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DETERMINATION OF BIOACTIVE COMPONENTS AND ANTIMICROBIAL ACTIVITY OF METHANOLIC EXTRACTS OF MISTLETOE LEAVES (*VISCUM ALBUM* L. SUBSP. *ALBUM* L.)

Dilek Keskin[1,*], Nur Ceyhan-Guvensen[2]

[1]Kosk Vocational High School, Aydin Adnan Menderes University, Aydin, Turkey
[2]Biology Department, Faculty of Sciences, Mugla Sitki Kocman University, Mugla, Turkey

ABSTRACT

The extract of ethanol, methanol, hexane, chloroform, isopropanol and water of mistletoe leaves (*Viscum album* L. subsp. *album* L.) were tested for antimicrobial activity against 11 bacteria and one yeast by disc diffusion method. In our study, methanolic extracts mistletoe of leaves showed the best inhibition zones against *Streptococcus faecalis* and *Bacillus subtilis* (22mm). Inhibition zones of the all of the extracts varied 6 to 22 mm against tested microorganisms. MICs of *V. album* subsp. *album* different extracts obtained by the broth serial dilution method, showed the lowest sensitivity to *S.faecalis* and *B.subtilis* with 4mg/ml concentration of methanolic extracts. Methanolic extracts of mistletoe were among the most active with the MIC values ranging from 4-256 mg/mL.

KEYWORDS:
Mistletoe leaves, *Viscum album* L. subsp. *album* L., extract, antimicrobial activity.

INTRODUCTION

Natural plant products have been used for therapeutic purposes since the time immemorial and their use is of a greater demand nowadays. The use of plant essential oils in both the food and the pharmaceutical industries has been developed interestingly, a systematic examination of plant extracts for these properties has become increasingly important. The use of natural antimicrobial compounds is important not only in the preservation of food but also in the control of microbial growth in the diseases condition [1-3]. Apart from these essential oils and extracts from several plant species are able to control microorganisms related to skin, dental caries and food spoilage, including gram-negative and gram positive bacteria [4]. Mistletoes therefore is one such plant which is reported to possess several medicinal properties. Its use as an anti-cancer, anti-diabetic, antihypertensive, and indeed as 'all-purpose herb' has been reported [5]. Many of these folkloric uses have already been investigated [6-8].

The objectives of this work were therefore to investigate the antimicrobial activities of six different solvent extracts from West Anatolian mistletoes and to determine the chemical compound content to find out the relationship between antimicrobial activity and the compound content. This is the first study about collected from antimicrobial activity of West Anatolian mistletoes. Therefore, we have tested antimicrobial effect against some microorganisms including opportunistic pathogens: Gram-negative bacteria; *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimurium* CCM 583,*Aeromonas hydrophila* ATCC 19570, *Klebsiella pneumoniae* CCM 2318.Gram-positive bacteria; *Staphylococcus epidermidis* ATCC 12228, *Bacillus subtilis* ATCC 6633, *Bacillus cereus* CCM 99, *Staphylococcus aureus* ATCC 6538/P, *Streptococcus faecalis* ATCC 8043,fungus; *Candida albicans* ATCC 10239.The antimicrobial activity was measured by using disc diffusion method and minimal inhibitory concentration (MIC).

MATERIALS AND METHODS

Samples. Mistletoe leaves (*V. album* subsp. *album*) were obtained from various retail outlets in Mugla province, West Anatolia, Turkey in 2013. The taxonomic identification of plant material was confirmed by botanist Prof. Dr. Aykut Guvensen, in the Department of Biology, Ege University, Turkey.

Preparation of mistletoe leaves (*Viscum album* L.) extracts. Plant leaves were washed with sterilized distilled water and air dried. Clean dry plant samples were stored in cotton bags. The materials were homogenized to a fine powder with the help of a mixer grinder. The 25grams portions of each dried powdered leaf material was soaked separately in 250ml ethanol, methanol, hexane, chloroform, isopropanol and water. The extraction was carried out by maceration for 7 days in each solvent at

room temperature (25±2°C). The solvents extracted material was filtered in separate flaks. All extracts were then dried and stored at 4°C until further analysis. The dried aqueous, ethanol, methanol, hexane, chloroform, isopropanol and water extracts were then dissolved in their respective solvents in a proportion of100mg/ml [9-11].

