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THE ENHANCING OF ANTIMICROBIAL, ANTIVIRAL, ANTIOXIDANT AND PRO-OXIDANT ACTIVITY IN SELECTED PLANT EXTRACTS USING INNOVATIVE MULTIFACTORIAL EXTRACTION AS A BASE FOR NEW FOOD ADDITIVES

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ABSTRACT

An original engine has been developed for the frugal extraction of plant extractive compounds with an accelerating effect. It operates on the principle of simultaneously combining several physical factors (sonication, temperature, and electrical field) to promote extraction. By applying this engine, plant extract samples were obtained with significantly enhanced dry matter content as well as bioactivity, particularly in antioxidant, antibacterial, enzyme inhibition, and anti-inflammatory activities. This device was utilized in the process of preparing four selected extract samples, which exhibited significant simultaneous antibacterial activity, inhibition activity on the main protease of SARS-CoV-2, antioxidant activity, as well as anti-inflammatory activity. Particularly, these activities were expressed mainly in the following extract samples: from the bark of oak (*Quercus robur*, L.), the flower of oregano (*Origanum vulgare* L.), grains of rapeseed (*Brassica napus subsp. oleifera*,) and grains of oat (*Avena sativa* L.). The activity enhancement factor (coefficient) varied in the interval of (2.8 - 6.2). The extraction products were prepared for the development of new food supplements for pandemic situations, aimed at enhancing the body's defenses. The analytical aspects as well as elicitation perspectives for the continuing research are discussed.

Keywords: Physical promotion, extraction process, bioactivity, food supplements

INTRODUCTION

The necessity of bioactive compounds as prospective drugs or agents with preventive/therapeutic effects for food supplements is evident and widely published (**Boy *et al.*, 2018**). The basic screening of bioactivity of compounds/medicinal plant extracts consists of several fundamental assays: screening for antioxidant activity, antimicrobial activity, anti-inflammatory activity, and basic enzyme inhibition activity, including anti-proteinase activity, as described by **Amigo-Benavent *et al.* (2021)**. A modern and promising method for determining antioxidant/pro-oxidant activity involves conducting simultaneous tests on a single microplate. (**Prieto *et al.*, 2012; Prieto *et al.*, 2015; Maliar *et al.*, 2023**). The commercial database SciFinder, one of the most widely utilized databases, reveals a significant shortage of scientific papers on pro-oxidants, especially papers describing the results of simultaneously tested antioxidant/pro-oxidant activity. The previous COVID-19 pandemic period heightened interest on papers concerning promising agents with antiviral activity, particularly SARS-CoV-2 3CL proteinase inhibitors, as well as those with antibacterial and anti-inflammatory properties (**Chassagne *et al.*, 2011; Kim, 2021; Kpemissi *et al.*, 2023**). The enhancement of bioactivity in medicinal plant extracts can be achieved through physical treatments, complementing reactive extraction methods (**Conde *et al.*, 2009; Saad *et al.*, 2014; Jesus *et al.*, 2020**). The main goal of this study was to conduct experiments aimed at enhancing the extraction yield of selected medicinal plant matter extracts, which exhibited interesting bioactivity. This goal was achieved through the application of various physical treatments such as temperature adjustment, ultrasonication, and exposure to electric fields, with air evaporation as oxidation source elimination.

MATERIAL AND METHODS

Chemicals

2,2-Diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$); 2,2-Diphenyl-1-picrylhydrazin (DPPH); 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ); $FeCl_2.4H_2O$; $FeCl_3.6H_2O$; 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid /TROLOX/; gold$^{(III)}$ chloride; M^{pro}, 3Cl Protease From Coronavirus Sars-Cov-2 and Mueller Hinton Broth (MHB) were purchased from Merck /Sigma/ (USA) and peptadecapeptide H_2N-EEEETSAVLQSGFRC-COOH was prepared by Thermo Fisher Scientific (USA).

