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Antimicrobial Activity of Extracts from the Callus Culture of *Rubia tinctorum* L.

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The *in vitro* antimicrobial activity of *Rubia tinctorum* callus extracts were studied against selected microorganisms by agar well diffusion assay. Calli were extracted using ethanol, n-hegzan and chloroform. Among the three solvents used, callus extracted in ethanol was found to be more effective against some microorganisms with inhibition zone between 10 and 24 mm. Extracts of chloroform showed poor inhibition than other two solvents. No activity was observed against *Proteus vulgaris* and *Enterococcus faecalis*. Thus, the positive results suggest that the *R. tinctorum* callus extracts should be further studied to determine the bioactive chemical compounds.

Keywords: *Rubia tinctorum*, Antimicrobial activity, Callus culture, Turkey.

The use of medicinal herbs in the treatment of infection is an age-old practice and several natural products are used as phytotherapic for treatment of many diseases[1]. Approximately 20% of the plants found in the world have been submitted to pharmacological or biological tests[2]. The systemic screening of antimicrobial plant extracts represents a continuous effort to find new compounds with the potential to act against multi-resistant pathogenic bacteria and fungi[3].

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The development of plant cell cultures, nowadays, is an important strategy for bioprospection of natural products. Thus, the large-scale production *in vitro* of bioactive compounds or extracts used as phytotherapics, pharmaceutical products, food additives and cosmetics should be encouraged because of their scientific, economical or ecological importance[1]. The past few decades have seen increasing scientific interest in the both growth of plant tissue culture and the commercial development of this technology as means of producing valuable phytochemicals[4].

Callus cultures from medicinal plants have been established under suitable conditions to enable production of antimicrobial substances *in vitro*[5, 6]. Recently, papers investigating the antimicrobial activity of extracts from calli of different medicinal plant species have been published[7, 8]. Although very few plant cell processes are operating commercially, the most successful commercial pharmaceuticals produced from undifferentiated cell cultures are antibiotic compounds[9]. *Rubia tinctorum* L. (Rubiaceae) is widely distributed in southern and southeastern

Europe, in the Mediterranean area, and in central Asia[10]. Its roots contain secondary metabolites; di- and trihydroxyanthraquinones, alizarin and purpurin and their derivatives, ruberythric acid (alizarin-primeveroside), pseudopurpurin and lucidin-primeveroside, rubiadin, munjistin, quinizarin, lucidin and 1,8-dihydroxy-anthraquinone were also identified from plant tissues[11]. Previous studies of *Rubia tinctorum* revealed analgesic, diuretic, aperient, astringent, antibacterial, antifungal and spasmolytic activity and facilitate the loosening of kidney concrements containing calcium and magnesium phosphates. In this connection, madder is used in medicine[12, 13]. Additionally, alizarin red is one of several histochemical stains that highlight calcium, particularly calcium deposits in soft tissues[14]. The underground parts of *R. tinctorum* have been used as a natural dye in Turkey[12].

The aim of this study was to determine the in vitro antimicrobial activity of crude ethanol, n-hegzan and chloroform extracts from callus cultures of *Rubia tinctorum* against some selected microorganisms using the agar well diffusion method.

MATERIAL AND METHODS

Plant material

Rubia tinctorum L. (Rubiaceae), seeds collected from Manisa, Turkey in 2009 and were identified in the Department of Biology, Ege University, Ýzmir. Voucher specimen was deposited in Department of Biology, Dumlupýnar University, Kütahya, Turkey. *Rubia tinctorum* L. callus cultures were established from micropropagated plants.

Callus culture

Leaves and stem segments from micropropagated plants were cut and placed on Murashige and Skoog's medium (MS)[15] containing 3% of sucrose and 1% (w/v) agar (Sigma). The medium was supplemented with 2,4 D (0,1mg/L), BAP (0,5 mg/L) and kinetin (0.5 mg/L). Prior to autoclaving at 120°C (1.5 Kg/cm^2) for 20 min, the pH of all media was adjusted to 5.8 with 0.1N NaOH. Cultures were maintained in permanent darkness at 25 ± 2°C which was under 16h light / 8h dark period. The initiated calli were routinely subcultured onto a fresh medium during 2 weeks, not only to produce large amounts of calli, but also to ensure a normal growth and to prevent deterioration of explants characterized by the appearance of a brown color. Calli were harvested at day 30 of cultivation and dried at 60ºC.

