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ANTIMICROBIAL AND ANTIPROTOZOAL EFFECT OF SWEET MARJORAM (*Origanum majorana* L.)

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Abstract. The objective of the present study that has been carried out in the Institute of Chemistry, Warsaw University of Life Sciences, was to investigate antimicrobial activity of a number of sweet marjoram extracts. Raw (fresh or dried) plant material from culinary or medicinal herbs may contain varying amounts of bacteria or protozoa, including some known human pathogens. The isolates, which are prepared by procedures involving the use of elevated temperature and/or organic solvents or other chemicals are expected to have considerably less or no such potentially harmful burden. Four sweet marjoram (*Origanum majorana* L.) fractions obtained by steam distillation, Soxhlet n-hexane extraction, extraction with aqueous ethanol, and with ethanolic ammonia solution were evaluated *in vitro* for activity against twenty Gram-positive or Gram-negative bacterial strains, six *Candida* sp. yeast strains and a single protozoan species *Pentatrichomonas hominis*. The n-hexane extract showed the highest antibacterial activity and inhibited growth of eight out of nine *Staphylococcus aureus* strains used. The other bacteria showed no substantial susceptibility to the extracts, except that *Acinetobacter baumannii* showed some inhibition by the aqueous ethanol extract. The isolates were also active against three out of six *Candida* sp. strains used, and the ethanolic ammonia extract reduced the number of viable *P. hominis* trophozoites by 50% at 160 $\mu g \cdot ml^{-1}$ concentration in 24 h cultures; the remaining extracts were considerably less or but marginally effective. These data warrant further study on identifying the components of the extracts with the highest activities.

Key words: Sweet marjoram, antibacterial activity, antiprotozoal activity, anti-yeast activity*, Pentatrichomonas hominis*

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INTRODUCTION

Essential oils from various condiment plants possess multiple biological activities, including antibacterial, antifungal and antioxidant actions [Baratta at al. 1998, Daferera at al. 2003, Zheng at al. 2009]. Sweet marjoram (*Origanum majorana* L., syn.: *Majorana hortensis* Moench) has been used for centuries both as a culinary and medicinal herb. It has been evidenced in a number of studies that essential oil from sweet marjoram contains mainly terpinen-4-ol, α- and γ-terpinenes, linalool and carvacrol, which are the basis for their antimicrobial properties [Sarer at al. 1982, Daferera at al. 2000, Vagi at al. 2005, Nurzyńska-Wierdak and Dzida 2009]. Essential oil from sweet marjoram reduced the growth rate of *Photobacterium phosphoreum* which is the specific organism responsible for spoilage of modified atmosphere packaged fillets of cod [Mejlholm and Dalgaard 2002]. Combination of essential oils of oregano and thyme, oregano with sweet marjoram and thyme with sage had the most effect against *Bacillus cereus, Pseudomonas aeruginosa, Escherichia coli* O157:H7 and *Listeria monocytogenes* [Tajkarimi at al. 2010]. Whereas other isolates were used rarely in microbiological studies, it has been shown that aqueous and methanolic extracts from sweet marjoram contain multiple compounds with considerable antimicrobial action, e.g. phenolic derivatives (phenolic acids, flavonoids as apigenin, luteolin, quercitin and their glycosides as rutin or isovitexin) [Janicsak at al. 1999, Fecka and Turek 2008]. Some sweet marjoram fractions could act as insecticides. For examplee, the essential oil from sweet marjoram has shown promise as a potential agent for the control of head lice [Yang at al. 2009] and German cockroach [Jang at al. 2005], whereas methanol extract is highly toxic to larvae of the known crop pest *Spodoptera littoralis* [Pavela 2004].

Recent studies on sweet marjoram isolates have produced more interesting findings. For instance, ursolic acid from sweet marjoram was reported to reduce amyloid β-protein neurotoxicity [Heo at al. 2002], methanol sweet marjoram extracts were shown to inhibit of blood platelet adhesion to laminin-coated wells, their aggregation and protein secretion [Yazdanparast and Shahriyary 2008]. Antiproliferative and strong antioxidative effects of aqueous ethanol sweet marjoram extracts were found in human lymphoblastic leukemia cell line (Jurkat) *in vitro* at non-cytotoxic concentrations [Abdel-Massih at al. 2009].

