Water soluble biomolecules from *Nepeta nuda* regulate microbial growth: A case study of apple juice preservation

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The following study was designed to explore antimicrobial properties of the by-product obtained in a hydro-distillation process of essential oil from *Nepeta nuda* L. We strived to develop a novel drink with antimicrobial self-preserving properties based on two components, *N. nuda* decoct and apple juice. By using 96-well plate microdilution assay it was shown that the *N. nuda* decoct has antimicrobial potential towards 8 bacterial and 6 fungal species, with the range of minimal inhibitory concentrations 10-300 mg/mL. By using actual food system, such as apple juice, in combination with and without short thermal treatment, we have shown that the decoct of *N. nuda* can inhibit the growth of food contaminant fungus *Penicillium aurantiogriseum*. It was determined that 3 volumes of decoct (500 mg/mL) and 22 volumes of apple juice should be mixed in order to obtain self-preserving drink resistant to *P. aurantiogriseum* contamination. Likewise, when thermal treatment (80 °C for 10 s) is included, self-preserving mixture of decoct and apple juice should be made in volume ratios 3:47, respectively. The designed product maintained the pleasant taste as determined by panelists during the sensorial evaluation. Chemical investigations (UHPLC–Orbitrap MS analysis) of *N. nuda* decoct showed that the most abundant compound was 1,5,9-epideoxyloganic acid (0.410 mg/g of dried decoct). Since *N. nuda* is traditionally used as a tea, we presented the novel formulation of the drink with antimicrobial properties based on the its decoct and apple juice.

Key words: *Nepeta nuda*; drink; antifungal; self-preserving; chemical composition

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1. INTRODUCTION

Apple juice is one of the most widely consumed juices due to its health beneficial and nutraceutical properties accompanied by a pleasant taste. Juice quality and safe consumption rely on methods like refrigeration and addition of chemical preservatives to keep the product safe. Unfortunately, disease outbreaks, related to consumption of food contaminated with microorganisms, increased (Gayán et al., 2013). Since fruit juices of high quality and safety are current demand worldwide it is of prime importance that edibles are free of spoilage microbes (Keyser et al., 2008). Pasteurization, sterilization and addition of synthetic antimicrobials can solve this issue but also can change products' characteristics like color, aroma, and vitamin content (Heinz et al., 2003). Heat treatment and addition of antimicrobial preservatives are frequently used tools to dispose of microorganisms and extend shelf life of foods and beverages (Gayán et al., 2013). However, application of artificial preservatives is associated with numerous undesirable effects (Fiolet et al., 2018). Therefore, there is a notable interest in using natural matrices as juice preservatives that would keep the product safe for use and extend its shelf-life. Majority of food spoilage is caused by yeasts, moulds species and some acid-tolerant bacteria. Soft drinks can be friendly environment for microorganisms which can grow in acidic pH with low concentration of oxygen (Wareing and Davenport, 2004). The most frequently found yeasts and moulds species responsible for the juice spoilage are *Pichia sp.*, *Candida sp.*, *Saccharomyces sp.*, and *Rhodotorula sp.* for yeasts and *Penicillium sp.*, *Cladosporium sp.*, *Aspergillus niger*, *A. fumigatus*, *Botrytis*

sp., and *Aureobasidium pullulans* for moulds (Aneja et al., 2014). Considering that in our previous work (Reis et al., 2012) *Penicillium aurantiogriseum* was isolated from contaminated food, it was chosen as representative strain of spoilage fungi to test antimicrobial self-preserving properties of newly designed drink based on *Nepeta nuda* L. decoct and apple juice.

