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RESEARCH ARTICLE

IN VITRO BIOLOGICAL ACTIVITY EVALUATION OF ETHANOLIC EXTRACT OF *MELICA UNIFLORA* LEAVES

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ABSTRACT

Although Melica uniflora is part of the omnivore and herbivore animal diets, there are not enough studies. The aim of the study is to determine the antimicrobial and antioxidant activity also total phenolic and total flavonoid amount of ethanolic extract of *M. uniflora* leaves from Yenice Forest, Karabük province, Turkey. In the study, we used the disk diffusion method to evaluate the antimicrobial activity with eight bacteria and two yeast strains; DPPH free radical scavenging activity and ferric reducing antioxidant power analysis were used determine antioxidant activity. Folin-Ciocalteu method for determining the total phenolic amount and $AlCl_3$ method for the total flavonoid content of the extract were tested. Mean diameters of inhibition zones (IZD) of the bacteria were found in the range of 19.02 mm to 26.32 mm. This value was measured as 16.43 mm and 21.38mm for yeasts. The total antioxidant activity value of the extract was calculated at 4.54 mg AAE/g. The IC_{50} value was calculated 18.798 mg/mL for DPPH free radical scavenging activity. The FRAP value indicated that the reducing power of 1 gram of sample was equivalent to 3.33 µmol of Trolox. The total phenolic content of ethanol extract of *M. uniflora* leaves was determined as 0.466 mg GAE/g, while the flavonoid content was calculated as 4.44 mgQE/g. According to the obtained results, the analyzed *M. uniflora* leaves ethanol extracts demonstrated that the biological activity level could be considered significant.

Keywords: Wood melick, forage selection, Yenice Forest, Actinomycetes, *Nocardia*

1. INTRODUCTION

Some plants, such as *Melica uniflora*, are indicator types that can indicate how old a wooded area is. However, these plants may differ from region to region because habitats, lands, and conditions change the current flux [1]. *M. uniflora*, which is one of the indicator species of ancient woodland, is an essential subdivision of forests like beech (*Fagus* sp.) and spruce (*Picea* sp.) forests and is an important part of the feeding of herbivorous animals like *Capreolus capreolus* (roe deer) [2]. We know that herbivores are critically linked to high-quality plants or plant organs [3]. It is also known that there is an interaction with earthworm species like *Lumbricus* and ground cover species like *M. uniflora*. According to Campana et al. (2002), total abundance and the distribution of *L. terrestris* and *L. rubellus* species were not random. Because earthworms can influence their environment by mucus and urine production and change the distribution of organic and mineral matter, they prefer to feed by unique plants like M. uniflora. So, earthworms' distribution affects the soil microflora and build-up the structure of the soil [4]. This interaction can also influence vegetation's future by force of dispersion or predation of seed, drilling of pores further used by roots, and changes in nutrient availability and finally the forest health.

According to “How does environmental variation influence body mass, body size, and body condition? Roe deer as a case study” named study, "*Fagus sylvatica*" was found to be concentrated in the regions where there were relatively few livers in the study named "roe deer" (*C. capreolus*). At the same time, *Brachypodium* spp., *Ruscus aculeatus*, *Hedera helix*, *Rubia peregrina*, and *M. uniflora*

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were reported to be dominant species. *Brachypodium* spp. nor *Ruscus aculeatus* is preferred by roe deer [5]. Probably one of the preferred roe deer forage in these periods is *M. uniflora*. Can and Togan (2009) also reported that *C. capreolus* species was in the Yenice Forests in the Karabük province [6].

From ancient times to the present, pathogenic bacteria are a big problem for plants, animals, and humans. Even today, we need plant materials cause of helping to cure. Therefore, studies about the antimicrobial activity of plant materials are favored. At this point, choosing plants that will work is a critical step. In this study, we focused on the herbivore's forage selection mentioned above.

For screening biological activity, antimicrobial activity is a critical stage. As far as we know, the antimicrobial activity of *M. uniflora* has not been reported in the literature. For this reason, we preferred to study some members of soil microflora and especially pathogenic ones. Actinomycetes are a filamentous Gram-positive bacteria group widely distributed in soil, water, and affiliation with plants [7]. *Nocardia* and *Micrococcus* are the well known pathogenic actinomycetes for animal species and humans [8-10]. Especially, *Nocardia* causes rare but clinically important diseases, and the infections require a long duration of antibiotics [11]. So we added four actinomycetes, including *Nocardia*, *Micrococcus*, and *Streptomyces* species, to the study.