The aim of this study was to compare antimicrobial actions of sweet marjoram isolates obtained with various extraction methods, using a number of yeast strains and Gram-positive and Gram-negative bacteria. Additionally, we tested potential antiprotozoal utility of the isolates, using *Pentatrichomonas hominis* as the model species. This protozoan is a non-pathogenic commensal of the large intestine in humans, which yet can cause stomach ulcers and chronic catarrhs of the large intestine under certain circumstances, and creates serious problems mostly in the developing countries [Yang at al. 1990, Saksirisampant at al. 2003]. Studies on antiprotozoal activity of essential oils and other plant extracts have started quite recently and are sparse [Tasdemir at al. 2008, Mori at al 2008, Roumy at al. 2009]. In particular, to the best of our knowledge, there is no report of the antiprotozoal activity of any sweet marjoram extracts.

MATERIAL AND METHODS

The experimental material consisted of the fresh sweet marjoram leaves collected at the beginning of flowering time (mid-July) from the garden-plot near Zamość in 2009 and dry marjoram of „Kamis" brand purchased from the local food store in Warsaw (date of validity to consumption – December 2010).

A) Volatile fraction was prepared by heating 30 g of ground fresh sweet marjoram leaves with 400 ml of distilled water. The 200 ml of distillate was collected and extracted thrice with methylene chloride (3 × 40 ml). The extracts were combined and dried with anhydrous $MgSO_4$, and the solvent was then removed in a rotary evaporator at +40°C (bath temperature) and 470–490 hPa pressure. It was obtained 57 mg of the oily residue.

B) The preparation of the n-hexane extract was performed in a Soxhlet apparatus with ground fresh sweet marjoram leaves (30 g) and 400 ml of n-hexane. After 4 h the crude extract was evaporated at 40°C and 230 hPa to give 320 mg of light green oily residue.

C) The aqueous ethanol extract was prepared by stirring 20 g of the dry sweet marjoram herb with 500 ml of aqueous ethanol (70%) in a water bath for 10 h at 50°C. Next, the pulp was filtered through filter paper, and the solvent from filtrate was removed to dryness in a rotary evaporator at +50ºC. To remove the residual water it was evaporated twice to dryness with ethanol-toluene mixture (1:3,v/v, 2 × 100 ml) to yield 8.7 g of the light brown powder.

D) The ethanolic ammonia extract was processed in the some way as the aqueous ethanol extract with the exception: a mixture of aqueous ethanol (70%) and 25% aqueous ammonia (95:5, v/v) was used instead of 70% aqueous ethanol. It was received 9.3 g of the light brown powder. Both (aqueous ethanol and ethanolic ammonia extracts) were also assayed for the phenolic compounds content by Folin-Ciocalteu method using gallic acid (Sigma-Aldrich) (GA) as a standard [Singleton and Rossi 1965]. The content of phenolic compounds in the aqueous ethanol extract responds 170 mg GA/g of the plant extract and in the ethanolic ammonia extract 180 mg GA/g of plant extract.

Sweet marjoram isolates were evaluated *in vitro* for activity against the standard bacterial strains: *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis* ATCC 6633, *Proteus mirabilis* NCTC 4635, *Escherichia coli* NCTC 8196, *E. coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 27863, *P. aeruginosa* NCTC 6749, *Stenotrophomonas maltophilia* ATCC 13637, *Burkholderia cepacia* ATCC 25916, *Acinetobacter baumannii* ATCC 19606, *Staphylococcus aureus* ATCC 6538P, *S. aureus* NCTC 4163, *S. aureus* ATCC 29213, *S. aureus* ATCC 25923: clinical isolates: *S. aureus* MRSA, *S. aureus* MLSB A17 (constitutively resistant), *S. aureus* MLSB A18 (with induced resistance), *S. aureus* AK126, and *S. aureus* AK127; *Candida* sp. yeast strains (*C. albicans* ATCC1023, *C. albicans* ATCC 90028, *C. tropicalis* IBA 171, *C. parapsilosis* ATCC 22019, *C. guilliermondii* IBA 155, and *C. krusei* IBA 161) and protozoal strain *Pentatrichomonas hominis* (syn.: *Trichomonas hominis*, *T. intestinalis*).

The microorganisms used in antimicrobial studies were obtained from the National Institute of Public Health – National Institute of Hygiene (Warsaw, Poland), The Children's Memorial Health Institute (Warsaw, Poland), and from the own collection of the

Department of Pharmaceutical Microbiology, Medical University of Warsaw (Warsaw, Poland). *Pentatrichomonas hominis* has been isolated from a diarrheic stool of an adult patient and was cultured at +37°C in 15 ml tubes containing liquid Pahm medium [Chomicz et al. 2009].

