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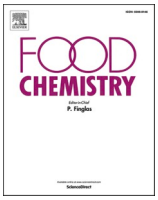

Untargeted phytochemical profiling and biological activity of small yellow onion (*Allium flavum* L.) from different regions of Romania

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ABSTRACT

This study examined the phytochemical profiles (mainly phenolics, carotenoids, and organosulfur compounds) and biological effects of hydroalcoholic extracts of *Allium flavum* (AF), a species of the *Allium* genus commonly known as small yellow onion. Unsupervised and supervised statistical approaches revealed clear differences between extracts prepared with samples collected from different areas of Romania. Overall, the AFFF (AF flowers collected from Făget) extract was the best source of polyphenols, also showing the highest antioxidant capacity evaluated through both *in vitro* DPPH, FRAP, and TEAC anti-radical scavenging assays and cell-based OxHLIA and TBARS assays. All the tested extracts exhibited α-glucosidase inhibition potential, while only the AFFF extract exhibited anti-lipase inhibitory activity. The phenolic subclasses annotated were positively correlated with the assessed antioxidant and enzyme inhibitory activities. Our findings suggested that *A. flavum* has bioactive properties worth exploring further, being a potential edible flower with health-promoting implications.

1. Introduction

Nutraceuticals represent a group consisting of different biologically active compounds, dietary fibers, pro- and prebiotics characterizing foods and dietary supplements. Nutraceuticals can help to low the risk of developing several diseases, including among the others, neurodegeneration, ailments caused by oxidative stress, diabetes, hypertension, and different types of cancer (Anand & Bharadvaja, 2022).

In recent years, the interest towards nutraceuticals has grown in both the scientific community and the food industry. In particular, several raw materials have been explored to develop novel food components showing potential health-promoting properties (Rocchetti et al., 2022; Sachdeva et al., 2020). According to scientific literature, wild plant species are of great interest as raw materials for the food industry, and the members of the genus *Allium* have received a special focus (Demir et al., 2022; Halder et al., 2022; Rocchetti et al., 2022). *Allium* is the largest genus of the Amaryllidaceae family, consisting of approximately 1000 species (POWO, 2023). Due to their pleasant taste, flavor and smell, some of these species are broadly used in human nutrition as spices and vegetables (*A. cepa, A. sativum, A. porrum, A. ascalonicum*) while being well-known medicinal plants rich in bioactive molecules such as organosulfur and organoselenium compounds, phenolics, steroidal saponins, vitamins and amino acids (Simin et al., 2019). A broad spectrum of biological activities has been found for both functional extracts and isolated compounds of *Allium* species, including strong antimicrobial, anti-inflammatory, antioxidant, anti-diabetic, hypolipidemic, anti-hypertensive, analgesic and immunoprotective effects.

Of interest, *A. flavum* L. is a wild-growing species of *Allium* genus

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commonly known as small yellow onion. In the last years, few studies have examined the beneficial properties exerted by *A. flavum* consumption and/or the exploitation of its functional extracts. In a previous work, Aleksandar et al. (2019) showed that methanol extracts were strongly antioxidant and synergistically increased doxorubicin anticancer activity against human hepatoma (HepG2) and lung carcinoma (A549) cells while protecting normal human fibroblasts (MRC-5) from doxorubicin cytotoxicity. Furthermore, a study by Mitić-Ćulafić et al. (2016) revealed that *A. flavum* extracts reduced tBOOH-induced DNA damage by up to 70%. Another study investigated the cytotoxicity of *A. flavum* extracts on colon cancer cells, reporting values of IC_{50} in the range 1.64–84 μg/mL. Combining plant extracts with Pd(II) complex caused lower IC_{50} values and better proapoptotic activity. Pd(II) complex determined a marked necrosis in a single treatment, but provided enhanced proapoptotic and lower necrotic activity in combination with plant extracts (Milutinović et al., 2015). Curcic et al. (2015) reported significant antibacterial activity, mainly when considering ethyl acetate *A. flavum* extracts. The best activity was shown against Gram-positive bacteria, *Staphylococcus aureus* ATCC 25923 and *Bacillus subtilis*, while *Escherichia coli* was classified among the less sensitive bacteria.