Plant phenolics contain phenolics acids, flavonoids, tannins, stilbenes, and lignans. The most abundant polyphenols in herbivore and omnivore diets are flavonoids. Spite of plant phenolics wide distribution, The researchers have been interested in plant phenolics because of their health effects, only in several decades. Polyphenols are interesting molecules because of their antioxidant potent, especially for researchers and food manufacturers. They can prevent oxidative stress in cells. Besides this, it has been reported that they can module the activity of many enzymes and cell receptors [12]. According to Provenza et al. (2015), herbivores do not graze at random but have marked food preferences, including selecting plants for self-medication [13]. For these reasons, we added our study total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity tests for a better evaluation. From this view, we perceive that identifying *M. uniflora* leaves' biological activity may help explain the reasons for animal forage selection or forest health.

2. MATERIALS AND METHODS

M. uniflora leaves were collected from Yenice Forests (1350 m altitude), Karabük province in Turkey during 2017 by Sevda Türkiş. The plant samples were stored in a zipper bag at + 4°C until analysis. The identification of this specimen was carried out using the Flora of Turkey by Sevda Türkiş [14] (Figure 1). The voucher specimen was deposited at the herbarium of Ondokuz Mayıs University, Samsun, Turkey (OMUB Herbarium No:8311) [15].

Figure 1. *M. uniflora*. The photo was taken by Sevda Türkiş [15]

Organic solvent extraction of the plant was prepared according to the methods by Wandscheer et al. [16]. Dried *M. uniflora* leaves were ground then extracted with ten-volume ethanol (95%) in the dark at room temperature for seven days. Extracts were filtered, and a rotary evaporator removed ethanol. Stok solution at 110 mg/mL was prepared using ethanol (70%) again as a solvent for bioassays.

Screening antimicrobial activity of the extract was individually tested against pathogenic strains of Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Proteus vulgaris* NRRL B-123), non filamentous Gram-positive bacteria (*Bacillus subtilis* NRRL B-209, *Staphylococcus aureus* ATCC 6538), filamentous Gram-positive bacteria (*Micrococcus luteus* NRRL B-1018, *Nocardia abscessus* DSM 44432, *Nocardia cyriacigeorgica* DSMZ 44484, *Streptomyces murinus* ISP 5091), yeast (*Candida albicans* ATCC 10231, *Saccharomyces cerevisiae* ATCC 9763). 50 µL ethanolic *M. uniflora* leaves extract was loaded on 6 mm sterile blank disks 30 min after Mcfarland adjustment (100 µL 0.5 Mcfarland organism). All bacterial type strains were cultured in the Mueller-Hinton Agar at 37°C; the others were cultured at 30°C. It was recorded the inhibition zone diameters of non-filamentous gram-positive and Gram-negative bacteria after 24 hours incubation period, and the others were recorded after 48 hours incubation period using a digital caliper. 70% ethanol, sulphafurazole (Sf 300), and nystatin were used as control groups. Antimicrobial activity tests were performed using disk diffusion methods to find antimicrobial sensitivities of selected pathogens on agar plates according to the CLSI procedures [17-19]. The disk diffusion test was repeated three times for each microorganism.

The phosphomolybdenum method was used to determine the total antioxidant content of the extract. The result was expressed as ascorbic acid equivalent (mg AAE/g dry extract) [20].
The scavenging activity (H+/e- transferring ability) of the extract against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was evaluated according to the method of Sanchez-Moreno et al. following the absorbance decline at 517 nm and expressed with SC_{50} (mg/mL) value (the extract amount that scavenges the 50% of the radicals in reaction medium) [21].
The scavenging activity value obtained for each different extract concentration was calculated using the following equation.

$$\text{Scavenging Activity (\%)} = (A_{blank} - A_{sample})/A_{blank} \times 100$$

SC_{50} value was also calculated using the graph drawn between activity and concentration values [21].
The ferric-reducing/antioxidant power (FRAP) of the extract was determined following the method based on the principle of reducing the Fe (III) - 2,4,6- tripyridyl-s-triazine (TPTZ) complex in the presence of antioxidants to form blue Fe (II) -TPTZ complex and measurement of maximum absorbance at 595 nm [22]. FRAP value of the *M. uniflora* extract was expressed as Trolox equivalent (µmol Troloxs/g extract).