Antimicrobial activities were assessed by the disk-diffusion method under standard conditions using Mueller-Hinton II agar medium (Beckton Dickinson) for bacteria, and RPMI agar medium (Sigma) supplemented with 2% glucose (Sigma) according to the guidelines established by the Clinical and Laboratory Standards Institute (formerly National Committee on Clinical Laboratory Standards) for yeast. Briefly, solutions of the tested extracts were prepared in a chloroform-methanol-DMSO mixture (4:1:1, v/v/v) to yield a concentration of 0.367%. Sterile filter paper (Whatman No. 3) disks of 9 mm diameter were dripped with 30 µl of the tested extracts solution or the solvent mixture containing no extracts (control), and were placed on the agar plates uniformly inoculated with the test microorganisms. The diameter of the clear zone surrounding the disk after 18 h incubation at +35ºC was the measure of antimicrobial activity of a given extracts (according to Clinical and Laboratory Standards Institute, Performance standards for antimicrobial disk susceptibility tests. Approved standard – ninth edition. CLSI document M2-A9, Wayne, PA, USA, 2006 and Clinical and Laboratory Standards Institute, Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard – seventh edition. CLSI document M7-A7, Wayne, PA, USA, 2006).

Before using the cultures of *Pentatrichomonas hominis* for antiprotozoal activity test, shape and motility of this protozoan were assessed microscopically in a Bürker chamber. Sweet marjoram extracts and volatile fraction were dissolved in a mixture of methanol and dimethylsulfoxide (DMSO) (1:1, v/v), and were diluted with the same vehicle to give the serial dilutions needed. After the transfer of 1 ml aliquots of *P. hominis* culture to individual 1.5 ml Eppendorf tubes, 10 µl of the tested fractions' serial dilutions were added to the respective tubes to give the final isolate concentrations of 40, 80, 120 and 160 µg ml^{-1}, than 10 µl of the methanol-DMSO vehicle was added to the control tubes. Next, all cultures were incubated at +37°C for 24 hours. The final viable (motile) protozoan counts were taken using the Burker chamber, and were expressed as percentages of the counts of motile protozoans in the respective control cultures.

Two-way analysis of variance (with "hanging" control group) with isolate type and isolate concentration as the main factors revealed significant effects of these factors ($F_{3,37} = 67.3$, $p < 10^{-6}$, and $F_{3,37} = 14.8$, $p = 2\times10^{-6}$, respectively) and a tendency for significant effect interaction ($F_{9,37} = 1.86$, $p = 0.090$).

All statistical analyses were performed using the Statistica software package v. 7.1 [StatSoft Inc, Tulsa, OK, USA].

RESULTS AND DISCUSSION

Of all bacteria tested only *Staphylococcus aureus* strains and *Acinetobacter baumannii* showed some growth inhibition by the isolates obtained by the Soxhlet n-hexane extraction and aqueous ethanol extraction. The majority of bacteria strains listed were resistant to all the fractions studied. (tab. 1).

Table 1. Antibacterial activity of sweet marjoram extracts (disk-diffusion test)[a]
Tabela 1. Aktywność przeciwbakteryjna ekstraktów z majeranku ogrodowego (metoda dyfuzyjno-krążkowa)

Bacteria strain Szczep bakteryjny	Inhibition zone diameter[b] Strefa zahamowania wzrostu, mm	
	Soxhlet n-hexane extract n-heksanowy ekstrakt Soxhleta	aqueous ethanol extract wodno-etanolowy ekstrakt
Staphylococcus aureus ATCC 29213	21	-[c]
Staphylococcus aureus ATCC 25923	19	-
Staphylococcus aureus ATCC 6538P	17	-
Staphylococcus aureus NCTC 4163	22	-
Staphylococcus aureus AK127	18	-
Staphylococcus aureus AK 126	16	-
Staphylococcus aureus MRSA	20	-
Staphylococcus aureus MLSB (constitutive, konstytutywny)	-	-
Staphylococcus aureus MLSB (induced, indukowany)	18	-
Acinetobacter baumannii ATCC 19606	-	17

[a]strains showing no susceptibility to extract, and extracts showing no growth inhibition effect on any bacterial strain tested were omitted from the table – szczepy bakterii niewrażliwe na działanie ekstraktów nie zostały umieszczone w tabeli
[b]taken from three independent experiments – dane z trzech niezależnych powtórzeń
[c]denotes no growth inhibition zone around disk – nie wykazano strefy zahamowania wzrostu wokół krążka

Table 2. Anti-yeast activity of sweet marjoram extracts (disk-diffusion test)
Tabela 2. Aktywność ekstraktów z majeranku ogrodowego wobec grzybów drożdżopodobnych (metoda dyfuzyjno-krążkowa)