Regarding the phytochemical profile, few recent studies investigated the natural compounds found in *A. flavum* matrices. Overall, 44 phenolic compounds were searched in methanol extracts of *A. flavum* by targeted LC-MS/MS, as reported by Simin et al. (2013), thus identifying 25 compounds, the most dominant being: ferulic, *p*-coumaric, caffeic, *p*-hydroxybenzoic, vanillic, protocatechuic and syringic acids, rutin, quercetin-3-*O*-glucoside and kaempferol-3-*O*-glucoside. Another study reports the isolation of three new spirostane-type glycosides and their cytotoxicity against a human cancer cell (Rezgui et al., 2014). However, no studies have comprehensively evaluated (e.g., by using untargeted omics-based approaches) the distribution of total carotenoids and sulfur compounds in flowers and stems of *A. flavum*.

Therefore, this study aimed to provide novel information and insights regarding the phytochemical profiles and biological effects exerted by hydroalcoholic extracts of flowers and stems of *A. flavum* collected from three areas in Romania. To the best of the authors' knowledge, this is the first research work that comprehensively compared flowers and stems of *A. flavum* collected from different geographical areas using an untargeted metabolomics approach.

2. Materials and methods

2.1. Plant material

In this work, the whole *A. flavum* L. specimens were collected from three different locations: (1) Piatra-Secuiului, Alba County, Romania (46.44273220652408, 23.58341925248172, alt. 919 m) in August 2019, voucher specimen No. 616/08.08.2019, (2) Făget, Cluj County, Romania (46.71360305485446, 23.547869092397907, alt. 633 m) in August 2020, voucher specimen No. 612/05.08.2019 and (3) Lăpuștești, Cluj County, Romania (46.715860709745144, 23.17987681194857, alt. 1073) in July 2019, voucher specimen No. 617/21.07.2019, at their maximum flowering period. Plant material was sorted, authenticated based on its botanical features, and separated into (a) stems (AFSF = *A. flavum* stems from Făget) and (b) flowers (AFFL = *A. flavum* flowers from Lăpuștești; AFFP = *A. flavum* flowers from Piatra-Secuiului; AFFF = *A. flavum* flowers from Făget). Next, all sample matrices were dried at room temperature in a dry place, to reach a constant weight. Afterwards, the dried material was kept in paper bags and stored in the herbarium of Pharmaceutical Botany Department of Iuliu Haţieganu (University of Medicine and Pharmacy Cluj-Napoca), until the following extraction step.

2.2. Extraction of bioactive compounds

A laboratory mill (Grindomix® GM 200, Retsch Gmbh., Germany) was used to powder the dry samples, and then manually sieved (1 mm standard sieve according to PhEur 10.6) to reach a particles' uniformity. Ethanol 70% (*v/v*) was used as extraction solvent and vortex apparatus (Velp Scientifica Classic, Bohemia, NY, USA) was used to homogenize the plant material. Thereafter, the extraction was promoted by an ultrasound-assisted method for 30 min at 50 °C and then the mixture was filtered under vacuum conditions. Next, ethanol was removed by evaporation using a rotary evaporator (Büchi R-210; Flawil, Switzerland) under reduced pressure, redissolved in ethanol and stored at 4 °C for further analyses (TPC, TFC and *in vitro* antioxidant assays). The extracts were lyophilized (Biobase® BK-FD18S, Biobase group, Jinan, Shandong, China) and placed in a desiccator until further steps. The dry extract was dissolved in 5% DMSO and the appropriate buffer to evaluate enzyme inhibition capacity. For the cell-based assays, the extracts were redissolved in PBS.

2.3. In vitro total phenolic content (TPC) and total flavonoid content (TFC)

The TPC assay was done according to the Folin-Ciocalteu method adapted to a microplate reader, previously reported by Mocan et al. (2016). The results were expressed as mg GAE/g extract. Similarly, the TFC was determined using the aluminum chloride method, as fully reported by Mocan et al. (2016), expressing the results as mg RE/g extract.