Total phenolic content of the ethanolic extract prepared from *M. uniflora* leaves was determined following the Folin-Ciocalteu method modified by Singleton & Rossi. The amount of phenolics was expressed as gallic acid equivalent (mg GAE/ g dry extract) [23]. The extract's total flavonoid content was tested by following the $AlCl_3$ method and calculated as quercetin equivalent (mg QE/g dry extract) According to the method, 1 mL of 2% $AlCl_3$ solution (prepared in methanol) was mixed with the same volume of extract. The extract absorbance was measured at 415 nm against the blank after 10 minutes [24].

The antibacterial activity data were analyzed on Statistical Package for the Social Science Predictive Analytics SoftWare Statistics (SPSS PASW Statistic) version 18. One-way analysis of variance followed by the Tukey honestly significant difference (HSD) test. The results were evaluated in the confidence limit of 0.05.

3. RESULTS AND DISCUSSION

Although trimethoprim-sulfamethoxazole (TMP-SMX) is the preferred antibiotic alone in treating *Nocardia* infections, in vitro susceptibility studies conducted in recent years, have revealed that sulfonamide resistance has been observed [25]. For this reason, research on alternative drugs has increased in recent years, especially for pathogenic actinomycetes [26]. As a result of the literature review, we found eight *Melica* species in Turkey (*M. altissima, M. ciliata, M. eligulata, M. minuta, M. uniflora, M. penicillaris, M. persica* and *M. pieta*). However, there was not any report about the antimicrobial effect of *Melica* species. Therefore this study is significant because of the first antimicrobial activity survey of *Melica*. For this aim, we used ten pathogens by disk diffusion method for antimicrobial activity assay (Figure 2 (a-d)).

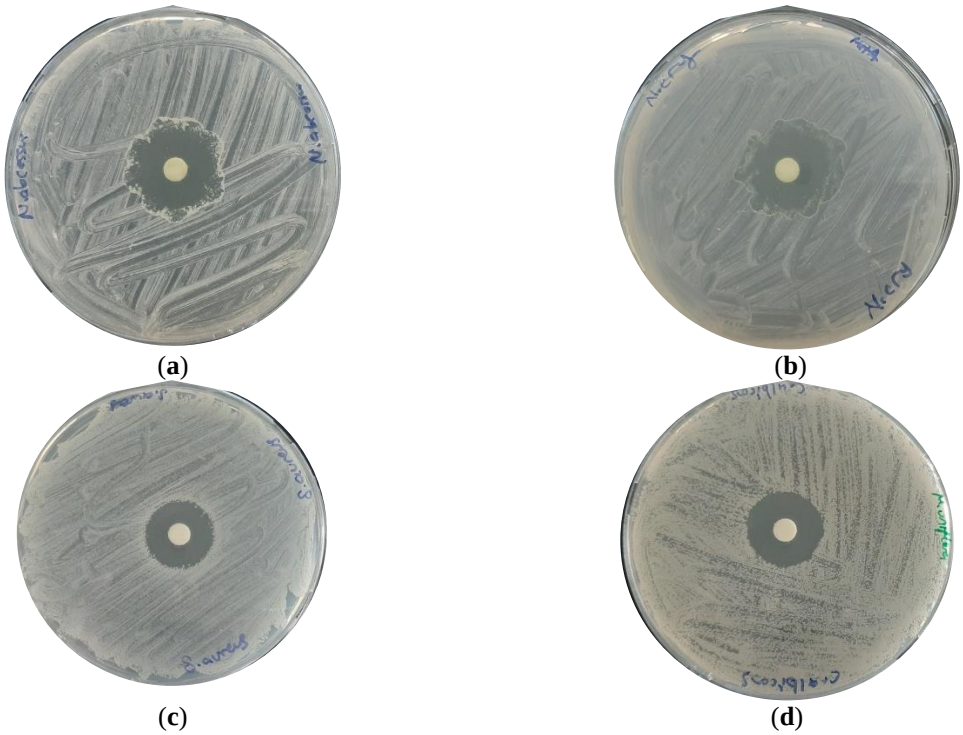

Figure 2. Disk diffusion photos of *M uniflora* leaves ethanolic extract (a) *N. abscessus* (b) *N. cyriacigeorgica* (c) *S. aureus* (d) *C. albicans.* The photos were taken by E. ÇİL

