

Agro-ecological cultivation, secondary metabolite characteristics and microbiological tests of lemon balm (*Melissa officinalis*) – the variety Citronella

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Lemon balm (*Melissa officinalis* L.) is a native of the eastern Mediterranean region southward to Israel and Syria and eastward through the Crimea, the Caucasus, and Northern Iran to the Tien Shan and the Pamirs. It appears to have been naturalized in the Central Europe including the Carpathian Ukraine and Eastern Slovakia. The objective of this study was characterization of the chemical composition and antimicrobial activity of essential oils from *Melissa officinalis* L. variety Citronella in the conditions of cultivation in a lowland region of the Ukrainian Carpathians. Plant material, balm dry leaves and leafy tops of the shoots, were grown in a small-scale cultivation ground at the Zakarpatian Institute of Agroindustrial Production in Velikaja Bakta, Carpathian-Ukraine. The Citronella variety is used for herb production. Essential oil from balm parts (herb and leaves) was isolated by distillation with boiled water. Hydro-distillation lasted for 4 hours, sample weights were 10 g of dry drug matter. A modified Cooching & Middleton distillation apparatus was used. Identification and evaluation of balm essential oil were carried out using the selected analytical method of HPLC-DAD analysis. The final balm extract was analyzed directly on a Dionex UltiMate 3000 system. The analysis of balm essential oil was carried out using a gas chromatograph Varian 3090, connected to a MS Saturn 2100T integrator. The sensitivity of microorganisms to plant extracts was determined by the disk diffusion test (with disk diameter of 6 mm). The quantitative and qualitative characteristics of balm essential oil (0.05–0.12% v/w) and its composition show very high content of α -citral (geranial) (44%), β -citral (neral) (29%) and citronellal (9%). Rosmarinic acid can be extracted from leaves and its content equals up to $1.7 \pm 0.1\%$. Composition of the natural substances depends on the balm taxon, origin, soil and climatic growing conditions, and methods of harvest and isolation and effective weed control which are used. Antibacterial effect of the oil showed low activity against all bacterial strains. Our results confirmed higher activity of EOs against Gram-positive bacteria than Gram-negative. The balm essential oil displayed antimicrobial effect especially towards *Candida glabrata* clinical strain. Thus, studies have shown that *Melissa officinalis* variety of Citronella in the conditions of cultivation in the lowland region of Carpathian-Ukraine according to biochemical characteristics and microbiological properties corresponds to world analogues.

Keywords: essential oil; chemical composition; antimicrobial activity; aromatic plant.

Introduction

The name balm comes from the Greek balsamon meaning “balsam”, an oily, fragrant resin. Since balm does not actually exude a balsam, it probably received its name from its fragrant aroma and its ability to soothe and calm the nerves. Balm has been known since antiquity for its medicinal qualities and for its use in bee-keeping (bees are particularly attracted to its pollen). The name *Melissa* of the genus of balsms is the Greek word for bee (Lust, 2014).

Lemon balm is a perennial plant that is common in the Mediterranean area and the Near East but is also naturalized in some places in Central Europe (Macura et al., 2011; Gurčik et al., 2012; Doğan et al., 2013). Mostly it is cultivated as a culinary herb, but it also grows in fields and gardens in Crimea, Ivano-Frankivsk, Lvov regions and Carpathian Ukraine. Essential oil obtained from *Melisa officinalis* L. has shown antioxidant (Helmy, 2006; Ondrejovič et al., 2012; Safaeian et al., 2016; Couladis & Koutsaviti, 2017), antibacterial (Rostami et al., 2012; Zazharskyi et al., 2019) and antitumour properties (De Sousa

et al., 2004; Queiroz et al., 2014). Lemon balm is used in various branches of industry such as medicine, cosmetics and food.