Yeast strain Szczep grzybów drożdżopodobnych	Inhibition zone diameter[a] Strefa zahamowania wzrostu, mm			
	A	B	C	D
Candida albicans ATCC 10231	-[b]	-	-	-
Candida albicans ATCC 90028	-	-	-	-
Candida tropicalis IBA 171	14	13	24	22
Candida parapsilosis ATCC 22019	-	-	-	-
Candida guilliermondii IBA 155	*[c]	*	14	11
Candida krusei IBA 161	*	*	14	13

[a] taken from three independent experiments – dane z trzech niezależnych powtórzeń
[b] denotes no growth inhibition zone around disk – nie wykazano strefy zahamowania wzrostu wokół krążka
[c] denotes trace activity with no regularly shaped inhibition zone around disk – wykazano ślad aktywności bez regularnego kształtu strefy zahamowania wokół krążka
A – frakcja z destylacji z parą wodną – steam distillation fraction
B – n-heksanowy extract Soxhleta – Soxhlet n-hexane exstract
C – ekstrakt wodno-etanolowy – aqueous ethanol exstract
D – ekstrakt wodno-etanolowy z dodatkiem amoniaku – ethanolic ammonia exstract

Table 2 shows the effects of the four sweet marjoram fractions on the growth of *Candida* sp. yeast. The most susceptible to all the fractions was *C. tropicalis*, while *C. parapsilosis* and two *C. albicans* strains were entirely resistant, and *C. krusei* and *C. guilliermondii* showed but moderate growth inhibition by aqueous ethanol extracts

only. Our data indicate that some components of sweet marjoram ethanol extracts, probably of polyphenolic structures, may have much stronger antifungal activity than those present in the volatile fraction obtained by water steam distillation. This fraction showed only a minor antifungal effect in our study, whereas 100% inhibition of *C. tropicalis, C. lipolytica* and *C. utilis* growth *in vitro* by the essential oil from Morocco-grown sweet marjoram at 5 ppm was reported in an earlier study [Charai at al. 1996]; the reasons for this disparity are unknown.

P. hominis showed no significant susceptibility to the fractions obtained by steam distillation or Soxhlet n-hexane extraction in this study. However, the ethanolic extracts at 40 μg ml^{-1} concentration significantly reduced the number of viable *P. hominis* trophozoites by about 20% after 24 h exposure. Increasing the concentration of the ethanol extract only marginally and nonsignificantly potentiated this effect, whereas the ethanolic ammonia extract reduced the number of viable trophozoites by 50% at 160 μg ml^{-1}

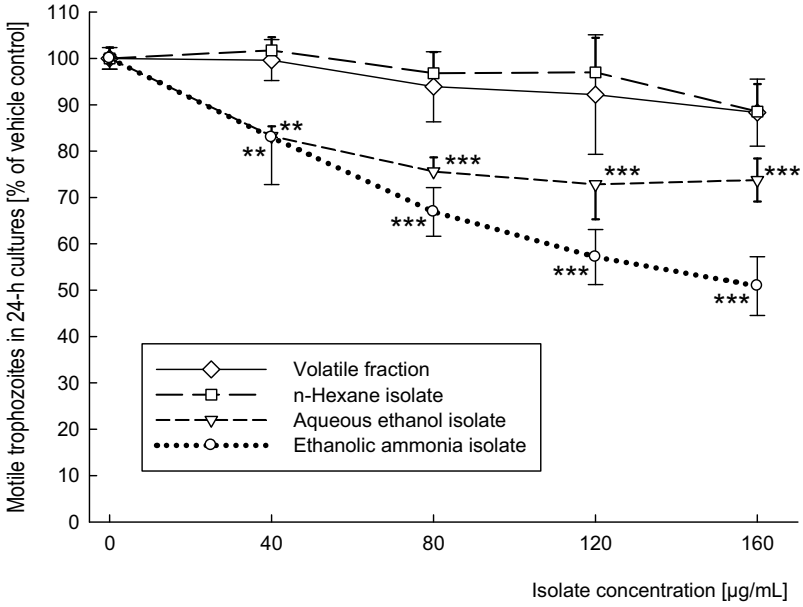

Fig. 1. Inhibition of *Pentatrichomonas hominis* growth *in vitro* by sweet marjoram isolates. Data points are means ± S.D. of three independent cultures, except that there were six independent control cultures (with vehicle added). ** $p< 0.01$, *** $p < 0.001$ vs. the control (no isolate in the culture medium), Dunnett's test; in all cases, "p" values less than 0.05 were considered significant.