Extract preparation

The dried and powdered calli were reduced to coarse powder. 25 g of calli powder was extracted with 150 mL of solvents (ethanol, n-hegzan 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 own solvents (1 mL)[16].

Test microorganisms and growth conditions

Test microorganisms included following bacteria: *Bacillus cereus* CM 99, *Bacillus subtilis* ATCC 6633, *Enterobacter aerogenes* ATCC 13048, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 39628, *Proteus vulgaris* ATCC 8427, *Staphylococcus aureus* ATCC 6538P and yeast *Candida albicans* ATCC 10231. Cultures of these bacteria were grown in Mueller Hinton broth (Oxoid) at 37^{0}C for 24h and the studied yeast was incubated in glucose yeast extract broth at 30^{0}C for 48h[17]. Test microorganisms were obtained from the culture collection of Ege University, Faculty of Science, Basic and Industrial Microbiology Department.

Determination of antimicrobial activity

In vitro antimicrobial 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 (µl) inoculum (containing approximately 10^5 bacteria per milliliter and 10^4 yeast per mL) was added to 25 mL melted Mueller Hinton Agar (MHA) and Potato Dextrose Agar (PDA) medium cooled at 45^{0}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 µL of extract concentration with a negative control (solvents, 60 µL) was loaded in the wells. The dishes were preincubated at 4^{0}C for 2 h to allow uniform diffusion into the agar. After preincubation, for bacteria the plates were

incubated at 37^{0}C for 24h and 30^{0}C for 48h for yeast[17]. The antimicrobial activity was evaluated by measuring the inhibition zone diameter observed[18]. In addition, commercial antibiotics [Penicillin G (10 IU), nalidixic acid (30 µg) and nystatin (10 µg)] were used as positive control to determine the sensitivity of the strains[19].

Statistical analysis

The mean values were statistically analyzed with the MINITAB Release 13.20 program by the general one-way (unstacked) analysis of variance (ANOVA) to find out the most effective extracts and the most sensitive test organisms. Similarity (%) of microorganisms in relation to their susceptibility to the callus extracts was analyzed by the multivariate cluster analysis according to the data obtained from well diffusion assay.

RESULTS AND DISCUSSION

The ethnobotanical screening tests of calli extracts of *Rubia tinctorum* in different solvents against some selected microorganisms using agar well diffusion technique are depicted in Table 1. The extracts are found to be more effective against bacteria than yeast. Chloroform extract showed poor inhibition on test microorganisms than other solvents. No activity was observed against *Proteus vulgaris* and *Enterococcus faecalis* (Table 1).

The callus extract of *R. tinctorum* in ethanol showed 24 mm inhibition zone against *Enterobacter aerogenes* likely nalidixic acid (26 mm). n-hegzan extract showed 20 mm inhibition zones against *Bacillus cereus* and *E. aerogenes*. The most antifungal activity was observed by n-hegzan extract against *Candida albicans* (14 mm), but nystatin showed 22 mm inhibition zone on *C. albicans* (Table 1).

Susceptibility of test microorganisms, in decreasing order was as follows: *E. aerogenes*, *B. cereus*, *B. subtilis*, *E. coli*, *C. albicans*, *S. aureus*, *P. vulgaris* and *E. faecalis* (Figure 1). Figure 2 summarizes the similarity of test microorganisms in relation to their susceptibility to the callus extracts.

The extracts of higher plants can be very good source of antibiotics against various fungal and bacterial organisms[20]. Plant based antimicrobial compounds have enormous therapeutical potential as they can serve the purpose without any side effects that are often associated with synthetic antimicrobials. Higher plants have also made important contributions in the areas such as cancer therapies[3].

Table 1. Inhibition zone values of callus extracts of *R. tinctorum* against some microorganisms (mm)

Microorganisms	Ethanol	n-hegzan	Chloroform	P	N	Na
E. coli (EC)	18.0	12.0	12.0	-	-	26.0
S. aureus (SA)	10.0			24.0	-	20.0
B. cereus (BC)	22.0	20.0	17.0	10.0	-	28.0
B. subtilis (BS)	22.0	16.0	18.0	-	-	30.0
E. aerogenes (EA)	24.0	20.0	20.0	-	-	26.0
E. faecalis (EF)	-	-	-	24.0	-	30.0
P. vulgaris (PV)	-	-	-	10.0	-	12.0
C. albicans (CA)	10.0	14.0	-	-	22.0	-

P: Penicillin G, N: Nystatin, Na: Nalidixic acid, -: No activity

In the previously study, extracts obtained from aerial parts and roots of *Rubia tinctorum* found to be effective against some selected microorganisms[12], such as root extracts were found to be more active against *E. aerogenes* and *B. cereus* than that of the control antibiotics.