The young shoots have a pleasant lemon aroma before budding. They are rich in vitamins, proteins, phytochemicals, microelements and other biologically active substances needed for the organism, especially after winter exhaustion. Lemon balm is used as a spicy herb in dry and fresh conditions. Fresh leaves can be put into any summer salads or vegetable soups. Dry leaves are used in the same meals in winter. Mixed with other spicy-aromatizing herbs, balm is very useful for preparing phyto-tea and stewed fruits (Babulka, 2005). Also, it could be added into homemade beer and kvass and also flavoured vinegar. Balm goes well with other herb spices in pickles, tinned foods, pickles of cucumber, tomatoes, food vegetable marrow, pepper, etc. In cookery, it is possible to sprinkle meat, or fish with powder from balm leaves as well as game some minutes before readiness. Lemon balm is a wonderful medical plant. It could be useful in treating all kinds of neuroses, stomach pain, problems in bowels that do not disappear under strict observance of keeping of diet, migraine and different headaches (Yarnell et al., 2013).

Tea from balm removes shortness of breath, attack of tachycardia, pain in heart, at the same time slows down breathing and rhythm of heart systoles, reducing blood pressure. Oil and tincture are used for applying to affected areas and bathes at rheumatism pain. Balm is very useful in bee-keeping because it is a good honey-bearing plant. Bees readily inhabit hives on the inside of which balm syrup was rubbed and that is why balm is named bee-grass.

The objective of this paper was characterization of the chemical composition and antimicrobial activity of essential oils from *M. officinalis*, variety Citronella, in the conditions of cultivation in the lowland region of the Ukrainian Carpathians.

Material and methods

Plant material, balm dry leaves and leafy tops of the shoots, were taken from plants grown in a small-scale cultivation ground in the Zakarpatian Institute of Agroindustrial Production in Velikaja Bakta, Carpathian-Ukraine. The Citronella variety is used for production of medicinal herbs. The variety has straight-standing, branch, slightly fluffy stem 0.7–0.8 m long. From the 4–5th years of vegetation the number of stems is 90 to 100. Leaves are opposite to each other, long-oval form, serrated, very aromatic. The lower part of leaf is lighter in colour than the upper one, which is fluffed with short hair. The length of leaf is 50 or 70 mm, the width ranges 30 to 50 mm. Flowers have two white or yellowish small lips, collected in clusters with 5–10 flowers. Seeds are dark-blue and white, dotted, shining smooth nuts. The weight of 1000 seeds was 0.7–0.8 g. Restoration of vegetation begins in late March or in early April and prolongs to 120 or 130 days in the first year and increases to 160 days in the following years. The number of days from restoration of vegetation to flowering fluctuates from 75 to 115 days. Flowering of the balm takes place from June to August and provides a long period for bees to collect honey. Seeds ripen from September to October. Balm is a drought- and frost-resistant, light-demanding herb. It does not depend on the soil type, but it grows better on loamy soils, rich with humus. It can grow in shade but in this case the yield is reduced. One plant can grow in the same place up to 5 years. In regard to varying soil-climatic conditions, the Western Ukraine Lowland provides heterogeneous conditions for variety of plant species, which are characterized by biodiversity and abundant gene pool material. This plain is also one of the most productive agricultural regions of Ukraine.

Balm powdered drug in amount of 1 g of the plant tissues was homogenized, 80 mL of ethanol (50%) R was added, boiled in a water-bath under reflux condenser for 30 min. Then the substance was left to cool and filtered. The filter was rinsed with 10 mL of ethanol (50% v/v) R. The filtrate were put into a volumetric flask and diluted to 100.0 mL using ethanol (50%) R (Murarikova et al., 2015).

Essential oil from balm parts (herb and leaves) was isolated by distillation with boiled water. Hydro-distillation lasted for 4 hours, sample weights were 10 g of dry drug matter. The modified Cooking & Middleton distillation apparatus was used. Identification and evaluation of the balm's essential oil were carried out using the selected analytical methods.

The analysis of the balm's essential oil was carried out using a Varian 3090 gas chromatograph connected to MS Saturn 2100T integrator. The following operating conditions were used: capillary column: RX-5MS, 30 m x 0.250 mm i.d., film thickness: 0.25 μ m, carrier gas: He₂, adjusted to a flux of 1.5 mL/min, injection and FID-detector temperatures: 220 °C respectively 250 °C, a capacity of sample injection: 2 μ L, MS-detector with automatic injector type 1177.

Components were identified by their GC retention times, and the obtained values were comparable to those mentioned in the literature (Ieri et al., 2017). Oil component standards for comparison were supplied by Extrasynthese, Merck, Fulka and Roth.

