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Antibacterial activities of *Calendula officinalis* callus extract

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Abstract: The purpose of this study was to determine the antibacterial activity of *C. officinalis* callus derived from cotyledon explants. Cotyledons excised from *in vitro* germinated seedlings were used as explants. Explants were transferred on MS medium supplemented with benzil amino purine (BAP; 2 mg l⁻¹), α -naphthalene-acetic acid (NAA; 2 mg l⁻¹) for callus studies. The cultures were maintained on the same media compositions and were sub-cultured at an interval of 4 weeks. Callus cultures were harvested at the end of the 16th week. Calli were dried at 40° C in the dark for antimicrobial studies. *Calendula officinalis* callus extracts were tested for their antibacterial activities by using agar well diffusion method. Ethanol and chloroform extracts from these plants were assayed against nine bacteria species (*Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 25922, *Bacillus cereus* ATCC 7064, *Bacillus subtilis* ATCC 6633, *Salmonella typhimurium* CCM 5445, *Proteus vulgaris* ATCC 6896, *Enterococcus faecalis* ATCC 29212, *Enterobacter cloacae* ATCC 13047, and *Kocuria rhizophila* ATCC 9341). The test antibiotics penicillin G, novobiocin, ampicillin, chloramphenicol and erythromycin were used for comparison. Callus formation was observed at the end of the 5th week on cotyledon explants. *C. officinalis* callus extracts showed 38 mm inhibition zone against *S. aureus*, and chloroform extracts showed 32 mm inhibition zone against *B. cereus*. These results are very close to the test antibiotics used and *C. officinalis* is found more effective on gram positive bacteria.

Keywords: *Calendula officinalis*, callus, antibacterial activity, BAP, NAA.

1. INTRODUCTION

Plants are able to synthesize substances called secondary metabolites in addition to the production of carbohydrates, proteins and fats, the primary building blocks of life, that is, primary metabolites. The discovery of the bioactive properties of secondary metabolites and their widespread use in many areas of industry such as medicine, cosmetics, paint making, and fragrance progressively increase the significance of medical and aromatic plants in the world markets [1, 2]. Biotechnological methods, especially callus culture of plant cell tissue cultures, appear as alternative methods in the production of secondary metabolites in order to solve encountered problems by wild environments [3]. In many studies callus cultures represent an alternative source for producing natural antimicrobial compounds and plant tissue cultures produce a variety of secondary metabolites, sometimes in higher percentage than the original

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plant, particularly in the polyphenolic class in which high yields have sometimes been obtained [4].

Calendula officinalis L., known for its ornamental plant characteristics, is a medicinal plant belonging to Asteracea family. It is annual or perennial taproot plant with 20-40 cm height and has 20 varieties [5]. The chemical compounds identified in methanol extracts of *C. officinalis* include polar compounds phenolic acids and flavonoid glycosides [6]. As a result of the many studies performed with *C. officinalis*, the anti-cancer [7], anti-microbial [8], anti-leishmanial [9], anti-HIV [10], antioxidant [11], cytotoxic, anti-tumor [12], anti-viral [13], anti-inflammatory [14], edema amplifier [15], hypoglycemic [16], uterotonic [17], effects and its utilization in the treatment of venous ulcers [18], as well as the biligenic [19] pharmacologic effects of this plant have been reported.

In this study, the effect of BAP and NAA plant growth regulators applied on *C. officinalis* cotyledon explants and the antibacterial effect of the obtained calluses have been investigated.

2. MATERIAL and METHODS

2.1. Plant material

Calendula officinalis seeds used in the study were obtained from Hekim Sinan Medicinal Plant Research Center of in the municipality of Kutahya, Turkey.

2.2. Medium & culture conditions

Murashige and Skoog medium was used as nutrient medium. For germination studies without any plant growth regulators and for callus studies MS medium supplement with 2 mg l⁻¹ BAP and 2 mg l⁻¹ NAA were respectively used [20]. They were supplemented with 100 mg l⁻¹ myo-inositol and 30 mg l⁻¹ sucrose. The pH of the medium was adjusted between 5.7-5.8 using 0.1 N NaOH or 0.1 N HCl before the addition of 0.7 % (w/v) agar and then the medium was autoclaved at 121°C for 15 min at 105 kPa. Cultures were maintained at 24 ± 2°C, under 16:8 photoperiod at a 4000 lux white fluorescent light, in a growth room.