Plant material and extract preparation

Two samples of plant material, oak bark and oregano dried plant (flowers and leaves) were purchased from company Calendula, Inc. Nová Ľubovňa, as dried matter in kilogram quantities in year 2023. Similarly, the grains of oat and rape seed were purchased from Research Plant Institute, Piešťany Slovakia, collected in year 2023, in kilogram quantities.
A total of 200 g of dried plant matter was disintegrated into small pieces (under 5 mm particle size). The plant matter was then extracted in the original engine for frugal extraction of plant extractive compounds with 2 dm^{-3} of a 50% (v/v) ethanol solution in the dark at room temperature for 2 hours under various regimens, applying one or alternatively several extraction-promoting factors (temperature, sonication, electric field, air evaporation (oxygen source). Afterwards, the extract was filtered, and samples were stored in tubes at 4°C in the dark. For each extract sample, the value of dry matter per 1 cm^{-3} of extract was determined gravimetrically, final output parameter is dry matter (DM).

Determination of antioxidant activity (AOXA) and prooxidant activity (PROOXA)

The DPPH method was employed to measure antioxidant activity, while the FRAP method was used to measure both antioxidant and pro-oxidant activity, both performed on microplates to ensure equal concentrations of key reagents (DPPH, TPTZ, $FeCl_3$) at 0.4 mmol.dm^{-3}. Detailed methodology description was previously published (**Maliar *et al.*, 2023**).
Briefly, 0.4 mmol.dm^{-3} DPPH$^{\bullet}$ radical solution was prepared in ethanol and FRAP reagent was prepared as separately solution A containing 0.0187 g TPTZ in 10 cm^{-3} of ethanol and solution B containing 0.338 g sodium acetate in 88.3 ml of water and 1.748 cm^{-3} of acetic acid, these solutions were mixed freshly before each

experiment. Additionally, 1.2 mol.dm^{-3} solutions of FeCl$_2$.4H$_2$O and FeCl$_3$.6H$_2$O were prepared freshly prior to the experiments.

The microplate template used for the assays is presented on Figure 1. Firstly, dilution of tested samples (prepared extracts) was realized on microplate testing field by dilution mode to 1/2 concentration of previous column. Secondly, conversion standards solutions were ad into the wells. Consequently, the microplate was initialized by adding150 µl of 0.4 mmol.dm^{-3} DPPH reagent to rows A, B and C and similarly, 100 µl of FRAP reagent was added to rows D, E and F Finally, 100 µl of reaction mixture was removed from the wells from rows A, B, and C.

The microplate was incubated for 10 minutes at room temperature, followed by measurement at 517 nm and 630 nm for DPPH and FRAP, respectively. The optical density (OD) data of the samples were adjusted by subtracting the background data and transformed into percentile values of conversion measurement using 0% and 100% conversion data. Final output parameters DPPH$_{50}$ and FRAP$_{50}$ (expressed in µmol.dm^{-3}) were calculated from the following function plot: percentage of conversion = f (concentration of dry matter weight of the extract sample) was used.

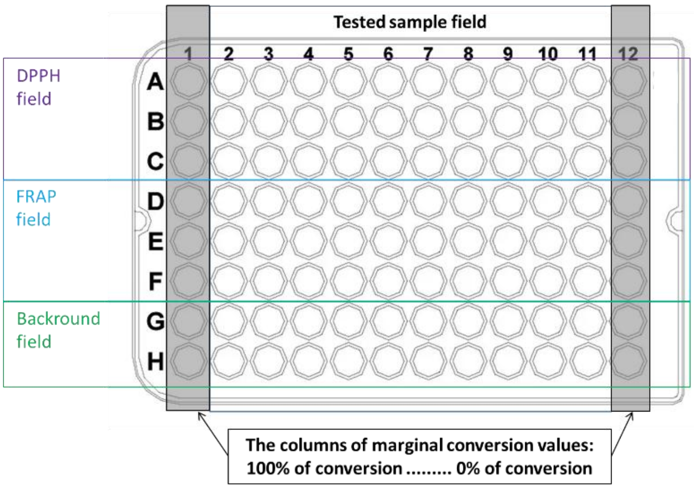

Figure 1 Microplate template. Organization of the microplate template, consisting of a tested sample field (wells A2-H12) and columns dedicated to standard, representing 100% conversion (wells A1-F1) and 0% conversion (wells A12-F12) for the assays