The final balm extract was analyzed directly on a Dionex UltiMate 3000 system. Stationary phase was Phenomenex Kinetex 100 A (C18, 5 μ m, 250 mm x 4, 6 mm). Mobile phase C was water: H₃PO₄–99.5: 0.5 (v/v), mobile phase D: methanol: H₂SO₄ 50:50 (v/v), with flow equaling 1.0 mL/min, mobile phase gradient: 0 min. – 100% C, 5 min. – 100% C, 25 min. – 100% D, 35 min. – 100% D, 40 min. – 100% C and temperature of thermostat was 30 °C. Detection wave length λ =

340 nm. Content of rosmarinic acid was determined by using the standard (\geq 98%) from Merck.

The sensitivity of microorganisms to plant extracts was determined by the disk diffusion test (Balouiri et al., 2016). The bacterium inocula 100 μ L in the physiological solution were adjusted to the equivalent of 0.5 McFarland standard, and evenly spread on the surface of Muller-Hinton agar (incubated at 37 \pm 2 °C for 24 hours); yeasts – on SDA agar (incubated at 35 \pm 2 °C for 48 hours). Sterile filter paper disks (6 mm in diameter) impregnated with 10 μ L of essential oil were placed on the dish plate previously inoculated with a microbial suspension. The diameters of the inhibition zones were measured in millimeters including the diameter of the disk. Each antimicrobial assay was performed at least three times. As test culture, we used the following microorganisms from the ATCC (American Type Culture Collection, USA) collection: *Candida albicans* ATCC 885-653, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, *Streptococcus pyogenes* ATCC 19615. Also we used clinical strains of bacteria and yeast, which were isolated from the oral cavity of patients with periodontal disease. As negative control were used DMSO.

Data obtained were expressed as mean \pm standard deviation (SD) of three measurements. Tukey's test was applied for comparisons of the values; differences were considered significant at P < 0.05.

Results

Crop management practice of balm cultivation in the Ukrainian Carpathians. Balm seedlings grow in greenhouses or hotbeds. Soil mixture is prepared from turf earth, peat and humus in proportion 1:1:1. If there is no peat, the mixture is prepared from turf and humus in proportion 50:30. Sand in amount of 10–30 kg/m² or chopped straw, or 2–10 kg of ash can be added per one square m. For improving the structure and aeration of the ground it is reasonable to add zeolite, but no more than the one fourth part. In this case, the germinating power of the seeds increases, the developing of root system and the upper elevated part of herbs improves. To improve the nutritious characteristic of the soil mixture, mineral fertilizers are added: superphosphate 50 g, ammonium saltpeter 20 g and potassium salt 18 g/m². Such fertilizers can be used as nitro-amino-phosphate 30–50 g/m². It is good to crush down the soil substrate into small lumps and make furrows every 50 mm. It is best to sow seeds processed with solutions of microelements such as cupric sulphate, potassium permanganate or boron acid in concentration of 1% or 2% during 6–8 hours. The norm of seeds sowing is 1.5–2.0 g/m². The seed germination power is increased after processing with microelements, the seeding develops better, the herb length and root system are increased and the number of leaves and circumference of the root collar are increased as well. The liquid complex humine fertilizer on the base of Zakarpatian lignite, 20–30 of solution per 10 L of water is used for watering seedlings together without root fertilization, with this procedure taking place 2–3 times during the growth of seedlings. Seeds are sowed at a depth of no more than one cm, and covered with soil mixture or sand. Under favourable conditions shoots appear in 10–15 days. When the first true leaves appear, thinning should be performed so the distance between the plants is 40–50 mm. On the 30–40th days of sowing they are planted in wide rows with living space of 0.7–0.3 m between the rows. Dense planting reduces quality of the raw material and seed productivity are reduced. The ground is prepared depending on the previously cultivated plants. If the precursors were cereal crops, then right after harvesting the stubbles at 10–12 cm depth should be removed and after the weeds are removed the field should be tilled. A total of 80–100 kg of nitrogen, 100–120 kg of phosphorus and 60–80 kg of potassium is added under the crop or during disking. Before sowing, the soil should be loosened to small particles.