Microbial strains and cultivation. Antimicrobial assays were carried out against eleven bacterial strains, five Gram-positive bacterial strains, including *Staphylococcus epidermidis* ATCC 12228, *Bacillus subtilis* ATCC 6633, *Bacillus cereus* CCM 99, *Staphylococcus aureus* ATCC 6538/P, *Streptococcus faecalis* ATCC 8043, six Gram-negative bacterial strains, including *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimurium* CCM 583, *Aeromonas hydrophila* ATCC 19570, *Klebsiella pneumoniae* CCM 2318, and yeast *Candida albicans* ATCC 10239.

The bacteria strains were inoculated on nutrient broth (Oxoid) and incubated for 24 h at 30±0.1°C, while the yeast was inoculated on yeast extract broth (Oxoid) and incubated for 48 h. Adequate amounts of autoclaved Müller-Hinton Agar (Oxoid) and Yeast Extract Agar (YEA) were dispensed into sterile plates, and allowed to solidify under aseptic conditions. The counts of bacteria and yeast strains were adjusted to yield approximately 1.0×10^7-1.0×10^8/ml and 1.0×10^5-1.0×10^6/ml, respectively, using the Standard McFarland counting method. Of the test organisms 0.1 ml was inoculated with a sterile swab on the surface of appropriate solid medium in plates. The plates containing the bacterial and yeast cultures were incubated at 25±0.1°C and 30±0.1°C, respectively, for 1 h [12].

Study of antimicrobial activity by disc diffusion method. For the first screening, the paper disc diffusion method was used to determine antibacterial activity, which is based on the method described previously [13]. Sterile paper discs (6 mm; Oxoid) were loaded with 50 µL of different amounts (0.25, 0.5 and 1 mg) of the extracts dissolved in dimethyl sulphoxide (DMSO) (Lab-Scan) and were left to dry for 12 h at 37 °C in a sterile room. Bacterial suspensions were diluted to match the 0.5 MacFarland standard scale (approximately 1.5×10^8 CFU/ml) and they were further diluted to obtain a final inoculum. After Mueller-Hinton agar (Merck) was poured into Petri dishes to give a solid plate and inoculated with 100 µl of suspension containing 1×10^8 CFU/ml of bacteria, the discs treated with extracts were applied to petri dishes, ampicilin (10µg/ml) (Oxoid), chloramphenicol (30µg) (Oxoid), nystatin (30µg/disc) (Oxoid) and erythromycin (10µg/disc)were used as positive controls and paper discs treated with ethyl acetate, methanol and DMSO were used as a negative control. The plates were then incubated at 35 °C for 24 h in an incubator. Inhibition zone diameters around each of the discs were measured and recorded at the end of the incubation time.

The minimal inhibitory concentrations (MICs). The MIC was determined by the tube macro-dilution method [14]. Solution of each extract was serially diluted two fold in Mueller-Hinton broth (Merck) so the final concentrations of the extracts in the medium were ranged from 0.008 to 256 mg/ml. Initial inoculants were prepared by suspending growth in a sterile saline and turbidity was adjusted to yield 0.5 McFarland standard and then diluted to 1:10 ratio. Prepared inoculum (0.1 ml) was added into each tube to obtain the final turbidity (approximately 104 colony-forming units (CFU) ml^{-1}). The MIC was defined as the lowest concentration of the plant extract at which visible growth is inhibited. The test tubes were incubated at 24°C/24 h. Each test included control, consisting of the substrate with the solvent. The MICs of erythromycin (Oxoid), chloramphenicol (Oxoid) and nystatin (Oxoid) were also determined.

TABLE 1
Antimicrobial activity of mistletoe leaves (*Viscum album* L.) extracts against test microorganisms by disc diffusion method

Microorganisms	M			E			H			C			I			W	
	M (mm)	W (mm)	D (mm)	E (mm)	W (mm)	D (mm)	H (mm)	W	D	C (mm)	W (mm)	D (mm)	I (mm)	W (mm)	D (mm)	W	D (mm)
S. faecalis	22	9	13	14	9	10	-			-	8	8	14	7	11		8
S. typhimurium	14	9	9	15	-	11	15			10	8	-	12	8	7		9
E. coli	17	11	12	14	-	13	-			13	-	7	15	-	10		-
P. aeroginosa	16	8	11	19	-	8	9			9	7	11	9	10	11		13
A. hydrophila	12	11	11	15	-	13	9			7	6	10	10	10	6		11
S. epidermidis	14	11	10	11	-	14	9			6	6	8	8	8	8		10
S.aureus	15	11	19	16	-	15	9			8	7	13	8	16	10		9
K. pneumoniae	21	13	13	18	-	11	-			7	-	8	10	9	8		-
B. cereus	13	9	21	16	-	12	-			12	-	10	13	8	9		13
B.subtilis	22	10	11	12	-	12	-			11	8	8	19	9	11		12
C. albicans	9	10	9	8	13	11	-			12	-	10	16	-	12		-