Determination of antibacterial activity /ABA/

Determination of antibacterial activity of tested extract samples on microplates was realized in according with CLSI (Clinical Laboratory Standard Institute) protocol using dilution mode to one half concentration /like methodology of determination of AOXA, PROOXA activity of tested samples. Briefly, dilution was realized by sterile Agua per injection, followed by addition of two-time concentrated volume MHB, both in volume – 100 µl and finally addition of 10 µl inoculum of eight following bacterial strains (each on separate microplate): carbapenemase producing *Klebsiella ssp*., ESBL *Enetrococcus aerogenes*, MDR *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, VRE *Enterococcus faecium* and MRSA *Staphylococcus aureus*. After 24 days of cultivation, proliferation was recorded by turbidimetric measurement. The last non-proliferating well represents the MIC (Minimal Inhibitory Concentration) parameter, expressed as both a titer and in milligrams of extract dry matter per cm^3. Finally, the average MIC value was calculated from the individual values for eight different strains. Final /ABA/ parameter is an average value of MIC$_{AVG}$ parameters for eight different bacterial strains.

Determination of M^{pro} inhibition activity as a parameter of antiviral activity (AVA)

For the determination of inhibition activity on the main protease of SARS-CoV-2 (3CL proteinase) was applied a method, described by **Garland *et al.* (2022)**. This method utilizes the inhibition of aggregation of pentadecapeptide-derived gold nanoparticles, was adapted for microplate. Briefly, 0.1 mmol.dm^{-3} 3CL^{pro} substrate peptide solution in the presence of 20 mmol.dm^{-3} Tris pH 6.5, 50 µmol.dm^{-3} EDTA, 10 µg.cm^{-3} BSA, 100 mmol.dm^{-3} NaCl and 0.05% for 30 min at 37°C unless otherwise stated. 10 µl digested peptide solution was then added to 190 µl citrate-stabilized AuNP solution (unless otherwise stated) under room temperature. After mixing, absorbance at 630 nm was measured on a microplate reader. The output parameter for /AVA/ is IC$_{50}$ value, calculated from the function: OD 630 nm = f (dry matter/cm^3 of extract).

Calculation of dimensionless variables

For each bioactivity, particularly AOXA, PROOXA, ABA and AVA, dimensionless variables in the form of Enhancement factor (EF) were finally calculated as follows: ratio output parameters (DPPH$_{50}$, FRAO$_{50}$, MIC$_{AVG}$ and IC$_{50}$), explained in text hereinbefore without and after applying of physical treatment. This indicates the decrease in the following parameters: DM, DPPH$_{50}$, FRAP$_{50}$, MIC$_{AVG}$, and IC$_{50}$ values after the physical factors promoting the extraction process using the original engine for frugal extraction of plant extractive compounds.

RESULTS AND DISCUSSION

For the realization of the promoted extraction, an original engine was developed for frugal extraction of plant extractive compounds with an accelerating effect. It operates on the principle of simultaneously combining several physical factors (sonication, temperature, and electrical field) to promote extraction. The scheme of this engine is presented in Figure 2.

The other part of the work was dedicated to the realization of the physically promoted extraction and preparation of the extract samples with enhanced bioactivity, as indicated by the EF. The results of the enhanced DM and bioactivity of the extracts from the flower of oregano (*Origanum vulgare* L.), bark of sessile oak (*Quercus petraea* Matt.), grains of rapeseed (*B. napus subsp. oleifera* L.), and grains of oat (*Avena sativa* L.) are presented in Table 1 and Table 2. The increase in extraction yields, expressed as AOXA, using all three physical factor treatments separately for the selected medicinal plant extracts are presented in Table 1. Table 2 presents the increase of extraction yield using all three physical factors simultaneously, along with the bioactivity for the selected medicinal plant extracts, all expressed as EF.