The genus *Nepeta* L. includes approximately 300 species and represents one of the largest genera in the Lamiaceae family, subfamily Nepetoideae. *Nepeta* species are mostly perennial, rarely annual plants (Asgarpanah et al., 2014), and are native to Europe, Asia, North Africa and North America with the greatest richness in Southwest Asia (mainly Iran) and Western Himalayas (Nargis Jamila, 2011; Süntar et al., 2018). Some species from this genus are used as food flavouring agents, including *N. ispahanica*, *N. binaloudensis*, *N. bracteata*, *N. pogonosperma*, *N. pungens* and *N. crispa* (Nargis Jamila, 2011; Süntar et al., 2018). There are different classes of bioactive compounds in *Nepeta* species, predominated by terpenes and phenolic acids (Salehi et al., 2018; Süntar et al., 2018). The majority of bioactive properties are ascribed to iridoid monoterpenoids nepetalactones, unique group of compounds for this group of plants (Süntar et al., 2018). Although abundant in *Nepeta* species, iridoid glucosides are largely neglected and their bioactivity has been scarcely investigated. Only recently, it has been demonstrated that the dominant iridoid glucoside in *Nepeta* species, 1,5,9-epideoxyloganic acid, is as equally potent antimicrobial as nepetalactones (Aničić et al., 2021). Being present as a glucoside this compound is less volatile than nepetalctones, and is thus more stable in food formulations.

The aim of the present study was to utilize *Nepeta nuda* decoct, by-product obtained during the distillation process of *N. nuda* essential oil, which is a rich source of 1,5,9-epideoxyloganic acid, to preserve apple juice. The idea was to extend shelf life and maintain quality of the juice without using chemical preservatives, while keeping organoleptic characteristics. Therefore, the aim of this work was to phytochemically characterize *N. nuda* decoct and investigate its *in vitro* antibacterial and antifungal activities. Additionally, the objective was to analyze the self-preserving potential of *N. nuda* decoct in blended apple juice on the growth of *Penicillium verrucosum* var. *cyclopium* in combination with thermal treatment. Literature survey revealed that that *Nepeta nuda* decoct is traditionally used internally against cystitis and prostate gland inflammation and externally for wound healing (Kozuharova et al., 2014) and also as a herbal tea for treating hysteria, melancholy and uterine cramps (Aćimović et al., 2020).

2. MATERIALS AND METHODS

2.1. Plant material and by-product of *N. nuda* essential oil distillation

The samples of wild growing *Nepeta nuda* L. were collected from mountain Tara, Serbia, in summer 2013, and authenticated by Dr. Milan Veljić (Faculty of Biology, University of Belgrade). The samples were lyophilized (LH Leybold, Lyovac GT2, Frenkendorf), reduced to a fine dried powder and stored in a desiccator, protected from light, until further analysis.

During essential oil hydro-distillation (for 3h) according to Gormez et al. (2013) in a Clevenger type apparatus a by-product from *N. nuda* was obtained. Boiled water containing *N. nuda* sample from hydro-distillation process was filtered (Whatman No 4) after cooling, and further frozen at -20 °C. Sample was subsequently lyophilized (LH Leybold, Lyovac GT2, Frenkendorf) until dryness. Dried decoct obtained as described above was used for all further investigations.

2.2. Microorganism

The antibacterial activity of *N. nuda* decoct was tested against eight bacteria species: Gram-positive *Bacillus cereus* (clinical isolate), *Listeria monocytogenes* (NCTC 7973), *Micrococcus flavus* (ATCC 10240) and *Staphylococcus aureus* (ATCC 6538) bacteria as well as Gram-negative *Enterobacter cloacae* (clinical isolate), *Escherichia coli* (ATCC 35218), *Pseudomonas aeruginosa* (ATCC 27853) and *Salmonella typhimurium* (ATCC 13311).
The antifungal activity of *N. nuda* decoct was tested against eight fungi: *Aspergillus fumigatus* (ATCC 1022), *A. niger* (ATCC 6275), *A. versicolor* (ATCC 11730), *A. ochraceus* (ATCC 12066), *P. funiculosum* (ATCC 8725), *P. ochrochloron* (ATCC 9112), *P. aurantiogriseum* (food isolate), and *Trichoderma viride* (IAM 5061).
All of the microorganisms were deposited at the Mycological Laboratory, Institute for Biological Research "Siniša Stanković", University of Belgrade, Serbia.