2.3. Establishment of the aseptic culture

Initially, *C. officinalis* seed pods were unshelled, separated, and washed using distilled water. Later surface sterilization was made in 70% ethyl alcohol for 3 min and 0.5% NaOCl for 5 min, the seeds were rinsed with sterilized distilled water to remove the traces of sterilant. They were transferred on MS medium for germination.

2.4. Callus culture & induction

Calendula officinalis L. cotyledon explants were used as explants for the induction of callus. Cotyledons were excised from aseptic seedling and were and cultured on MS medium supplemented with; 2 mg l⁻¹ BAP and 2 mg l⁻¹ NAA for callus studies. The cultures were maintained on same medium compositions and sub-cultured in the same medium every four weeks. The calli were harvested at the end of 16th week and were dried at 40°C under the dark for antimicrobial studies.

2.5. Test microorganisms and growth conditions

Test microorganisms included following bacteria: *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 25922, *Bacillus cereus* ATCC 7064, *Bacillus subtilis* ATCC 6633, *Salmonella typhimurium* CCM 5445, *Proteus vulgaris* ATCC 6896, *Enterococcus faecalis* ATCC 29212, *Enterobacter cloacae* ATCC 13047, and *Kocuria rhizophila* ATCC 9341. Cultures of these bacteria were grown in Mueller Hinton broth (Oxoid) at 37°C for 24h [21].

Test microorganisms were obtained from the culture collection of Ege University, Faculty of Science, Basic and Industrial Microbiology Department.

2.6. Antibacterial activity assay

The dried and powdered plants were reduced to coarse powder. Two g of powder was extracted with 20 ml of ethanol and chloroform at room temperature with stirring for 3 days (125 cycles/minute). The solvents were evaporated to dryness after extraction progress. Sample solutions were prepared by dissolving the extracts in same solvents (1 ml). *In vitro* antibacterial studies were carried out by the agar well diffusion method against test microorganisms. Bacterial strains grown on nutrient agar at 37°C for 24 h were suspended in a saline solution (0.85% NaCl) and adjusted to a turbidity of 0.5 MacFarland standards [10^6 Colony Forming Units (CFU)/ml]. Briefly, 50 microlitres (μ l) inoculum (containing approximately 10^5 bacteria per milliliter) was added to 25 mL melted Mueller Hinton Agar (MHA) medium cooled at 45°C. This was then poured into 90 mm diameter Petri dishes and maintained for 1h at room temperature. Small wells (6 mm diameter) were cut in the agar plate using a cork borer; 60 μ l of extract concentration with a negative control (ethanol and chloroform, 60 μ l) were loaded in the wells. The dishes were preincubated at 4°C for 2 h to allow uniform diffusion into the agar. After preincubation, the plates were incubated at 37°C for 24h. The antibacterial activity was evaluated by measuring the inhibition zone diameter observed. In addition, commercial antibiotics (penicillin G, novobiocin, ampicillin, chloramphenicol and erythromycin) were used as positive control to determine the sensitivity of the strains [22]. All experiments were performed in triplicate.

3. RESULTS and DISCUSSIONS

Plant tissue culture techniques enable the production of plant tissue or cells in sterile environments under controlled conditions, allowing the growth and development of the cells or tissues to be manipulated for a variety of applications. One of these methods callus cultures, could be produced pharmacologically active molecules at the desired amount and constant quality at any time in laboratory conditions [23, 24]. In this study, the antimicrobial effect of the calli obtained from the cotyledon explants of the *C. officinalis* plant was examined.

It is more feasible to take explants that will be used in tissue culture studies from plants grown in *in vitro* conditions. The risk of contamination for plants which grow under these conditions is almost nonexistent, and they regenerate much easier and can be used directly as explants [25]. Thus, the explants to be used for this study were obtained from the plantlets produced from seeds that were germinated on MS media. At the end of the surface sterilization procedure, which was performed by incubating the seeds in 70% ethyl alcohol for 3 min, in 0.5% bleach for 5 min, and rinsing 3 times with incubation for 3 min in distilled water, sterile plantlets were successfully obtained. The cotyledones to be used as explants were isolated from these plants.