Ryc. 1. Wpływ izolatów z majeranku na wzrost *in vitro Pentatrichomonas hominis*. Przedstawione wartości są średnimi ± S.D. z trzech niezależnych hodowli, z wyjątkiem wartości kontrolnej, która jest średnią z sześciu niezależnych hodowli (prowadzonych w obecności nośnika). ** $p < 0,01$, *** $p < 0,001$ w stosunku do wartości dla hodowli kontrolnej (bez izolatu w podłożu), test Dunnetta. We wszystkich przypadkach za próg istotności statystycznej przyjmowano $p < 0,05$.

without the effect reaching a plateau (fig. 1). These results indicate that some sweet marjoram herb components with a presumably acidic character have much stronger antiprotozal activity than those extracted under non-alkaline conditions. The susceptibility of *P. hominis* to the ethanolic ammonia extract may be of some practical importance, as this trichomonad can develop resistance to antiprotozoal drugs and commonly used disinfectants.

Our data show highly diversified antimicrobial activity of different sweet marjoram extracts, which is understandable, as different extraction methods provide material enriched in various substances. The volatile fraction obtained by steam distillation contain mostly isoprenyl-derived volatile compounds, whereas n-hexane extraction yields also other hydrophobic substances beside the isoprenoids. *Origanum majorana* essential oil was reported to be rich in terpenoic hydrocarbons, alcohols and phenolic compounds [Daferera at al. 2000]. Extraction of sweet marjoram with methanol provides mainly phenolic acid (caffeic acid, chlorogenic acid, rosmarinic acid) and flavonoids as apigenin, luteolin, quercetin and rutin [Sellami at al. 2009]. Notably, both the composition and yields of marjoram fractions are also greatly affected by the geographic region origin of the raw material [Baranauskiene at al. 2005] and the choice of harvesting date [Zawiślak 2008, Nurzyńska-Wierdak and Dzida 2009]. The results of the present investigation warrant further study on identifying of the components of the isolates with the highest antimicrobial activities.

CONCLUSION

1. The n-hexane extract showed the highest antibacterial activity and inhibited growth of eight out of nine *Staphylococcus aureus* strains tested.
2. All of the fractions were active against three out of six *Candida* strains.
3. The ethanolic ammonia extract reduced the number of viable *P. hominis* trophozoites by 50% in 24 h cultures.

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PRZECIWBAKTERYJNA I PRZECIWPIERWOTNIAKOWA AKTYWNOŚĆ MAJERANKU OGRODOWEGO (*Origanum majorana* L.)

Streszczenie. Celem badań prowadzonych w Katedrze Chemii Szkoły Głównej Gospodarstwa Wiejskiego w Warszawie było zbadanie aktywności przeciwdrobnoustrojowej otrzymanych wyciągów z majeranku. Materiał roślinny może być często zanieczyszczony różnymi bakteriami lub pierwotniakami, stanowiących zagrożenie dla zdrowia. Należy oczekiwać, że ekstrakty uzyskane w podwyższonej temperaturze i/lub z użyciem rozpuszczalników organicznych oraz innych substancji chemicznych będą istotnie mniej zanieczyszczone mikrobiologicznie. Zbadano aktywność przeciwdrobnoustrojową czterech izolatów z majeranku ogrodowego (*Origanum majorana* L.) otrzymanych poprzez destylację z parą wodną, ekstrakcję n-heksanem w aparacie Soxhleta, ekstrakcję suchego ziela majeranku wodno-etanolowym roztworem i wodno-etanolowym z dodatkiem amoniaku, wobec dwudziestu Gram-dodatnich szczepów bakterii, sześciu szczepów grzybów drożdżopodobnych z rodzaju *Candida* i pierwotniaka *Pentatrichomonas hominis*. Największą aktywność przeciwbakteryjną wykazał wyciąg n-heksanowy, który hamował wzrost ośmiu z dziewięciu badanych szczepów *Staphylococcus aureus.* Pozostałe szczepy bakterii nie były wrażliwe na ekstrakty stosowane w badaniu, z wyjątkiem szczepu *Acinetobacter baumannii,* którego wzrost hamował ekstrakt przygotowany z mieszaniny etanolu z wodą z dodatkiem amoniaku. Etanolowe wyciągi wykazały także aktywność wobec trzech z sześciu szczepów *Candida,* a wodno-etanolowy ekstrakt z dodatkiem amoniaku, redukował przy stężeniu 160 $\mu g \cdot ml^{-1}$ o 50% liczbę *P. hominis*; pozostałe wyciągi były znacznie mniej efektywne.

Słowa kluczowe: ziele majeranku, aktywność przeciwbakteryjna, aktywność przeciwpierwotniakowa, aktywność przeciwgrzybicza*, Pentatrichomonas hominis*

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