2.3. Antibacterial activity

The antibacterial assay was done by microdilution method (CLSI, 2015; Tsukatani et al., 2012) utilizing 96-well microtiter plates to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The bacterial suspensions were adjusted with sterile saline solution until the concentration of 1.0×10^8 CFU/mL. *N. nuda* decoct was dissolved in sterile distilled water and added to Tryptic Soya Broth medium and after inoculated with bacteria in final concentration 1×10^6 CFU/well (100 μL). The microplates were incubated for 24 h, at 37 °C. The lowest concentrations without visible growth of bacteria under the optical microscope were defined as the minimum inhibitory concentrations (MICs). Furthermore, the MICs of the samples were detected following the addition of 40 μL of iodonitrotetrazolium chloride (INT) (0.2 mg/mL) and incubation at 37 °C for 30 min. The lowest concentration that produced a significant inhibition (around 50 %) of the growth of the bacteria in comparison with the positive control was identified as the MIC. MICs, obtained from the susceptibility testing of various bacteria to tested extracts were determined also by a colorimetric microbial viability assay based on the reduction of the INT color and compared with a positive control for each bacterial strain. MBC was determined by serial sub-cultivation of 2 μL into microplates containing 100 μL of TSB. The lowest concentration that showed no growth after this sub-culturing was read as the MBC. The results were expressed in mg/mL. Sodium benzoate (E211) was utilized as positive control (1 mg/mL in sterile saline solution). Sterile distilled water was used as negative control.

2.4. Antifungal activity

A modified microdilution technique was utilized to investigate the antifungal activity (Daouk et al., 1995; Espinel-Ingroff, 2001). The fungal spores were washed with sterile saline solution at 0.85 % containing polysorbate-80 (0.1 %). The spore suspension was adjusted with sterile saline solution to a concentration of 1.0×10^5 in a final volume of 100 μL per well. The inoculums were stored at 4 °C for posterior utilization. The inoculum dilutions were cultivated in malt extract agar to verify the absence of contamination and validate each inoculum.
MIC was determined by serial dilution technique using 96-well microtiter plates. Dissolved decoct of *N. nuda* was added to a malt extract broth with fungal inoculum. The microplates were incubated for 72 h at 28 °C. The lowest concentrations without visible microbial biomass growth under optical microscope were defined as the concentrations that completely inhibited fungal growth.

Minimum fungicidal concentration (MFC) was determined by a 2 μL serial sub cultivation of the tested compound dissolved in a cultivation medium, and inoculated during 72 h in microtiter plates containing 100 μL of broth per well and with incubation for 72 h at 28 °C. The lowest concentration without visible biomass concentration was defined as MFC indicating the death of 99.5 % of the original. The commercial fungicide bifonazole (Srbolek, Belgrade, Serbia) was used as positive control (1–3500 μg/mL).

2.5. In situ self-preservation of formulated drink

2.5.1. Apple juice

Apples (Granny Smith) were purchased on the local market. Apple was cleansed in alcohol after which skin was peeled off and seeds were discarded. Juice was prepared in a sterile blender (10 g of apples and 100 mL of sterile water), filtered through filter paper and centrifuged. Supernatant was used as apple juice. pH of the juice was measured and was 3.5. The apple juice obtained in such manner was cultivated on laboratory broths to check the absence of contamination with bacteria or fungi.

2.5.2. Formulation of effective antifungal concentration of N. nuda decoct in apple juice combined with thermal treatment

N. nuda decoct was dissolved in sterile distilled water (500 mg/mL) and combined with apple juice in different ratios to explore the most effective concentration. Firstly, MIC and MFC concentrations of *N. nuda* decoct (in prepared apple juice used as medium base) were determined as described previously in the section 2.4. Furthermore, apple juice was used solely to investigate fungal growth with *P. aurantiogriseum* (1 $\times$ 10^5 CFU/mL) used as contaminant species. It was determined that the fungus grew and sporulated in apple juice. The volume ratios of *N. nuda* decoct (500 mg/mL) and apple juices used in the experiment were as follows 48:52, 24:76, 12:88, 6:94 and 3:97. The experimental flasks were kept at room temperatures for 5 days. After determination of MIC and MFC in this self-preserving system, it was further explored if heat pasteurization (at 80 °C in water bath for 10 s and 30 s) of the inoculated mixtures may reduce MIC and MFC concentrations for *P. aurantiogriseum*. MIC and MFC concentrations of *N. nuda* decoct were determined in all of the experimental conditions, as well as volume ratios of *N. nuda* decoct (500 mg/mL) and apple juice necessary for self-preservation under different conditions (with or without thermal treatment).