Result of some studies revealed that auxins played an important role in the callus induction. Furthermore, they showed that cytokinins facilitated the effect of auxins in callus induction [26]. It has been reported that callus formation is high in many tissue culture studies in which plant growth regulators, namely BAP from among cytokines and NAA from among auxins are applied [27-29]. In the present study, 2 mg l⁻¹ BAP ve 2 mg l⁻¹ IBA cytokinins that were applied caused a swelling of the explants after 5–7 d of culture, and callus induction was observed on the cotyledon explants within 5th wk of culture (Figure 1).

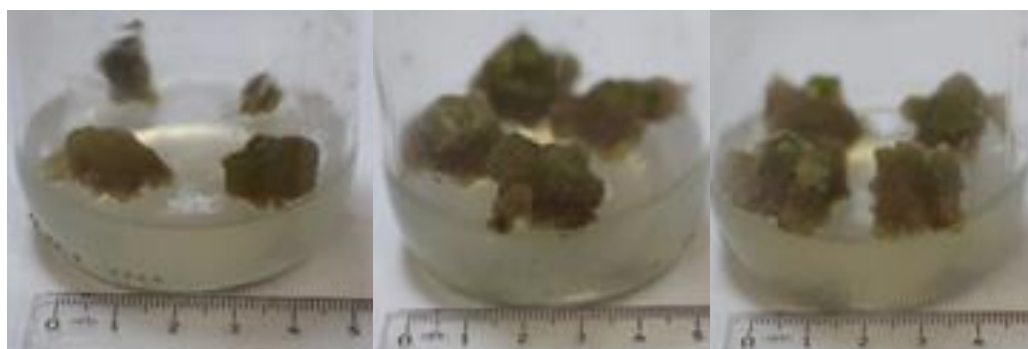


Figure 1. Calli obtained from *C. officinalis* cotyledon explants

Table 1. Screening for antibacterial activity of callus from cotyledons of *C. officinalis* extracts against standart microorganisms.

Bacteria	Zone of inhibition (mm)						
	Extracts		Antibiotics				
	Eth.	Chl.	Pe. G	Nov.	Amp.	Chlor.	Eryt.
<i>S. aureus</i>	38	26	24	32	20	18	12
<i>E. coli</i>	12	8	6	6	26	9	28
<i>B. cereus</i>	28	32	10	25	28	12	15
<i>B. subtilis</i>	24	20	8	13	32	11	30
<i>S. typhimurium</i>	14	10	6	40	6	13	28
<i>P. vulgaris</i>	18	12	10	26	12	9	22
<i>E. faecalis</i>	20	18	24	28	30	18	16
<i>E. cloacae</i>	10	10	12	22	12	11	30
<i>K. rhizophila</i>	16	13	20	28	10	12	15

Eth: Ethanol; Chl: Chloroform; Pe: Penicillin; Nov: Novobiocin; Amp: Amphotericin; Chlor: Chloramphenicol; Eryt: Erythromycin

When literature is reviewed, it is evident that many studies have been carried out in recent years on the secondary metabolite production [30-32] and on the antimicrobial effects of these metabolites in addition to studies such as cell culture, microbial growth, organogenesis, embryogenesis by callus culture method [33-37]. However, no literature has been found on the antimicrobial effect of *C. officinalis* calli.

In the present study, we observed *in vitro* antibacterial activities of ethanol and chloroform extracts of the anti-bacterial activity of cotyledon derived calli of *Calendula officinalis* and standard antibiotics (Table 1.).

The extracts of *C. officinalis* showed various antibacterial activities against the test bacteria. All extracts studied in this work showed antibacterial activity against at least one of the test microorganisms with inhibition zones ranging from 8 to 38 mm (Table 1). Ethanol extract of *C. officinalis* callus showed 38 mm inhibition zone against *Staphylococcus aureus* and chloroform extract showed 32 mm zone against *Bacillus cereus*. These results are very close to the test antibiotics used and *C. officinalis* found more effective on gram positive bacteria.

The results of the current investigation clearly indicate that the antibacterial activity vary with the *C. officinalis*. Further, the active phytochemicals of this plant against some bacteria should be characterized and their toxicity should be evaluated *in vivo*.

Conflict of Interests

Authors declare that there is no conflict of interests.

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