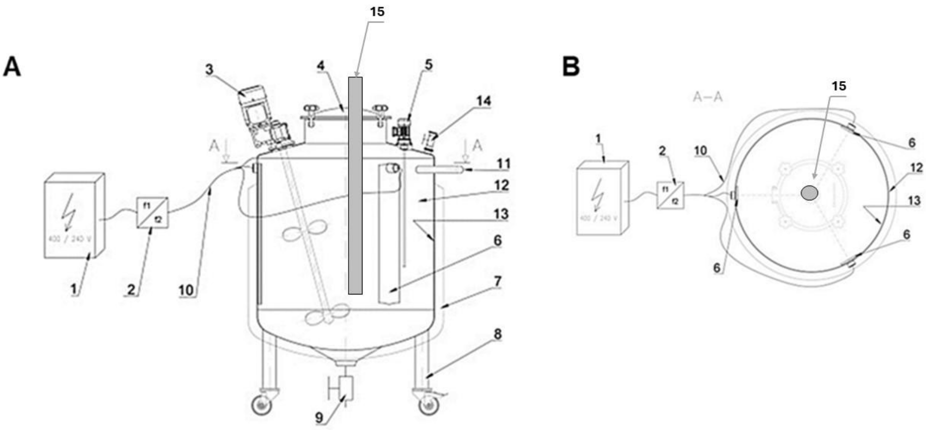

Figure 2 The scheme of constructed engine for extraction, promoted by physical factors treatment, **A** frontal view, **B** floor plan view. Legend: **1** AC+ electricity source, **2** frequency alternat, **3** stirrers with electrical gear case, **4** engine enter neck, **5** super sound source, **6** electrodes, **7** engine isolation, **8** engine legs, mobility concept, **9** bottom product outlet, **10** electricity distribution, **11** grap handle, **12** engine box corpus, **13** dielectric surface performance, **14** vacuum connection, **15** ultrasonic finger.

From the achieved results in Table 1, it is evident that all three physical factors contribute to the antioxidant activity AOXA of the evaluated extract samples. The partial contribution to the enhanced bioactivity is relatively similar or almost equal. However, the AC+ electric field promotion seems to be a more significant factor of enhancement, not only for AOXA, but also for other bioactivity parameters (PROOXA, ABA and AVA, partial results are not presented). Table 2 presents the increase in "extractivity" using all three physical factor treatments simultaneously and the bioactivity for selected (medical) plant extracts, expressed as EF. For all four samples and for all bioactivity parameters, significant increases in EF values were observed, especially for the sessile oak (*Q. petraea* Matt.) bark extract, which could be a very promising component for nutraceuticals suitable for pandemic viral and bacterial infections. Our findings could be supported by published papers, from two different points of view. Firstly, the selected medical plants and prepared extracts are the subject of the current research for its bioactivity, particularly oregano (**Lu *et al.*, 2021**), oak bark (**Dedrie *et al.*, 2015**), rapeseed (**Wang *et al.*, 2021**) and oat (**Kulichová *et al.*, 2018**). Secondary, the enhancement of physical treatment is widely published, as mentioned in the Introduction (**Conde *et al.*, 2009; Saad *et al.*, 2014; Jesus *et al.*, 2020**).

The increase of bioactivity with a coefficient over 5 is indeed significant, indicating that the prepared extracts are highly valuable from both a biological and therapeutic potential standpoint. This approach appears to be promising for the preparation of more active extracts for the field of food additives or functional food. The subject of the following study will involve testing other chemical (reactive extraction) and physical treatments separately and simultaneously for the mentioned plant material, as well as other prospective medicinal plants.