Into the open soil, the seeds can be sown in spring and autumn. In the conditions of the lowland zone of Transcarpathian region sowing in autumn is best carried out in September–October. For sowing, mainly fresh harvested seeds are used or seeds stored for no more than two years, and after the analysis of sprouting has been made. Before sowing the seeds are processed by stimulators of growth or microelements. Processing with humin fertilizers should be made in proportion of 30 g per

one liter of water, keeping the seeds in it over 6–8 hours. After this the seeds are dried till friable condition. For sowing large plots, sowing machines of CKON-4 type or CO-4 types are used. Sowing of a small plot is performed manually, making distances between the rows 0.7 m; the norm of sowing is 3–4 kg seeds/ha, the depth of sowing was down to 10 mm.

During the first year of vegetation, balm grows slowly, therefore over this period the plant requires particular care and watering (if needed), but it is important not to allow it to be overgrown with weeds. During the vegetation, 2–3 interrow weedings should be performed. In the second and the following years, it is reasonable to make extra fertilization with 60–90 kg of active substance of NPK fertilizer. For our conditions these norms are optimal and higher norms do not cover the expense. Complex fertilizer should be applied during the two periods – the first one in spring during the restoration of vegetation and the second after the first harvesting. Herbs of balm are not damaged by pests or diseases, but they could fall in waterlogged places. While growing lemon balm increasingly occupies the area. The plant density was 752 ± 83 individuals/100 m² with total of 2.320 ± 355 seedlings/100 m² in 2014.

The raw material for sale is above-ground biomass of balm, harvested before the appearance of the flower buds. At this term, it is reasonable to pay special attention because composition changes into worse side. The first cutting is made at the beginning of June, the second one – in late July or in early August. If the climatic and weather conditions are favourable, a third harvest is possible. The yield of green mass from the first harvest ranges from 500–800 kg/ha and 1.500–1.900 kg/ha from the second. In the yield structure, stems compose 35–45% and leaves 55–65%. Raw material is dried in dark and well ventilated buildings (Table 1). In our experimental cultivation, the total dry biomass yield was 3.346 ± 252 kg/ha in the first year and 3.388 ± 171 kg/ha in the second year. The seed productivity was 300–330 kg/ha, the seed weight per 1000 was 0.7–0.8 g.

Light yellow or colourless pure essential oils were obtained from 10 g of dry herb sample. Yield ranged 0.05–0.12% (v/w) on a dry weight basis. On the other hand, fresh leaves contained about 0.10% volatile oil (Small, 1997). The results during the first and second cutting of balm herbs showed that the essential oil content varied greatly withing the range 0.12–0.25% (0.048–0.100 g/plant) (Khalid & Ahmed, 2011). The biochemical composition of the essential oil had improved by the second harvest; these results coincide with results of studies on other essential oil bearing plants (Hendawy & Khalid, 2005).

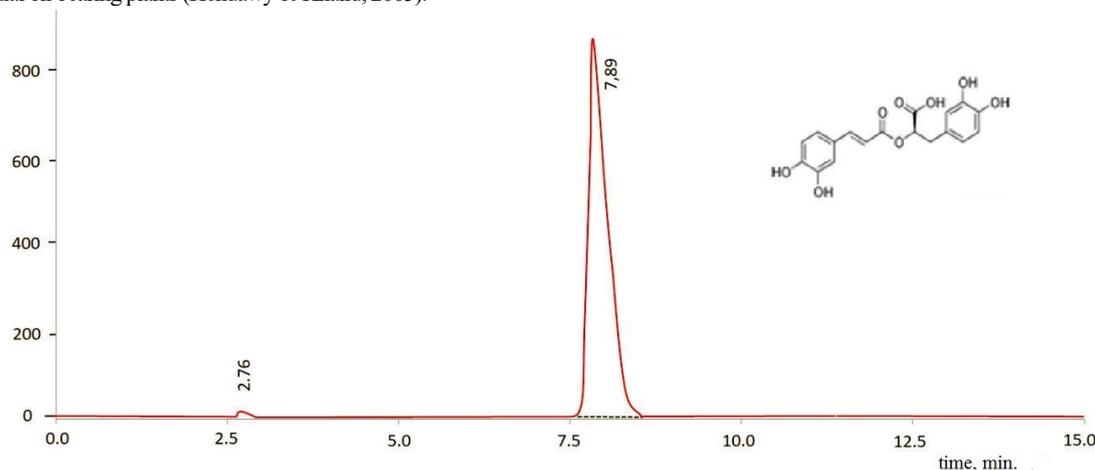


Fig. 1. HPLC DAD analyses of rosmarinic acid of lemon balm leaves

Antimicrobial activity of the oil was tested against bacterial and fungi strains responsible for nosocomial infections: *S. aureus*, *E. coli*, *E. faecalis*, *S. pyogenes*, *C. albicans*, *C. glabrata*. Antibacterial effect of the oil showed low activity against all bacterial strains tested but high antimutic effect (Table 3).