2.5.3. Sensory evaluation

Sensory evaluation of apple juice mixed with NND (as described in the section 2.5. for the final formulation) was assessed by a group of 10 untrained panellists. Panellists were selected among students and staff of the Institute for Biological Research "Sinisa Stankovic" – National Institute of Republic of Serbia, University of Belgrade. The panellists were asked to evaluate overall acceptance of the drink sample on a scale from 5 to 1; indicating decreasing taste. Overall acceptance was evaluated using a 5-point scale, according to a previous report, where 1 = extremely dislike, 2 = dislike, 3 = neither like nor dislike, 4 = like; 5 = extremely like. Results were expressed as average grades given by 10 panellists.

2.6. Chemical characterization of *N. nuda* decoct

2.6.1. UHPLC–Orbitrap MS analysis

Dried *N. nuda* decoct was diluted in 96 % methanol (w:v=1:10) and extracted in an ultrasonic bath for 10 min. Following 10 min centrifugation at 10,000 g supernatant was filtered through 0.2 μm cellulose filters (Agilent Technologies, Santa Clara, CA) and stored at 4 °C until use.

Chemical characterization of *N. nuda* decoct was performed using ultra-high performance liquid chromatographic (UHPLC) system consisting of a quaternary Accela 600 pump, Accela Autosampler (ThermoFisher Scientific, Bremen, Germany). Syncronis C18 (100 $\times$ 2.1 mm, 1.7 μm particle size) at 40 °C was used as analytical column for separation. The mobile phase consisted of (A) water with 0.1 % formic acid in ultrapure water and (B) acetonitrile with 0.1 % formic acid. The linear gradient program was used: 0.0-1.0 min 5 % B, 1.0-16.0 min from 5 % to 95 % (B), 16.0-16.1 min from 95 % to 5 % (B), then 5 % (B) for 4 min (Vasić et al., 2019). The injection volume for all samples was 5 μL and the flow rate was 0.3 mL/min.

UHPLC system was coupled to a linear ion trap - OrbiTrap hybrid mass spectrometer (LTQ OrbiTrap MS) equipped with heated electrospray ionization probe (HESI-II; Thermo Fisher Scientific, Bremen, Germany). The mass spectrometer operated in negative ion mode and MS spectra were acquired by full range acquisition covering 100 – 1000 *m/z*. Parameters of the ion source was as in Gašić et al. (2015). Resolution was set at 30,000 for full scan analysis. The data-dependent MS/MS events were always performed on the most intense ions detected in the full scan MS. The ions of interest were isolated in the ion trap with an isolation width of 5 ppm and activated with 35 % collision energy levels (CEL).

Xcalibur software (version 2.1) was used for instrument control, data acquisition and data analysis. The molecule editor program, ChemDraw (version 12.0), was used as a reference library to calculate the exact (monoisotopic) masses of compounds of interest. The tentative identification of compounds for which standards are not available was achieved using previously reported MS fragmentation data found in literature.

2.6.2. UHPLC/-HESI-MS/MS quantitative analysis

Quantitative analysis was performed using Dionex Ultimate 3000 UHPLC system (Thermo Fisher Scientific, Germany) connected to TSQ Quantum Access Max triple-quadrupole mass spectrometer (Thermo Fisher Scientific, Switzerland), operating in a negative ionization mode. Chromatographic separation of nine targeted compounds in *N. nuda* samples was performed at 30 °C on Hypersil gold C18 column (50 $\times$ 2.1 mm) with 1.9 m particle size (Thermo Fisher Scientific, USA). Mobile phases, gradient elution program and the settings of mass spectrometer were previously described in Mišić et al. (2015). Selected reaction monitoring (SRM) mode of the mass spectrometer was utilised for the quantitative analysis, with collision-induced fragmentations performed using argon, and collision energy (cE) set to 30 eV. Xcalibur software (version 2.2) was used for the instrument control, data acquisition, and analysis.

Compounds were quantified based on the calibration curves of commercial standards: caffeic acid, rosmarinic acid and quercetin, all purchased from Sigma Aldrich (Steinheim, Germany). Regression was calculated for the calibration curves, and they all showed good linearity (r=0.999, P<0.001). The total amount of each targeted compound was evaluated by the calculation of peak areas and is expressed as μg per g of dry weight (μg/g dw). Nepetanudoside, tuberonic acid glucoside, 1,5,9-epideoxyloganic acid, and caffeoylglycolic acid were quantified relatively, using the calibration curve of rosmarinic acid.