No effect

(-): No inhibition

M: Methanol W: Water D: % 10 DMSO E: Ethanol H: Hegzan C: Chloroform I: Isopropanol

TABLE 2
Antimicrobial activity of ethanol, methanol, chloroform, isopropanol and DMSO against tested bacteria by disc diffusion method.

Microorganisms	Inhibition zone diameters (mm)					
	Methanol	Ethanol	Hexane	Chloroform	Isopropanol	DMSO
S. faecalis	14	13	-	9	11	-
S. typhrium	-	-	-	-	-	-
E. coli	-	-	-	11	15	-
P. aeroginosa	10	10	-	-	13	9
A. hydrophyla	7	8	-	7	10	9
S. epidermidis	-	-	-	-	-	-
S.aureus	17	14	-	-	21	16
K. pneumoniae	9	10	-	8	23	10
B. cereus	-	9	8	-	12	8
B.subtilis	17	17	11	-	12	11
C. albicans	18	11	-	-	12	10

(-): No inhibition

TABLE 3
Inhibition zone diameters of the reference antibiotics against test microorganisms

Microorganisms	Inhibition zone diameters (mm)				
	Ampicilin	Penicillin G	Erythromycin	Chloramphenicol	Nystatin
S. faecalis	28	30	22	25	-
S. typhimurium	25	32	27	23	-
E. coli	29	32	24	33	-
P. aeruginosa	28	33	22	28	-
A. hydropyhila	27	31	25	26	-
S. epidermidis	23	30	23	25	-
S. aureus	29	27	24	20	-
K. pnemuoniae	28	29	26	25	-
B. cereus	26	30	25	24	-
B. subtilis	32	35	16	30	-
C. albicans	-	-	-	-	12

(-): No inhibition

RESULTS AND DISCUSSION

The extract of methanol, ethanol, hexane, chloroform, isopropanol and water of mistletoe leaves were tested for antimicrobial activity against 11 bacteria and one yeast by disc diffusion method. In our study, methanolic extracts mistletoe of leaves showed the best inhibition zones against *S.faecalis* and *B.subtilis* (22mm)in table 1. Inhibition zones of the all of the extracts varied 6 to 22 mm against tested microorganisms in Table 1. Besides, the inhibition zone diameters of the tested extracts against the test microorganisms were shown Table 2. Among the tested extracts, isopropanol showed the best inhibition zones against *K.pneumoniae* (23mm) in Table 2. All of the tested extracts showed no inhibition zones against *S.typhimurium* and *S.epidermidis* in Table 2.

In this study, 5 reference antibiotics were used as positive control. These include, ampicilin (10µg/disc), penicillin G (10µg/disc), erythromycin (15µg/disc), chloramphenicol (30µg) and nystatin (30µg/disc). Ampicilin, penicillin and chloramphenicol very strongly inhibited the growth of *B.subtilis* whereas, erythromycin exhibited a very big zone of inhibition against *S. typhimurium* and *K. pneumoniae*. Nystatin weakly inhibited the growth of *C. albicans* (Table 3).

Erturk and his friends [15] reported that different concentrations of n-hexane extract were tested using the agar diffusion technique against 6 bacteria (*B.subtilis, S.aureus, E.coli, P.aeruginosa, Enterobacter cloacae* and *Proteus vulgaris*), and 1 fungus (*C.albicans*). It was displayed that fractions 6 and 7 of n-hexane extract of *V. album* subsp. abietis showed antimicrobial activity against the microorganisms tested.

Hussain and his friends [16] reported that the ethyl acetate, chloroform, ethanol, and methanol crude extracts of selected plant parts had significant antimicrobial activities on both gram positive and gram negative bacteria. The ethyl acetate and methanol crude extracts of leaves and twigs of *V. album* exhibited prominent activities against gram positive and gram negative bacteria used in comparison to other extracts which had moderate activity against all the tested bacteria. The antimicrobial activities of the crude extracts of the selected plant parts were more active against gram negative bacteria than gram positive bacteria.