Discussion

Balm essential oil can contains up to 40–44% of α -citrale (geranial). Therefore, this natural substance is an aroma compound used in perfumery for its citrus aroma. Citralis also used as a flavouring and for

Table 1
Quantitative changes of dry biomass (kg/100 m²) of balm during two years of cultivation

Component of dry biomass	First year	Second year
Underground biomass – roots	10.82 \pm 0.81	12.47 \pm 0.45
Total overground biomass	33.46 \pm 2.52	33.88 \pm 1.71
Overground biomass – stems	16.61 \pm 3.48	19.58 \pm 2.73
Overground biomass – leaves	16.85 \pm 1.53	18.30 \pm 0.79
Ration of underground /over ground biomass	1 : 3.09	1 : 3.04

Composition of essential oil from lemon balm determines its aromatherapy effects. The most relevant component is α -citrale (geranial), content of which depends on the chemotype of the selected plant, which was the variety Citronella (Table 2, Fig. 1).

Table 2
Comparison of the qualitative and quantitative characteristics of lemon balm essential oils from Ukraine ($x \pm SD$, n = 3)

Component, % of essential oil*	rt, min**	Ukraine	Slovakia
		variety Citronella	variety Citra
D-limonene	15.2	1.70 \pm 0.10	0.55 \pm 0.10
citronellal	23.2	9.34 \pm 0.55	16.00 \pm 2.04
methyl citronellate	25.2	1.30 \pm 0.15	0.80 \pm 0.02
β -caryophyllene	26.7	0.62 \pm 0.03	9.50 \pm 2.05
β -citrale (neral)	28.4	29.90 \pm 2.00	16.00 \pm 1.02
germacrene D	29.4	0.61 \pm 0.02	0.21 \pm 0.01
α -citrale (geranial)	29.6	44.34 \pm 3.55	18.00 \pm 3.03
geranyl acetate	29.8	2.98 \pm 0.50	1.41 \pm 0.12
caryophyllene oxide	35.7	2.70 \pm 0.32	1.85 \pm 0.41

Note: * – data are expressed as area % on the total area of the identified peaks; ** – retention times.

Rosmarinic acid is a natural polyphenolic compound and was first discovered in 1958 by the Italian scientists M. L. Scarpatti and G. Oriente (Friedman, 2015). The qualitative and quantitative compositions of the main aromatic and polyphenolic constituents were examined and compared in the leaves of lemon of two years cultivation. The results demonstrated total hydroxycinnamic compounds equaling $6.2 \pm 0.5\%$, contents of rosmarinic acid – $1.4 \pm 0.1\%$ (in 2010) and $1.7 \pm 0.1\%$ (in 2014, Fig. 1) and total flavonoid compounds – 0.5% .

increasing the odour of the essential oil. It also has strong antimicrobial properties and pheromone effects on insects. Citralis is used in the synthesis of vitamin A, ionone, and methyl ionone, to mask the smell of smoke (Burdock, 2009). Among the next constituents, the active are β -citrale / neral ($29.9 \pm 2.0\%$), citronellal ($9.3 \pm 0.5\%$), geranyl acetate ($2.9 \pm 0.5\%$), caryophyllene oxide ($2.7 \pm 0.3\%$), D-limonene ($1.7 \pm 0.1\%$), methyl citronellate ($1.3 \pm 0.1\%$) and less than 1% content is β -caryophyllene, germacrene D etc. The composition of essential oils from several varieties of peppermint *Melissa* is different, and the same tendency can be observed for Citra variety cultivated in parallel field in Eastern Slovakia (Table 1).