Table 1 The increasing of extraction yield, expressed as AOXA parameter $DPPH_{50}$, using all three physical factor treatments for plant extracts, expressed as Enhancement Factor (EF)

plant extract	EF (temperature)	EF (sonication)	EF (AC+ electric field) promotion)	EF (all treatments - sum of three contributions)
Origanum vulgare L.	2.0	1.2	2.5	5.1
Quercus petraea Matt.	2.1	1.5	2.5	6.0
Brassica napus subsp. oleifera	1.8	1.5	1.9	4.0
Avena sativa L.	2.9	4.5	5.0	4.5

Table 2 The increase in extraction yield, using all three physical factors simultaneously and the bioactivity for plant extracts, expressed as Enhancement Factor (EF)

Plant extract	EF (DM) (dried matter)	EF (AOXA) scavenging of $DPPH^{\bullet}$ radical	EF (PROOXA, the measure of conversion of Fe^{3+} to Fe^{2+})	EF (ABA, MIC_{AVG})	EF (AVA, inhibition of 3CLpro SARS-Cov-2)
Origanum vulgare L.	3.1	5.1	4.5	3.6	3.1
Quercus petraea Matt.	4.2	6.0	6.2	4.3	4.3
Brassica napus subsp. oleifera	2.8	4.0	4.5	3.6	3.1
Avena sativa L.	2.9	4.5	5.0	3.9	3.5

The study selected plant extracts that were the result of screening from previous research. All four extract samples demonstrated significant simultaneous activities, namely parallel antioxidant or pro-oxidant activity, antibacterial activity, and inhibitory activity against the M^{pro} enzyme of the COVID-19 virus (unpublished data). The samples were also selected, based on different source materials as drugs with simultaneous multi-effects.

Dried *O. vulgare* L. herb was chosen from approximately 50 dried herbs of plant taxa. Similarly, the bark of *Q. petraea* Matt. was selected from 20 tree bark drugs based on its highest activity. Finally, the seeds of agricultural crops *B. napus subsp. oleifera* and *A. sativa* L. were chosen due to their surprising bioactivity from a collection of agricultural crops and cultivable plant seeds with n=50.

The Lotus Database on natural metabolites presents 200 secondary metabolites for *O. vulgare* L., predominantly monoterpenes (such as terpinene, α-myrcene, thymol, carvacrol, and others), which undoubtedly carry antibacterial activity. On the other hand, polyphenolic acids (like rosmarinic acid, 3,4-dihydroxycinnamic acid, vanillic acid, ferulic acid, and syringic acid) and flavonoids (including quercetin, apigenin, luteolin, kaempferol, chrysoeriol, eriodictyol, taxifolin, vicenin-2, apigetrin, luteolin, isorhamnetin, disometin, galangin, and hesperetin) and salvionolic acid, or lithopsermic acid should be responsible for antioxidant/prooxidant activity as well as M^{pro} inhibitory activity.

For the tree *Q. petraea* Matt., the Lotus Database shows 44 records of secondary metabolites. Besides tannins, flavonoids (like isoquercetin and quercetin with their derivatives) and polyphenolic acids (such as gallic acid, syringic acid, vanillic acid, and ellagic acid) could contribute to antibacterial and antioxidant/prooxidant activities, as well as M^{pro} inhibitory activity. Aromatic aldehydes (vanillin, syringaldehyde, coniferylaldehyde) and coumarins (esculetin, scopoletin) are also noted.

For rapeseed (*B. napus subsp. oleifera*), the Lotus Database presents 271 records. In addition to expected sulfur compounds typical for the *Brassicaceae* family, such as glucobrassicin derivatives known for their antibacterial effects, polyphenols that exhibit M^{pro} inhibitory activity and antioxidant/prooxidant effects due to their aromatic hydroxyl groups are noted. These include polyphenolic acids (like cinnamic acid, synapic acid, caffeic acid, ferulic acid, p-coumaric acid, syringic acid, gentisic acid, trans-2-hydroxycinnamic acid, p-hydroxybenzoic acid, p-hydroxycinnamic acid), flavonoids (quercetin, apigenin, kaempferol, luteolin, myricetin), lignans (secoisolariciresinol, matairesinol), low-molecular aldehydes (vanillin, phenylacetaldehyde, 2-aminobenzaldehyde), as well as carotenoids (zeta-carotene, luteoxanthin, flavoxanthin) and glycosylated seco-iridoids (oleuropein).