Kang and Chung [17] reported that ethyl ether fraction against *B.cereus* showed stronger activities than benzoic acid (2.5 mg/ml). The MIC of Korean

TABLE 4
MICs of the ethanolic extracts of leaves of mistletoe, erythromycin, chloramphenicol, nystatin against test microorganisms

Microorganisms	MIC (mg/ml)			
	Methanol	Antibiotics		
		Erythromycin	Chloramphenicol	Nystatin
S. faecalis	4	4	2	-
S. typhimurium	64	0.016	4	-
E. coli	16	2	0.008	-
P. aeruginosa	32	4	0.016	-
A. hydrophila	128	2	0.16	-
S. epidermidis	64	4	0.16	-
S.aureus	32	2	8	-
K. pneumoniae	8	2	2	-
B. cereus	64	2	4	-
B.subtilis	4	32	0.008	-
C. albicans	256	-	-	16

(-): No inhibition

mistletoe extract and slovent fractions were in the range of 6.25-25 mg/ml. The MIC (6.25 mg/ml) of ethyl acetate fraction onto *Staphylocossus aureus* was the lowest among them. Ethyl ether fraction which showed the strongest antioxidant activities by DPPH (1,1-diphenyl-2-picryl-hydrazyl) and FRAP (ferric ion reducing antioxidant power) methods had the highest total phenolic contents. It is suggested that Korean mistletoe could be utilized as natural preservative material through the study of the active compounds from ethyl ether fraction. Also Cebovic and his friends [18] reported that *Viscum album* L. grown on plums exhibited significant antioxidative and hepatoprotective potential against in vivo CCl4-induced oxidative stress in experimental animals. The mechanism appeared mostly to be mediated by induction of antioxidant enzymes activities. In addition, applied mistletoe extract might also possess beneficial effects on restoration of impaired oxidative balance in normal tissues as an efficient antioxidant. Their data suggest that "plum's" mistletoe may be potentially useful in the prevention of the liver injuries caused by oxidative tissue damage, but emphasise the need of further elucidation of mechanisms of its action. Cebovic and his friends [19] reported that non-polar CO2 extract of Viscum album leaves and selected constituents of the extract have cytotoxic activities against EAC and AS30D cells in vivo, although the extract appears to be more active. The extract and its selected constituents also exhibited certain antioxidant activity, and it was suggested that the possible mechanism of their action may be due to altering the antioxidant status in tumour cells. Cebovic and Popovic (2007) [20] reported that 5% aqueous extract of *Viscum album* leaves may be potentially useful in the prevention of tumor development, but emphasise the need of further elucidation of its action mechanisms, as well as qualitative and quantitative analysis of this specific extract. Miguel and his friends [21] reported that *Berberis vulgaris* and *Viscum album* were the most promising as antioxidants, anti-inflammatory and acetylcholinesterase inhibitors. The capacity for scavenging ABTS and DPPH free radicals of *V. album* and *Chamaerops humilis* extracts were also not significantly different from those of the positive controls. *Viscum album* extract had also an important capacity for scavenging hydroxyl radicals.

MICs of mistletoe different extracts obtained by the broth serial dilution method, showed the lowest sensitivity to *S.faecalis* and *B.subtilis* with 4 mg/ml concentration of methanolic extracts in table 4. Methanolic extracts of mistletoe were among the most active with the MIC values ranging from 4-256 mg/ml in Table 4.

CONCLUSION

On the basis of present investigations, it is concluded that there exists a great potential in the search of new and more potent antimicrobial substances from the natural sources. The potential for developing antimicrobials from plants appears rewarding as it will lead to the development of phytomedicines to act against microbes.

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REFERENCES

[1] Baratta, M.T., Dorman, H.J.D., Deans, S.G., Figueiredo, A.C., Barroso, J.G., Ruberto, G. (1998) Antimicrobial and antioxidant properties of some commercial essential oils. Flavour and Fragrance Journal. 13, 235–244.

[2] Seenivasan, P., Jayakumar, M. and Ignacimuthu, S. (2006) In vitro antibacterial activity of some plant essential oils. BMC Complementary and Alternative Medicine. 6, 39.

[3] Nazia, M., Chaudhry, A. and Tariq, P. (2006) Antimicrobial Activity of Cinnamonum cassia against diverse microbial flora with its nutritional and medicinal impacts. Pakistan Journal of Botany. 38(1), 169-174.