2.4. Screening of the phytochemical profile by UHPLC-HRMS analysis

The resulted *Allium* extracts (10 mg) were dissolved in 200 μL of ethanol 70% (*v/v*), centrifuged at 6000 × g (considering 10 min at 4 °C). Afterwards, the obtained supernatants were filtered using 0.20 cellulose-syringe filters and analysed for untargeted phytochemical profiling. This latter was done by high-resolution mass spectrometry (HRMS) using a Q-Exactive™ Focus Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific, Waltham, MA, USA) coupled to a Vanquish ultra-high-pressure liquid chromatograph (UHPLC). All the details regarding UHPLC and HRMS analysis (mass resolution of 70,000 FWHM at 200 *m/z*) can be found in a previous published work by our research group (Babotă et al., 2022; Rischer et al., 2022). The raw data generated by HRMS were processed using the software MS-DIAL (version 4.70). The putative annotation (level 2 of confidence typical of untargeted metabolomics-based experiment) was reached via spectral matching against the comprehensive databases FooDB and Phenol-Explorer, using a tolerance for mass accuracy of 5 ppm. Finally, all the phytochemicals identified were grouped into different and typical subclasses and then semi-quantified according to hydroalcoholic standard solutions of pure standard compounds (Extrasynthese, Lyon, France) analyzed under the same instrumental conditions. Overall, 7 phenolic compounds, namely ferulic acid (for phenolic acids), quercetin, catechin, cyanidin and luteolin (for different flavonoid subclasses), resveratrol (stilbenes), oleuropein (other remaining phenolics), beta-carotene (carotenoids), and alliin (organosulfur compounds) were used for this purpose. The results were finally expressed as μg equivalents (Eq.)/g lyophilized extract (n = 3).

2.5. In vitro biochemical assays for evaluating the antioxidant potential

Six complementary *in vitro* assays were used to evaluate the antioxidant potential of the different *Allium* extracts, namely DPPH, FRAP (ferric reducing antioxidant power), TEAC (indicating radical scavenger activity), superoxide radical ($O_2^{\cdot-}$) scavenging activity assay, thiobarbituric acid reactive substances (TBARS) formation inhibition assay, as well as oxidative hemolysis inhibition (OxHLIA) assay. All protocols were extensively described previously (Les et al., 2017; Martins et al., 2015; Mocan et al., 2016; Babotă et al., 2021; dos Santos et al., 2019).

2.6. In vitro biochemical assays for evaluating the enzyme inhibition potential

The enzyme-inhibitory capacity of extracts was assessed considering different enzymes, namely α-glucosidase (α-Glu), tyrosinase (Tyr), acetylcholinesterase (AChE), and pancreatic lipase, using *in vitro* methods, as fully and comprehensively described in previous works (Kim et al., 2010; Les et al., 2017; Gowri et al., 2007).

2.7. Statistical and correlation analyses

The different assays were carried out considering triplicate values and the results were finally expressed as mean values ± SD (standard deviation). Additionally, Pearson's correlation coefficients were calculated between the different phytochemical classes and biological activities ($p < 0.05$), using the software R-studio.

Regarding untargeted metabolomics experiments, the multivariate data analysis was done into MetaboAnalyst 5.0 (Pang et al., 2021) and SIMCA 13 (Umetrics, Malmo, Sweden), considering both unsupervised and supervised models, being hierarchical cluster analysis (HCA, Euclidean distance) and principal component analysis (PCA), followed by orthogonal projections to latent structures discriminant analysis (OPLS-DA), respectively. Besides, the goodness of fitting and prediction, together with permutation testing, cross-validation of the model and absence of significant outliers was checked in SIMCA 13. Finally, the ability of compounds in discrimination was checked trough variables importance in projection (VIP) approach, considering a minimum VIP score > 1.