3. RESULTS AND DISCUSSION

3.1. Antimicrobial activity

The results of antibacterial activity are presented in the Table 1. *Escherichia coli* and *Pseudomonas aeruginosa* were the most resistant species of bacteria to inhibitory potential of *Nepeta*

nuda decoct with MIC of 20 mg/mL and MBC of 40 mg/mL. The other investigated species of bacteria such as *Micrococcus flavus*, *Salmonella typhimurium*, *Enterobacter cloacae*, *Listeria monocytogenes*, *Staphylococcus aureus* and *Bacillus cereus* showed the same reaction to inhibitory and bactericidal effect of the *N. nuda* decoct (MIC 10 mg/mL; MBC 20 mg/mL). From the results given above, it is evident that the antibacterial effect was not related with the difference in cell wall of G+ and G- bacteria, but the effect was dependent on the bacteria used for the investigation. Regarding positive control – E211, the antibacterial effect was stronger when compared with the effect of *N. nuda* decoct. This was expected as well, since the commercial preservative represents one single active compound, while decoct is a mixture of different compounds that might exhibit synergistic or even antagonistic effects.

Table 1. Antimicrobial activity of *Nepeta nuda* decoct in mg/mL.

	NND[a,b]		E211[c]	
Fungal/bacterial strain	MIC	MBC	MIC	MBC
Micrococcus flavus	10	20	2	4
Salmonella typhimurium	10	20	1	2
Escherichia coli	40	80	1	2
Enterobacter cloacae	10	20	2	4
Pseudomonas aeruginosa	40	80	1	2
Listeria monocytogenes	10	20	1	2
Staphylococcus aureus	10	20	4	4
Bacillus cereus	10	20	0.5	0.5
Aspergillus flavus	150	300	1	2
Aspergillus niger	>300	>300	1	2
Penicillium funiculosum	37.5	75	1	2
Penicillium aurantiogriseum	37.5	75	2	4
Aspergillus versicolor	150	300	2	2
Penicillium ochrochloron	300	>300	1	2

[a] NND stands for *Nepeta nuda* decoct
[b] MIC and MBC stand for Minimum Inhibitory and Minimum Bactericidal Concentration, respectively.
[c] E211 - sodium benzoate was utilized as positive control.

Regarding antifungal activity of the investigated decoct, the results are presented in the Table 1. The most sensitive microfungi to decoct antifungal effect were *Penicillium funiculosum* and *Penicillium aurantiogriseum* with MIC of 37.5 mg/mL and MFC of 75 mg/mL. *Aspergillus niger* was the most resistant fungal strain to which antifungal effect was not recorded up to 300 mg/mL. Antifungal effect of the commercial drug bifonazole was prominent when compared to decoct antifungal effect, as expected.

Although the antimicrobial effects were less pronounced than those recorded for commercial preservatives, *N. nuda* showed an interesting antimicrobial potential, which might be exploited to protect apple juice from spoilage. As decoct is the by-product of essential oil distillation, and is usually discarded, the present study offers the new perspective to rationally utilize the plant material, in a cost-effective way. To our best knowledge this is the first report on the antimicrobial activity of *N. nuda* decoct. There is only some information about antimicrobial activity of *N. nuda* essential oil (Gormez et al., 2013) and tincture (Smiljković et al., 2018).

3.2. Self-preserving properties of newly designed drink and sensory evaluation

Penicillium aurantiogriseum is able to contaminate apple juice, and at the same time was one of the two most sensitive fungal species to *Nepeta nuda* decoct (NND). This strain was used as a model system for the formulation of self-preserving drink based on NND and apple juice. Firstly, we have established effective inhibitory and fungicidal concentrations of NND in apple juice as a medium. It was shown that MIC and MFC were higher when compared to the *in vitro* experiment, suggesting that apple juice is more complex medium than simple laboratory broth used for *in vitro* experiment. MIC was determined at 60 mg NND/mL of apple juice and MFC at 120 mg NND/mL of apple juice. Therefore, we were able to formulate volume ratios of NND (500 mg/mL) and apple juice (prepared as described in Material and Methods section) (Table 2).