Singh and Sudarshana[21] tested the aqueous and ethanolic extracts of *Baliospermum axillare* callus against bacteria like *X. campestris*, *P. syringae* and the fungi *Fusarium solani* and *F. oxysporum*.

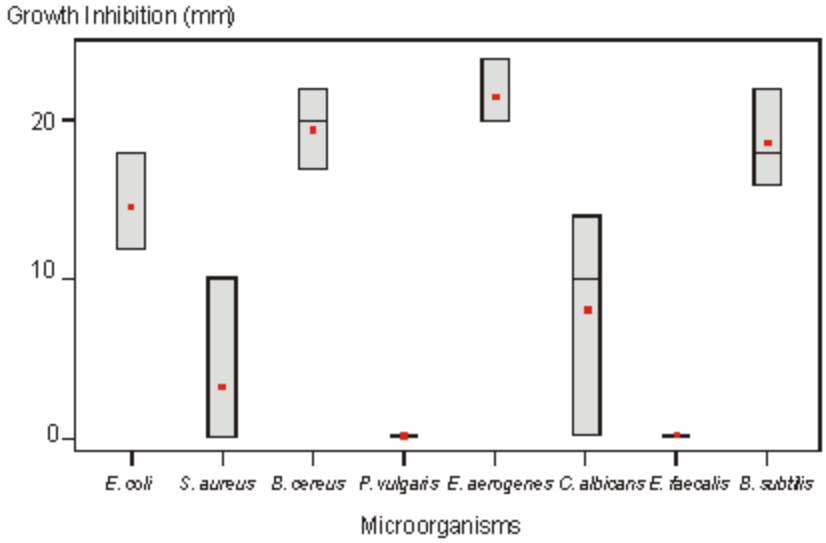

Fig. 1. Mean values of microorganisms in relation to their susceptibility to the plant extracts. Means are indicated by solid circles.

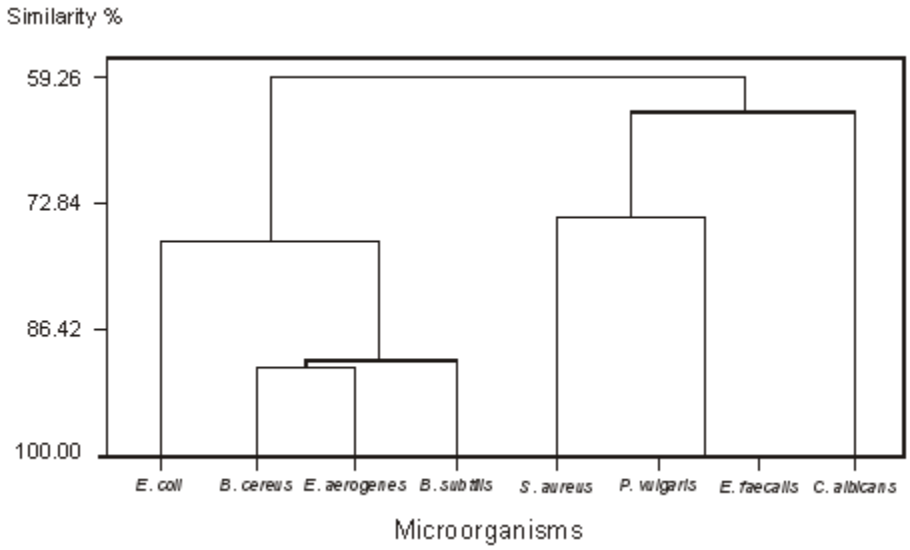

Fig. 2. Similarity (%) of microorganisms in relation to their susceptibility to the callus extracts.

Extracts from cell cultures of *Rhus coriaria*, *Peganum harmala*, *Thymus* and *Allium* species did not show any antimicrobial activity in Sökmen *et al.*[7]. The ethanolic, methanolic and chloroformic extracts of *Nerium oleander* leaf and root showed considerable antimicrobial activity against *Bacillus pumillus*, *B. subtilis*, *S. aureus* and *E. coli*[22].

In conclusion ethanol, n-hegzan and chloroform extracts of callus culture of Rubia tinctorum inhibited bacterial and fungal growth at various degrees. Further work is needed to isolate the active principle from the plant extracts and to carry out pharmaceutical studies.

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