	D	Orange–white
10	1 2,4-D + 1 BAP	288±13.7i	85	0.3	D	Orange–brown
11	2.5 2,4-D + 1 BAP	215.29±12.9gh	70	0.24	D	Orange–violet
12	5 2,4-D + 1 BAP	32.68±3.3a	30	0.23	D	Orange–white
13	0.1 2,4-D + 2 BAP	84.84±1.1bcd	40	0.16	D	Orange–white
14	1 2,4-D + 2 BAP	264.08±2.02hi	70	0.14	D	Orange–white
15	2.5 2,4-D + 2 BAP	37.46±1.8ab	70	0.21	D	Violet–white
16	5 2,4-D + 2 BAP	107±3.9cde	75	0.2	D	Brown–white

Means±SE. Means with the same letters within columns are not significantly different at $p>0.05$

L loose, *D* dense

Table 2 Minimum inhibitory concentrations (MICs) and inhibition zone diameter (in millimeter) ±SE against *C. albicans*

Extracts and components	MIC ($mg\,cm^{-3}$)	Inhibition zone diameter (mm)
Plant extract	20	12.3±0.12
Callus extract (treatment 10)	20	11.97±0.08
Other callus extract	>20	–
Gallic acid	2.5	12.4±0.06
TMEG	0.312	10.2±0.17

Discussion

Media which supplemented with 0.1, 1, and 2.5 $mg\,dm^{-3}$ 2,4-D + 0.5 $mg\,dm^{-3}$ BAP were the most effective combination to produce callus from seedlings segments. The callus formation was not observed in the MSM under treatment no. 4; moreover, medium with treatment nos. 12 and 13 callus formations were 30 and 40 %, respectively. It was indicated that the presence of BAP in close concentration with 2,4-D is essential for the callus production in *L. salicaria*. Increasing the callus, (wet–dry) weights, densities, and brightening of the calli colors were directly attributed to cytokinin concentration. Wild plant and callus extracts from treatment no. 10 and pure phenolic substances showed antifungal activity, although there is no significant difference between phenolic contents of the plant aerial parts and the callus which developed under treatment no. 10. Consider to the results, it is found that phenolic compounds in both wild plant and callus extracts display an important role in antifungal activity. In the previous study, ellagic acid has also been shown to possess activity against *C. albicans* [24, 25]. Among the hormonal combinations tested, those involving treatment nos. 10 and 14 were more effective on phenolic components generation than other combinations. Other MSM which included 2,4-D (1, and 2.5 $mg\,dm^{-3}$) without BAP did not produce phenolic compounds, so the combination of these two plant regulators is necessary in order for phenolic substances generation. Wild plant moreover developed higher amount of phenolics. Lack of differentiation in cells of plant tissue culture could justify lower ranges of secondary metabolites, whereas plant organs exposed to a wide range of environmental stresses can usually induce secondary metabolites. Literature reviews showed that total phenol content was lower in callus of *Delonix elata* L. same as *L. salicaria* and higher amount was reported in their respective seedlings explants [26]. The calli of *Gardenia jasminoides* Ellis exhibited lower amounts of phenolics and antioxidant activity than its mother plant. They also reported that the shootlet culturing in all treatments increased the total phenolic content, compared to the control and mother plant. Moreover, for callus induction, 2,4-D was the auxin of choice [27]. The results of the study on callus formation of *Citrullus colocynthis* concluded that MSM supplemented with 2,4-D and kinetin produced higher contents of total phenolics, total flavonoids, and antioxidant activities. The high concentration of 2,4-D over KIN in media yielded the highest phenolics content [28]. The obtained results in this study suggested different facts that equal concentration of 2,4-D and BAP (1 $mg\,dm^{-3}$) is critical for the induction of phenolic components production in *L. salicaria*. In general, the results may be attributed to the important effect of cytokinin on the biosynthesis of secondary metabolic in in vitro growth culture. As shown in Table 1, increasing the BAP concentration (0, 0.5, and 1 $mg\,dm^{-3}$) with constant concentration of 2,4-D (1 $mg\,dm^{-3}$) resulted in a higher amount of phenolics contents.

In conclusion, the obtained results of this study support the hypothesis which confirm the role of phenolic compounds in anti-*Candida* activity of *L. salicaria* and represent the optimum media culture treatments for this plant.

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