Table 2. MIC, MFC [mg/mL] of *N. nuda* decoct (NND) and volume ratios of NND and apple juice after 5 days incubation of *P. aurantiogriseum* without or with heat pasteurization (80 °C).

	MIC[a]	Volume ratio	MFC[b]	Volume ratio
NND[c]	60	6:94	120	12:88
NND (10 s)	30	3:97	60	6:94
NND (30 s)	30	3:97	60	6:94

[a] MIC stands for Minimum Inhibitory Concentration.
[b] MFC stand for Minimum Fungicidal Concentration.
[c] NND stands for *Nepeta nuda* decoct

In the light of that fact, 3 volumes of NND (500 mg/mL) and 22 volumes of apple juice should be mixed in order to obtain self-preserving drink resistant to *P. aurantiogriseum* contamination. Furthermore, we investigated how the short thermal treatment would influence the antifungal effect of NND (Table 2). There were no significant differences between thermal treatments for 10 s and 30 s. The short thermal treatment at 80 °C for 10 s was the best choice in order to enhance fungicidal properties of NND and lower its amount in the final mixture. The final formulation of self-preserving mixture of NND and apple juice should be made in volume ratios 3 (NND): 47 (apple juice) and thermally treated at 80 °C for 10 s, in order to have the best effect against *P. aurantiogriseum* contamination. Since *Nepeta nuda* is used as a tea in some traditional medicines (Sharma et al., 2021), together with some other *Nepeta* species, we considered it appropriate for combination with apple juice to make a novel drink with functional properties. Furthermore we have investigated overall acceptability of the newly designed drink formulation as described above for the final formulation. The results for sensorial evaluation indicated that overall acceptability of the drink was high with average grade 4.4 suggesting that the product was likable for the panelists.

3.3. Chemical composition of *N. nuda* decoct

To determine major bioactive principles, LC/MS characterization of *N. nuda* decoct was performed and it resulted in the detection of nine compounds. Among identified compounds, three were confirmed using available standards (caffeic acid, rosmarinic acid, and quercetin), while the others were identified using high resolution mass spectrometry (HRMS) with multi stage mass spectrometry (MSn). The base peak chromatogram of *N. nuda* decoct is presented in Figure 1. The main LC/MS data of identified compounds are summarized in Table 3.

Compounds 1 and 4 with pseudomolecular ion ($[M+HCOOH–H]^-$) at 435 *m/z* were marked as two isomers of nepetanudoside. In the first stage of fragmentation, formic acid (HCOOH, 46 Da) is lost, which, in this case, was an adduct bound to the molecular ion. Thus a fragment

Table 3. Tentative identification of the compounds in *N. nuda* decoct.

Peak[a] No.	t_R[b] min	Compound name	Molecular formula $[M–H]^-$ [m/z]	Calculated mass $[M–H]^-$ [m/z]	Exact mass $[M–H]^-$ [m/z]	Δ ppm[c]	MS^2 Fragments [m/z] (% Base Peak)	MS^3 Fragments [m/z] (% Base Peak)	MS^4 Fragments [m/z] (% Base Peak)
1	5.45	Nepetanudoside isomer + HCOOH	$C_{18}H_{27}O_{12}$-	435.1508	435.15026	1.24	225(5), **227**(100), 228(10), 365(5), 387(5), 389(5)	**101**(100), 127(10), 209(5)	–
2	5.66	Tuberonic acid glucoside	$C_{18}H_{27}O_9$-	387.16606	387.16563	1.10	163(55), **207**(100), 318(15), 321(10), 341(15), 369(15)	**163**(100)	–
3	5.75	Caffeic acid[d]	$C_9H_7O_4$–	179.03498	179.03471	1.51	**135**(100)	135(60), 117(15), **107**(100), 91(55), 79(15)	–
4	5.79	Nepetanudoside	$C_{18}H_{27}O_9$-	435.1508	435.15051	0.66	**227**(100), 228(15), 388(30), 389(65), 390(10)	69(5), **101**(100)	–
5	6.16	1,5,9-Epideoxyloganic acid	$C_{16}H_{23}O_9$-	359.13476	359.13461	0.42	**197**(100), 153(65), 135(20), 109(15)	**153**(100), 135(10), 109(30)	**135**(100)
6	6.40	1,5,9-Epideoxyloganic acid isomer	$C_{16}H_{23}O_9$-	359.13476	359.13449	0.75	**197**(100), 153(20), 135(20)	**153**(100), 135(5), 109(20)	–
7	6.73	Caffeoylglycolic acid	$C_{11}H_9O_6$–	237.04046	237.04044	0.08	209(5), 193(5), 191(20), **161**(100)	**133**(100), 105(10)	–
8	7.46	Rosmarinic acid[d]	$C_{18}H_{15}O_8$–	359.07724	359.07648	2.12	223(10), 197(30), 179(40), **161**(100), 133(10)	**133**(100)	**105**(100)
9	8.74	Quercetin[d]	$C_{15}H_9O_7$-	301.03538	301.03531	0.23	271(50), 255(20), **179**(100), 151(80), 107(5)	**151**(100)	**107**(100), 83(10)