3. Results and discussion

3.1. Profiling of A. flavum extracts by UHPLC-HRMS

3.1.1. Phenolic compounds, total carotenoids, and organosulfur compounds

Because of their alleged positive effects on health, phenols have received a lot of academic consideration. Rapid screening of analyzed extract through Folin-Ciocâlteu (for determination of total phenolic compounds) and $AlCl_3$ methods (for determination of total flavonoid compounds) revealed that AFFF and AFSF extracts have the highest content of these compounds (**SM1**). As far as the quantification of the different bioactive compounds is concerned, the results (Table 1) revealed that AFFF extract was the best source of total phenolic compounds, recording a total of 2362.5 μg/g DM, followed by AFSF (1277.9 μg/g DM), AFFP (559.1 μg/g DM), and AFFL (493.7 μg/g DM). The results followed the same trend as the preliminary spectrophotometric findings obtained through the Folin-Ciocalteu assay, as shown in the **SM1** file. Therefore, it was evident that *A. flavum* samples from Făget area (AFFF and AFSF) were the most promising in terms of phenolic profile and potential bioactivity. Regarding a comparison between the different flower extracts, it was interesting to notice that AFFF was significantly ($p < 0.05$) higher in lower-molecular-weight phenolics (classified as "other phenolics") when compared with other extracts. In particular, they were abundant in juglone (an aromatic compound belonging to the subclass of naphthoquinones, VIP score = 1.04), followed by the hydroxybenzaldehydes *p*-anisaldehyde (VIP score = 1.01) and gallic aldehyde (VIP score = 1.06) (**SM1**). Regarding the other phenolic classes detected in the different extracts, our findings are in agreement with those reported by Simin et al. (2013), highlighting some phenolic acids (e.g., ferulic, *p*-coumaric, caffeic, *p*-hydroxybenzoic, vanillic, protocatechuic and syringic acids) and flavonoids (e.g., rutin, quercetin-3-*O*-glucoside and kaempferol-3-*O*-glucoside) as the dominant compounds in *A. flavum* subsp. *flavum* collected from three different locations in Serbia. The preliminary findings on flavonoid levels in extracts of *A. flavum* are reported in **SM1** file. These results indicate that the AFFF and AFFP extracts possess the highest concentration of flavonoid compounds. The most abundant flavonoids detected using UHPLC-HRMS were isorhamnetin, quercetin 3-rutinoside, 4"-*O*-methylepigallocatechin 3-*O*-gallate, luteolin, glycosidic forms of luteolin, and apigenin (**SM1**). Interestingly, besides naphthoquinones and hydroxybenzaldehydes, the VIP selection revealed that 12 flavonoids (including flavonols, flavanols, and flavones) and 7 phenolic acids (mainly hydroxycinnamics and hydroxybenzoics) were exclusive markers of AFFF extracts (**SM1**). Some of the exclusive markers found in AFFF could be those compounds accounting for the bioactivity reported in the next paragraphs (i.e., *in vitro* antioxidant and enzymatic inhibition capacities).

Table 1
Quantification of the main phytochemicals (i.e., phenolics, carotenoids, and organosulfur compounds) characterizing the four *A. flavum* extracts. Results are expressed as mean (μg standard equivalents/g dry matter) ± standard deviation ($n = 3$).

Class (μg Eq./g DM)	AFFF	AFSF	AFFL	AFFP
Flavones	54.4 ± 5.5^{c}	33.2 ± 1.1^{b}	18.7 ± 2.1^{a}	17.6 ± 2.7^{a}
Flavan-3-ols	27.3 ± 2.5^{c}	16.6 ± 0.5^{b}	3.7 ± 1.7^{a}	15.8 ± 1.3^{b}
Flavonols	216.1 ± 15.9^{d}	168.2 ± 2.7^{c}	60.3 ± 0.6^{a}	88.6 ± 0.5^{b}
Phenolic acids	437.8 ± 22.2^{d}	369.1 ± 10.6^{c}	170.9 ± 10.4^{a}	215.4 ± 15.5^{b}
Other phenolics	1626.9 ± 59.7^{c}	690.8 ± 11.3^{b}	240.2 ± 4.8^{a}	221.5 ± 12.1^{a}
Total phenolics	2362.5 ± 67.7^{c}	1277.9 ± 24.7^{b}	493.7 ± 6.7^{a}	559.1 ± 28.9^{a}
Carotenoids	128.7 ± 28.6^{c}	14.8 ± 1.6^{a}	46.5 ± 2.5^{b}	25.3 ± 6.3^{ab}
Organosulfur compounds	298.5 ± 16.7^{a}	263.6 ± 5.5^{a}	518.9 ± 74.4^{b}	261.8 ± 42.5^{a}

The different superscript letters within the same row indicate significant differences in the extracts according to ANOVA ($p < 0.05$) and using a Duncan's post-hoc test. Abbreviations: Eq. = Equivalents; DM = dry matter. AFFL = *A. flavum* flowers from Lăpuștești; AFFP = *A. flavum* flowers from Piatra-Secuiului; AFFF = *A. flavum* flowers from Făget; AFSF = *A. flavum* stems from Făget.