The most famous cultivars such as Citra and Citronella are cultivated in Presov (Eastern Slovakia). Their lemon balm oil composition can be characterized by the following compound: α -citral (26.5%), β -citral (17.5%), citronellal (16.5%) and β -caryophyllene (11.7%) respectively β -caryophyllene (23.3%), caryophyllene oxide (18.4%), α -citral (10.6%) and β -citral (7.2%) (Fejér & Gruřová, 2017). GC/MS analysis of herbage essential oil from lemon balm grown in Turkey revealed the following main components: citronellal (36.6% to 43.7%), citral (10.1% to 17.4%), thymol (0.4% to 11.9%) and β -caryophyllene (5.9% to 7.2%) (Cosge et al., 2009).

Table 3

Antimicrobial activities of the balm essential oils against typical and clinical strains, zones of inhibition (mm, $\bar{x} \pm SD$, $n = 3$)

Test-culture	Balm essential oil
<i>S. aureus</i> ATCC 25923	9.33 \pm 0.58 ^d
<i>S. aureus</i> (clinic)	9.17 \pm 0.76 ^d
<i>E. coli</i> ATCC 25922	8.50 \pm 0.50 ^{cd}
<i>E. coli</i> (clinic)	0.00 \pm 0.00
<i>E. faecalis</i> ATCC 29212	10.33 \pm 0.58 ^c
<i>S. pyogenes</i> ATCC 19615	9.50 \pm 0.50 ^d
<i>S. pyogenes</i> (clinic)	9.00 \pm 0.35 ^d
<i>C. albicans</i> ATCC 885-653	14.33 \pm 0.58 ^b
<i>C. albicans</i> (clinic)	14.00 \pm 0.33 ^b
<i>C. glabrata</i> (clinic)	19.33 \pm 0.58 ^a

Note: data in the column followed by different letters are significantly different at $P < 0.05$ according to Tukey's HSD test.

Nurzyńska-Wierdak et al. (2014) determined presence of 106 compounds, representing 100% of the oil constituents, using GC-MS and GC-FID. The predominant components were geraniol (45.2% and 45.1%) and neral (32.8% and 33.8%). However, the age of lemon balm plants affected the concentration of other constituents and the proportions of the following compounds were the subject to especially high fluctuations: citronellal (8.7% and 0.4%), geraniol (trace amounts and 0.6%), and geranyl acetate (0.5% and 3.0%), as well as isogeraniol, E-caryophyllene, caryophyllene oxide, germacrene D and carvacrol among others.

The anti-inflammatory effect of rosmarinic acid is well documented. The 2011 study is one of many examples where the inflammation was reduced (Gamaro et al., 2011). The use of this caffeic acid ester was also studied in the 2014 study – on two mice used in the test the supplementation with rosmarinic acid demonstrated anti-inflammatory effect against acute and chronic inflammation resulting from injury. The study suggests that the natural substance has potential for the treatment of pain and inflammation (Boonyarikipunchai et al., 2014). In a study published in 2015, rosmarinic acid has shown effectiveness in reducing the inflammation resulting from injury by modulation of the NF- κ B inflammatory response in rats (Rocha et al., 2015).

In respect to the qualitative–quantitative analyses of lemon balm essential oils originating from Egypt, Serbia and Poland, which were made in the past years, the same sufficient parameters were confirmed (Patora et al., 2003; Mimica-Dukić et al., 2004; Meftahzade et al., 2010). Essential oils in the context of the constantly growing trend towards the formation of antibiotic resistance are promising agents of action against bacteria and microscopic fungi that belong to the transient microbiota of the human organism (Salamon et al., 2017). The reasons for their use are their antimicrobial activity, high antioxidant, aromatic, anti-inflammatory properties. At the same time essential oils cause no imbalance in microbial conenoses of the human body, they are natural preservatives and have a multi-directional positive effect on a number of organs and the systems of the body (Kryvtsova et al., 2017; Kryvtsova et al., 2018; Salamon et al., 2018).

Conclusion

Thus, the studies have shown that *Melissa officinalis* L. variety Citronella grown in conditions of Western Ukraine is characterized by biochemical composition and antimicrobial activity that corresponds to the world analogues. Low antibacterial and high antimycotic activity of essential oils was established, and this determines the prospects for fur-

ther research and development of cosmetic and hygienic agents for the prevention of mycoses.

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