[a]Number of peaks (#) which corresponds to the peak numbers on Figure 1.

[b]t_R – retention time.

[c]Δ ppm – mean mass accuracy.

[d]Compounds confirmed using available standards.

Table 4. UHPLC/(±)HESI-MS^2 quantification of targeted compounds in by-product of *Nepeta nuda* essential oil distillation. Relative intensities of the main diagnostic MS^2 fragments utilized in SRM (Selected Reaction Monitoring)

Peak[a] No	t_R[b], min	Compound name	$[M–H]^-$ [m/z]	MS^2 fragments, [m/z](% Base Peak)	Concentration [μg/g dw]
1	2.45	Nepetanudoside isomer + HCOOH	435	398(5), 227(100)	156.41 ± 8.74
2	2.55	Tuberonic acid glucoside	387	163(50), 207(100)	161.51 ± 9.03
3	2.60	Caffeic acid	179	135(100), 134(85)	226.44 ± 15.15
4	2.61	Nepetanudoside	435	398(50), 227(100)	1265.48 ± 70.74
5	3.39	1,5,9-Epideoxyloganic acid	359	197(20), 153(100)	409.87 ± 6.22
6	4.07	1,5,9-Epideoxyloganic acid isomer	359	197(100), 153(10)	113.76 ± 3.12
7	4.10	Caffeoylglycolic acid	237	161(50), 133(25)	185.88 ± 5.46
8	4.19	Rosmarinic acid	359	161(100), 133(25)	272.08 ± 15.21
9	4.78	Quercetin	301	179(<5), 151(100)	3.54 ± 0.07

[a]Number of peaks (#) which corresponds to the peak numbers on Figure 1.

[b]t_R – retention time.

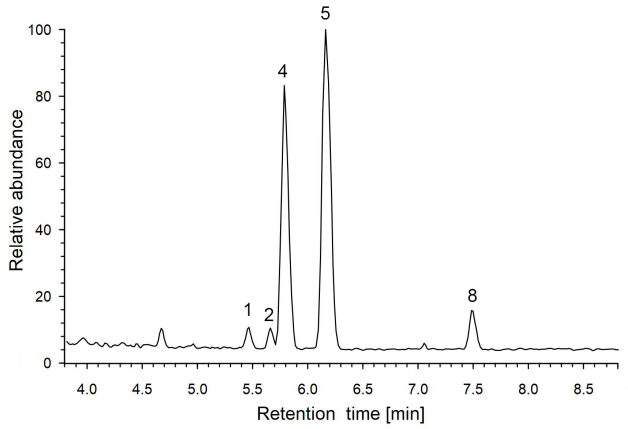

Fig. 1. UPLC/Orbitrap MS base peak chromatogram of *N. nuda* decoct.