Overall, *A. flavum* is also known as "small yellow onion" because of the color characterizing flowers and mainly due to the presence of carotenoids. Under our experimental conditions, four carotenoids have been structurally confirmed, namely 10′-apo-beta-caroten-10′-al, apo-10′-zeaxanthinal, apo-8′-lycopenal, and dehydro-beta-carotene (**SM1**). Interestingly, the apocarotenoid apo-10′-zeaxanthinal was extremely discriminant for the AFFF extract (VIP score 1.04), thus contributing to its higher total carotenoid content (i.e., 128.7 μg/g DM) reported in Table 1. On the other hand, total carotenoids were lower ($p < 0.05$) in *A. flavum* samples from Lăpuștești and Piatra Secuiului, recording 46.5 and 25.3 μg/g DM, respectively, while AFSF sample was characterized by the lowest total carotenoids content, 14.8 μg/g DM. Finally, as far as the distribution of typical organosulfur compounds is concerned, AFFL extract was the most abundant source (i.e., 518.9 μg/g DM). At the same time, no significant differences were recorded when considering the other extracts analyzed (Table 1). The untargeted screening allowed us to identify typical *Allium* metabolites like allixin (**SM1**) and alliin derivatives. Allixin is classified as an organic compound belonging to pyranones and derivatives. According to the comprehensive database FooDB, this compound has been detected in different foods such as garlic (*Allium sativum*), onion-family vegetables, garden onion (var.), pigmented onion, and garden onions (*Allium cepa*). However, based on a literature review very few articles have been published on allixin. Multiple studies have shown that water-soluble and lipid-soluble organosulfur compounds, including allixin and other compounds distributed in raw and aged garlic, display antioxidant properties and prevent oxidative damage to the LDL and the ensuing atherogenic events (Baliga et al., 2013). In this work, the organosulfur compounds had a quite good

prediction ability (VIP score > 1), recording 19 discriminant metabolites mainly belonging to alliin- and disulfide-derivatives (**SM1**), with (E)-methylated disulfide derivatives being the most important compounds for discrimination purposes.

3.1.2. Discrimination of the different Allium extracts by multivariate statistics

This work used an untargeted metabolomics approach to provide novel information regarding the functional composition and phytochemical profile of small yellow onion samples (wild *A. flavum* species). To this aim, UHPLC-HRMS allowed us to identify 296 compounds, exploiting two comprehensive databases, namely FooDB and Phenol-Explorer. Accordingly, several typical subclasses of compounds were identified, namely flavonoids (including dihydrochalcones, flavanones, flavones, isoflavonoids, flavan-3-ols, and flavonols), phenolic acids (such as hydroxycinnamics, hydroxybenzoics, hydroxyphenylacetics, and hydroxyphenylpropanoics), other phenolics (including lower molecular weight compounds), carotenoids, and organosulfur compounds (consisting of 37 compounds, such as alliin derivatives and disulfides). As far as an overview of the remaining annotated compounds is concerned, the enrichment analysis carried out on the software MetaboAnalyst 5.0 revealed that the most represented and significant super classes were organic acids (14 compounds), organoheterocyclic compounds (9 compounds), benzenoids (17 compounds), and polyketides (17 compounds). The compounds annotated and structurally confirmed under our experimental conditions can be found in the Supplementary Material 1 (SM1) file, together with other annotation parameters.