In Table 1, inhibition zone diameter values have been given by the mean of three replicates (mm) (mean ± Standard Deviation; n = 3) followed by different letters mean significant differences at $p < 0.001$ according to Tukey HSD test. Results can be classified into three levels qualitatively as sensitive (inhibition zone diameter ≥ 20 mm), intermediate (15 mm ≤ inhibition zone diameter ≥ 19 mm) and resistance (inhibition zone diameter ≤ 14 mm) based on zone size interpretative chart of Clinical and Laboratory Standards Institute [18]. As in the present work, we verified that the smallest inhibition zone for bacteria was *E. coli* (ATCC 25922T) with 19.02. mm, and for yeast was *S. cerevisiae* with 16.43 mm and also showed moderate antimicrobial activity. The others showed vigorous antimicrobial activity, particularly the most sensitive bacteria was *M. luteus* for sulphafurazole (41.18 mm) and the ethanolic extract (26.32 mm). Many studies reported that generally, plant extracts more effective on Gram-positive bacteria than Gram-negative bacteria because of the cell wall contents [27]. The high lipid content of the Gram-negative bacteria can prevent antibiotics from entering. Our study results supported these reports.

Table 1. Antimicrobial activities of *M. uniflora* leave ethanolic extract against tested microorganisms based on inhibition zone diameters.

Tested Microorganisms		Inhibition zone diameters (mm) of the extract	Sf300*	Nystatin (0.5 mg/mL)
Gram-positive filamentous bacteria (Actinomycetes)	*M. luteus*	26.32±0.8^{a}	41.18	No inhibition zone
	N. abscessus	23.24±0.1^{c}	32.56	No inhibition zone
	N. cyriacigeorgica	22.43±0.1^{bc}	31.45	No inhibition zone
	S. murinus	21.73±0.1^{b}	31.82	No inhibition zone
Gram-positive non-filamentous bacteria	*B. subtilis*	23.7±0.2^{ab}	34.10	No inhibition zone
	S. aureus	21.68±2.3^{bc}	29.73	No inhibition zone
Gram-negative bacteria	*E. coli*	19.02±1.6^{d}	36.51	No inhibition zone
	P. vulgaris	24.88±1.5^{ab}	30.96	No inhibition zone
Yeast	*C. albicans*	21.38±0.2^{bc}	No inhibition zone	33.12
	S. cerevisiae	16.43±0.1^{d}	No inhibition zone	30.40

*Oxoid Sulphafurazole Antimicrobial Susceptibility Disks 300µg

According to the obtained results from actinobacterial activity studies, *M. uniflora* leaves ethanolic extract is highly effective on Gram-positive bacteria, including filamentous and non-filamentous ones. In literature, antibacterial assays are generally limited to non-filamentous bacteria. So this study contributes new data for further studies, especially filamentous Gram-positive bacteria. When the previous studies are examined, we did not find many studies about antibacterial extracts on pathogenic actinomycetes. Çil and Türkiş had investigated antibacterial effects of *Taxus baccata* L. leaves from Yenice Forest [28]. Inhibition zone diameters from *Taxus baccata* ethanolic leaf extract were reported for *M. luteus* 16.61 mm, *N. cyriacigeorgica* 24.66 mm, *N. abcessus* 12.98 mm, and *S. murinus* 20.10 mm. When the results are compared, the antibacterial effect of *M. uniflora* leaves' ethanolic extract is more effective than *Taxus baccata* ethanolic leaf extract, especially on *N. abcessus*.

We know that phenolics can affect a broad spectrum of biochemical activities and gene expression, and antioxidants can be used to threaten or prevent diseases [29]. Cui et al. (2016) reported that anti-nutritional contents as antioxidants are also crucial in determining dietary selection in yaks [30].

On the other hand, the studies, including screening antioxidative potentials of *M. uniflora*, are rare in literature. So, the present study provides a significant contribution to the literature (Table 2).

Table 2. Total phenolic and flavonoid contents and antioxidant screening of *M. uniflora* leaves

Assays (measurement unit)	Values
Total phenolic content (mg GAE/g dry extract)	0.466
Total flavonoid content (mg QE/g dry extract)	4.44
Total antioxidant activity (mg AAE/g dry extract)	4.54
DPPH (IC_{50};mg/mL)	18.798
FRAP (µmol of TX/g dry extract)	3.33

Badridze and Kacharava [31] had investigated the antioxidant contents of leaves of some plants including *M. uniflora* as an ornamental plant and soluble phenols content had been calculated as 1.25

mg/g dry weight. Furthermore, the extract prepared using 200 mg of the dry plant had dsisplayed 24.2% inhibition on DPPH radical. The exciting thing is that it has the lowest antioxidant activity among the other 17 plants such as *Primula saguramica*, *Lamium album*, *Crataegus kyrtostyla*, etc., tested in the same study. This report supports the low phenolic content and antioxidant activity of *M. uniflora* from Yenice forests, while other plant species such as *Euonymus latifolius* or *Taxus baccata* have higher total phenolic content and antioxidant activity. Some researchers have stated that plants located in high altitudes have a higher antioxidant capacity than low altitude plants [32-34]