is formed at 389 *m/z*, which corresponds to the mass of nepatanudoside. However, the most intense ion (MS^2 base peak) found at 227 *m/z*, was generated by further loss of hexosyl residue (162 Da). This iridoid, nepetanudoside, got its name from the fact that it was isolated for the first time from the aerial part of *Nepeta nuda* ssp. *albiflora* (Takeda et al., 1995). Tuberonic acid glucoside (compound 2) found at 387 *m/z* showed MS^2 base peak at 207 *m/z* (loss of hexose, 180 Da). MS^3 base peak was found at 163 *m/z*, generated by the loss of CO_2 (44 Da). This compound was already identified in the extract of *Calamintha nepeta* (L.) Savi (Pacifico et al., 2015). The same reference explains the MS fragmentation pathway of this compound, which is fully consistent with our conclusions.
Compound 5 eluting at 6.16 min and displaying pseudomolekular ion $[M–H]^-$ at 359 *m/z* with MS^2 base peak at 197 *m/z* (loss of hexosyl group – 162 Da) was identified as 1,5,9-epideoxyloganic acid (Table 4). The MS^3 fragmentation showed base peak at 153 *m/z* obtained by loss of CO_2 group (44 Da), while the MS^3 secondary peak at 109 *m/z* was formed by further of CO_2. The MS^4 spectrum showed fragment ion at 135 *m/z*, formed by elimination of water (18 Da) from MS^3 base peak 153 *m/z*. Compound 6 at 6.40 min showing very similar MS fragmentation data was identified as an isomer of compound 5. Proposed fragmentation pathway of these two compounds is in accordance with the literature data (Aničić et al., 2021; Li et al., 2018). 1,5,9-epideoxyloganic acid is known to be present in the plants belonging to the genus *Nepeta* (Aničić et al., 2021; Takeda et al., 1996), and it was already identified in the *N. nuda* acetone extract (Dienaitė et al., 2018).
Compound 7 eluting at 7.20 min, with molecular ion $[M–H]^-$ at 237 *m/z*, and MS^2 base peak at 161 *m/z*, was tentatively identified as caffeoylglycolic acid. It gave MS^3 base peak at 133 *m/z*, generated by the loss of CO_2 (28 Da). Proposed fragmentation pathway of compound 7 is depicted in Figure 2. This compound was already isolated and identified in *Nepeta cataria* (Snook et al., 1993).

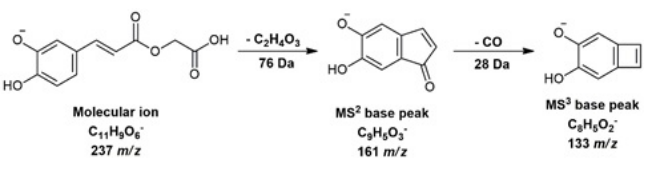

Fig. 2. Proposed fragmentation pathway of compound 7.

UHPLC/(-)HESI-MS^2 quantitative analysis (Table 4) revealed that the major constituent in *N. nuda* decoct was nepetanudoside (compound 4) with concentration of ∼1250 μg/g rosmarinic acid eqivalents per g dw. The concentration of 1,5,9-epideoxyloganic acid (5), was ∼410 μg/g dw. Rosmarinic (8) and caffeic acid (3) were also present in significant amounts, around 272 and 226 μg/g dw, respectively. The other compounds were less abundant. Caffeic acid, rosmarinic acid and quercetin have previously been recorded in *N. nuda* leaves (Aras et al., 2016). Caffeoylglycolic acid is here reported for the first time in *N. nuda*.
Iridoid glucosides and phenolic acids which predominate the bioactive compounds pool of the *N. nuda* decoct, obviously provide the preservative properties to apple juice formulated within the present study. Some of the identified constituents have previously been highlighted as the potent antimicrobials, including 1,5,9-epideoxyloganic acid (Aničić et al., 2021), rosmarinic acid (Aničić et al., 2021; Nadeem et al., 2019), caffeic acid (Magnani et al., 2014), and quercetin (Batiha et al., 2020). Thus, each constituent of decoct alone, and the interaction of the individual decoct components (e.g. antagonistic, synergistic, additive) with the constituents of the apple juice, contribute to the overall self-preserving properties of product. Apple juice is a rich source of natural oxidants itself, and the addition of *N. nuda* decoct enriches the phenolic acids pool and, most importantly, supplies the source of iridoid glucosides which offer a new perspective to the preservation and prolongation of the shelf-life without using synthetic preservatives. Besides antimicrobial protection, decoct constituents might also improve the antioxidant properties to the apple juice, thus giving the additional value to quality and health-benefits of the product.

CONCLUSION

Nowadays customers are more and more seeking for the beverages based on natural products without the artificial additives. In this study we have developed drink based on *Nepeta nuda* decoct and apple juice that is able to self-preserve. This formulation could provide the customers with drink that is resistant to *P. aurantiogriseum* contamination which confirmed our hypothesis.

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