As the next step, multivariate statistics were used to group *A. flavum* extracts according to their different nutraceutical profiles, using both unsupervised and supervised statistical models. Interestingly, the hierarchical clustering (Fig. 1A) and PCA score plot (Fig. 1B) revealed clear differences between the extracts; in particular, AFFF and AFSF samples showed the most exclusive phytochemical profiles compared with AFFL and AFFP. Accordingly, it was interesting to notice from the HCA heat map that some clusters of metabolites were up-accumulated in AFFF and AFSF extracts (Fig. 1A). Additionally, the PCA score plot (Fig. 1B) showed that two principal components could cumulative explain 76% of the total variability, thus confirming the effectiveness of untargeted metabolomics for discrimination purposes. Interestingly, the unsupervised statistics pointed out the impact of higher altitudes characterizing Piatra Secuiului (919 m a.s.l.) and Lăpuștești (1073 m a.s.l.) areas, compared to Făget area (633 m a.s.l.) on the phytochemical profile recorded. Besides the genetic background, it is known that plants are affected by environmental factors (including light, temperature, and biotic or abiotic stresses), then activating signaling pathways able to modulate their metabolic processes and promoting the biosynthesis of secondary metabolites (Lucini et al., 2020). Therefore, the unsupervised discrimination observed could be due to a different biosynthesis and/or accumulation of secondary metabolites (such as polyphenols) and other bioactive compounds.

Starting from the unsupervised findings by HCA and PCA plots, the supervised multivariate statistical analysis, based on OPLS-DA modeling, was used to discriminate *Allium* extracts maximizing the covariance between groups (Fig. 2). The model showed excellent accuracy and prediction parameters, with R^2Y (cum) = 0.999, R^2X (cum) = 0.797, and Q^2 (cum) = 0.972. Furthermore, after a cross-validation step, the overfitting was excluded together with the presence of significant outliers (data not shown). Overall, the OPLS-DA score plot findings confirmed what was previously reported for HCA and PCA plots; in particular, AFFF extracts differed from AFFL and AFFP extracts, with the prediction model successfully discriminating the different botanical parts (stems *vs* flower for *A. flavum* from Făget).

After that, the VIP selection method extrapolated the discriminant compounds driving sample grouping. The list of VIP discriminant compounds is reported in SM1, together with the corresponding VIP scores (prediction abilities). The VIP selection method provided 191 discriminant compounds, being 3 carotenoids, 23 flavonoids, 19 organosulfur compounds, 5 lower-molecular-weight phenolics, 12 phenolic acids, and 129 remaining metabolites (SM1). Among the most important phenolic compounds we listed gallocatechin (VIP score = 1.28), followed by 5-feruloylquinic acid (VIP score = 1.27) and 3-*O*-methylrosmarinic acid (VIP score = 1.25). Additionally, the most discriminant carotenoid was apo-8′-lycopenal (VIP score = 1.12), while the most important organosulfur compound was represented by (E)-methyl 3-(methylsulfinyl)-1-propenyl disulfide (VIP score = 1.26).

3.2. In vitro antioxidant activity of the different A. flavum extracts

Allium species are rich sources of compounds with antioxidant activity. In this study, we assessed the antioxidant activity exerted by