Similarly, 21 different plant species such as *Rosa canina, Rubus sanctus* Schreb, and *Primula vulgaris* Huds have been studied in a study involving *Hedera helix*; another species thought to be predominant in areas fed by *C. capreolus*. According to the study results, the inhibition ratio on DPPH radical and total phenolic content of the *H. helix* extract had almost the lowest values among the obtained values for other species tested [35]. As *H. helix*, *Ruscus* species are also preferred as a nutrition source for *C. capreolus* and hoping to find new resources; a study was designed to exhibit *Ruscus* species' antioxidant activity in Serbia. The IC_{50} value for DPPH scavenging activity of the ethanol extract of aerial parts of the *Ruscus hypoglossum* L. had been calculated as 1.632 mg/mL. Total phenolic and flavonoid contents were also reported as 8.569mg GAE/g and 0.125 mg routine/g, respectively [36].

It is claimed that these values are compatible with obtained findings for *M. uniflora*. Total phenolic and flavonoid contents of the extract prepared from *M.uniflora* leaves were investigated, and the values were calculated as 0.466 mg GAE/g and 4.44 mgQE/g, respectively. After these analyzes giving information about the presence of phenolics in small quantities and sufficient flavonoids. Total antioxidant activity (TAA) assay and antioxidative activity tests based on DPPH radical scavenging activity and reducing power on Fe^{3+} to Fe^{2+} were carried out with the thought that the extract may have antioxidant activity. Antioxidant activity was detected at a not very high rate, which is proportional to the extract's phenolic content. TAA value was found as 4.54 mg AAE/g. IC_{50} value as an indicator of DPPH free radical scavenging activity was calculated by taking advantage of the graph drawn between extract concentration and activity as 18.798 mg/mL. Furthermore, as a result of the FRAP assay, it can be concluded that the reducing power of 1 gram of sample was equivalent to 3.33 μmol of Trolox.

We believe that *M. uniflora* may be an important forage for roe deer in Yenice Forests due to its antibacterial properties, both phenolic and antioxidant content. Results showed that ethanolic extract from fresh leaves of *M. uniflora* inhibited both Gram-positive and Gram-negative bacteria and also yeasts. The phenolic compounds can dissolve within the bacterial membrane and penetrate the cell, where they interact with cellular metabolic mechanisms [37, 38]. In summary, our firm belief that the study of this natural product can be used as an antimicrobial agent in the pharmaceutical industry is necessary for animal and human health or in the cosmetic industry.

4. CONCLUSIONS

In 1999, the World Nature Fund (WWF) identified the Yenice Forests as one of the 100 global forests, including the Hedge and Protection Areas of Kavaklı. In Turkey, there are nine of them need urgent protection from such forests. These areas, defined as "Hotspots of European Forests," are among the forests with the World's highest biodiversity. Turkey, a part of three phytogeographical biodiversities of the 34 events detected, is the only country in the World [39]. We think that we should have more knowledge about the forests of Yenice and plan different studies about the region to take care of our biological richness and deserve this worthy sight. *M. uniflora* (wood melick, local name in Turkish is seyrek inci otu) is a vital understory element of coppice forest, beech, and spruce forests. It also is mainly found in old forests where rare old species are found, is a character species in aged forest areas like Yenice Forests [40]. Furthermore, in Turkey *M. uniflora*'s IUCN categories are NE. We also suggest to ecologists evaluate these plant species according to IUCN red list criteria.

This study provides evidence that the ethanolic extract obtained from the leaves of the *M. uniflora* plant, which is in the diet of the species found in different trophic levels in the forest, has antibacterial activity on different microorganisms. It could be a relationship between biological activity and forage selection. It should be investigated in further multidisciplinary studies.

With promising revealed antioxidant and antibacterial properties, *M. uniflora* leaves have great potential to be developed into natural preservatives and herbal products, applicable to the food and forage industries. Also, we can say *M. uniflora* is a medicinal plant with a pharmaceutical aspect thanks to antimicrobial and antioxidant properties. It could be used as a medicinal substance in the cosmetic or pharmaceutical industry. This study is also a preliminary study for the role of forest or animal health, and for this purpose, this is a basis for further studying cytotoxic and genotoxic effects of *M. uniflora*.

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