[4] Sartoratto, A., Machado, A.L.M., Delarmelina, C., Figueira, G.M., Duarte, M.C.T., Rehder, V.L.G. (2004) Composition and antimicrobial activity of essential oils from aromatic plants used in Brazil. Brazilian Journal of Microbiology. 35, 275-80.

[5] Kafaru, E. (1993) Herbal remedies. The Guardian. Thursday, 3, 24.

[6] Obatomi, D.K., Aina, V.O. and Temple, V.J. (1996) Effect of African Mistletoe on blood pressure in spontaneously hypertensive rats. International Journal of Pharmacognosy. 34(2), 124-127.

[7] Osadebe, P.O. and Ukwueze, S.E. (2004) A comparative study of the phytochemical and antimicrobial properties of the Eastern Nigeria species of African Mistletoe (Loranthus micranthus) sourced from different host trees. Journal of Biology Research and Biotechology. 2(1), 18-23.

[8] Ukwueze, S.E. (2008) An evaluation of the Antibacterial Activities of Leaf Extracts from *Loranthus micranthus* L, Parasitic on *Persea americana*. Science African. 7(1), 51-55.

[9] Ushimaru, P.I., Mariama, T.N., Luiz, C., Di Luciano, B. and Ary, F.J. (2007) Antibacterial activity of medicinal plant extract. Brazilian Journal of Microbiology. 38, 717-719.

[10] Hussain, M.A., Khan, M.Q., Hussain N. (2011) Antimicrobial Screening of *Viscum album* L. Extracts. 2nd International Conference on Environmental Science and Technology. IPCBEE vol.6.

[11] Tripathi, S., Ray, S., Das, P.K., Mondal, A.K., Verma, N.K. (2013) Antimicrobial Activities of Some Rare Aerial Hemiparasitic Taxa of South West Bengal, India. International Journal of Phytopharmacology. 4(2),106-112.

[12] Bradshaw, L.J. (1992) Laboratory microbiology. 4th Ed., Saunders College Publishing. Fort worth.

[13] Ali, N.A., Julich, W.D., Kusnick, C., Lindequist, U. (2001) Screening of Yemeni medicinal plants for antibacterial and cytotoxic activities. J Ethnopharmacol. 74, 173–179.

[14] NCCLS. (1997) Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved Standard M7-A4. National Committee for Clinical Laboratory Standards, Wayne PA, USA.

[15] Erturk, O., Katı, H., Yaylı, N., Demirbag, Z. (2003) Antimicrobial Activity of *Viscum album* L. subsp. abietis (Wiesb). Turkish Journal of Biology. 27, 255-258.

[16] Hussain, M.A., Khan, M.Q., Hussain, N. and Habib, T. (2011) Antibacterial and antifungal potential of leaves and twigs of *Viscum album L.* Journal of Medical Plants Research. 5(23), 5545-5549.

[17] Kang, S.J., Chung, S.K. (2012) Antioxidant and Antimicrobial Activities of Korean Mistletoe (*Viscum album* var. *coloratum*) Extracts against Food Poisoning Bacteria. Korean Journal of Food Preservation. 19(6), 919-924.

[18] Cebovic, T., Spasic, S., Popovic, M., Borota, J. and Leposavic, G. (2006) The European mistletoe (viscum album L.) grown on plums extract inhibits CCL_4-induced liver damage in rats. Fresen. Environ. Bull. 15, 393-400.

[19] Cebovic, T., Popovic, M., Rovcanin, B. and Gojkovic, Z. (2012) Evaluation of the cytotoxic and antioxidant effects of non-polar viscum album L. extract (collected from juniperus communis). Fresen. Environ. Bull. 21, 1454-1460.

[20] Cebovic, T. and Popovic, M. (2007) Effect of European mistletoe (viscum album L.) grown on plums under antioxidant status of ehrlich tumor cells in vivo. Fresen. Environ. Bull. 16, 435-442.

[21] Miguel, M., Bouchamaa, N., Aazza, S., Gaamoussi, F., Lyoussi, B. (2014) Antioxidant, anti-inflammatory and anti-acetylcholinesterase activities of eleven extracts of *Moroccan* plants. Fresen. Environ. Bull. 23, 1375–88.

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CORRESPONDING AUTHOR

Dilek Keskin
Kosk Vocational High School,
Adnan Menderes University,
Aydın – Turkey

e-mail: dkeskin@adu.edu.tr