The Lotus Database shows 117 records of secondary metabolites for common oat (*A. sativa* L.). In addition to significant avenacins (sterol-based substances) that may exhibit M^{pro} inhibitory activity - polyphenolic acids (ferulic acid, syringic acid, caffeic acid, 4-hydroxyphenylacetic acid, diferulic acid, 3,4-dihydroxybenzoic acid, vanillic acid), aldehydes (vanillin, p-hydroxybenzaldehyde, heptanal, 2-heptenal, nonanal), lignans (pinresinol, matairesinol, secoisolariciresinol, lariciresinol), flavonoids (naringenin, among others), as well as various cyclic and open-chain avenanthramides and other nitrogen-containing substances (putrescine, spermine) could also be responsible for additional biological activities.

The measured biological activity pertains to specific samples, which were purchased in the form of dried herbs from Calendula, Inc. Nová Ľubovňa and as native seeds from the Research Institute of Plant Production in Piešťany during 2023. This means that the given herbs may exhibit even higher biological activity, depending on the cultivar and growing year, which is determined by both biotic and abiotic stress factors as well as meteorological conditions (**Nawaz *et al.*, 2023; Vaičiulyté *et al.*, 2016**). All these determinants manifest in the content of secondary metabolites and, consequently, the observed level of bioactivity. A significant factor may also be the elicitation by selected elicitors that potentiate specific biosynthetic pathways. These may involve certain biosynthetic polyketides, for example, the phenylpropanoid biosynthetic pathway (**Vogt, 2010**), that starts from phenylalanine or tyrosine, with 4-coumaroyl-CoA as a key intermediate. This pathway leads to the biosynthesis of derivatives of cinnamic acid, lignols, flavonoids, isoflavonoids, catechins, coumarins, aurones, stilbenes, and other compounds. Another important biosynthetic pathway is the shikimic acid pathway (**Ghosh, *et al.*, 2012**), which begins with the condensation of phosphoenolpyruvate and erythrose-4-phosphate to chorismic acid, which is further metabolized on shikimic acid. This pathway synthesizes not only folates and aromatic amino acids but also C6-C1 aromatic carboxylic acids. The nitrogenous secondary metabolites of plants mentioned in the text above are formed through the metabolic derivatization of amino acids. Avenanthramides are produced by the condensation of anthranilic acid derivatives with C6-C1 (benzoic acid) and C6-C2-C1 (cinnamic acid derivatives) aromatic acids.

The content of targeted secondary metabolites in specific metabolic pathways can be stimulated by applying several elicitors, such as the family of jasmonic acid derivatives, which includes methyl jasmonate and methyl dihydrojasmonate (**Rani and Murali-Baskaran, 2025**). Plants produce jasmonic acid and methyl jasmonate in response to many biotic and abiotic stresses (in particular, herbivory and wounding), which build up in the damaged parts of the plant. The methyl jasmonate can be used to signal the original plant's defense systems or it can be spread by physical contact or through the air to produce a defensive reaction in unharmed plants.

CONCLUSION

The aim of this study was to highlight the perspectives for higher yields of plant extracts from biological material by simultaneously applying several physical factors. This approach to determining higher yields can be combined with the optimization of extraction by selecting the chemical nature of the extraction environment. With higher yields of secondary plant metabolites, the bioactivity of the obtained extract and the concentrate created from it logically increases. When isolating such concentrates, an internal atmosphere or evacuation of air is suitable/desirable to prevent unwanted oxidative processes. Extracts or concentrates, with higher potential for bioactive substances, are highly sought after, applicable directly as food additives or as semi-finished products for finalization into dietary supplements or even functional foods. This trend of applying natural substances in food is currently very relevant.

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