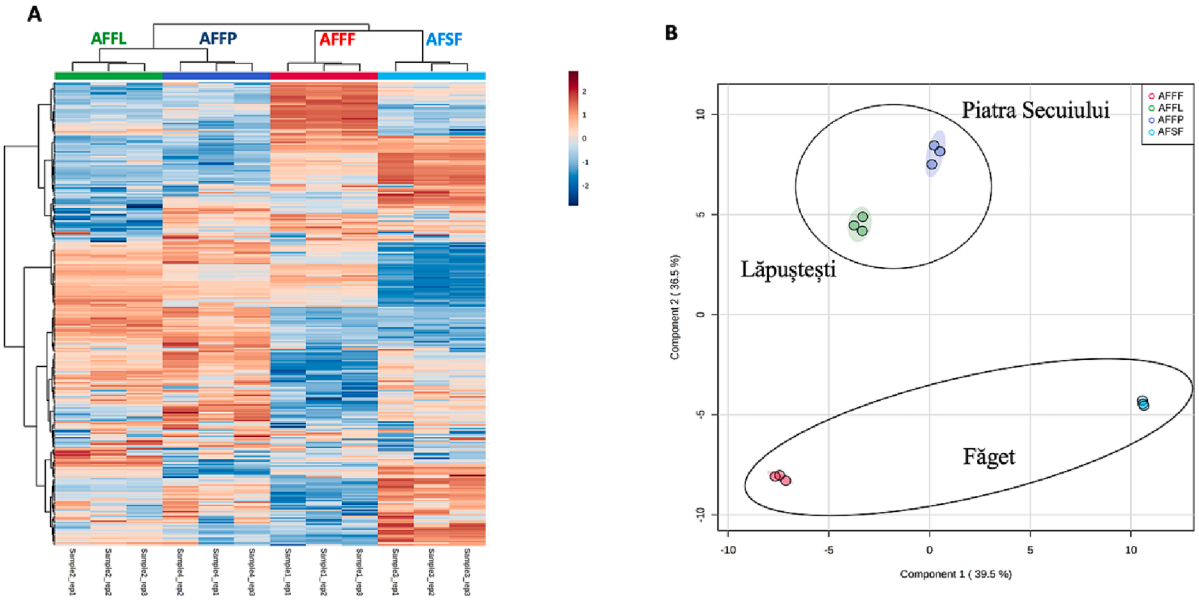

Fig. 1. Heat map from unsupervised not-averaged hierarchical cluster analysis (A) and score plot from Principal Component Analysis (B), both realized considering the untargeted phytochemical profiles of the different *A. flavum* extracts.

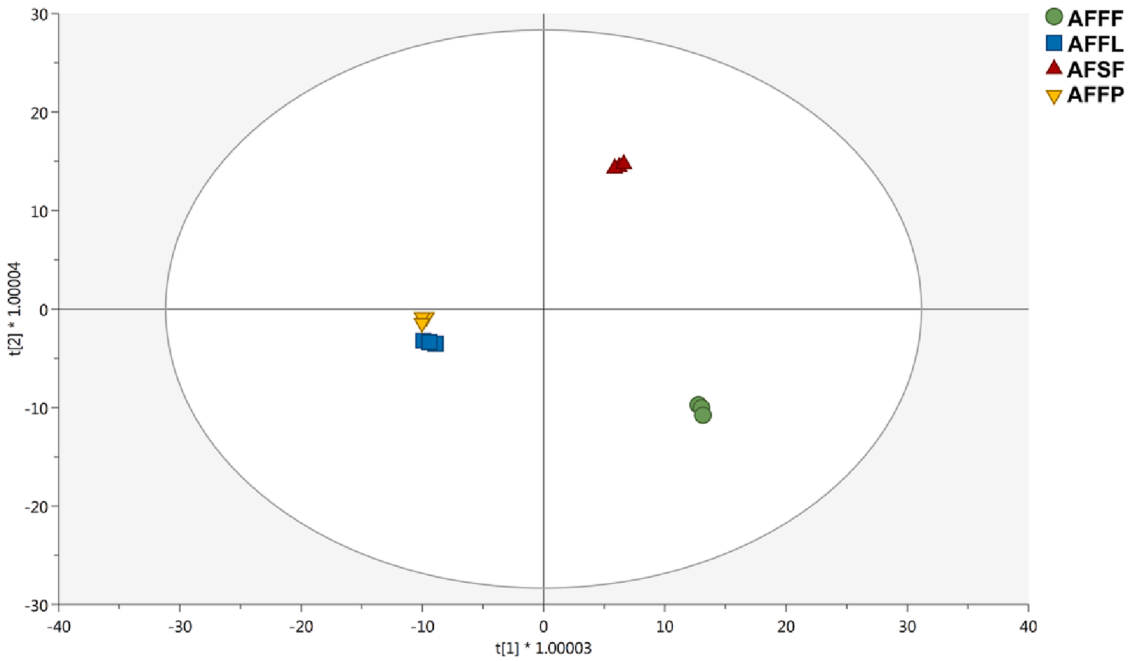

Fig. 2. OPLS-DA score plot built considering the untargeted phytochemical profiles of the different *A. flavum* extracts.

A. flavum extracts *via* DPPH, FRAP and TEAC assays. Correlated with the high values in terms of flavonoids and total phenolic compounds, the extract of flowers of *A. flavum* collected from Făget exerted the highest antioxidant capacity (40.01, 6.99, and 43.23 mg TE/g dw for TEAC, FRAP and DPPH assay, respectively, Table 2). In line with these results, a study by Simin et al. (2013) revealed that the methanolic extract of whole *A. flavum* plant exerted an IC_{50} of 117.38 μg/mL, whereas the aerial parts exerted a more potent antioxidant capacity, namely IC_{50} of 42.12 μg/mL. Another study by Aleksandar et al. (2019) reported a value of IC_{50} = 72.3 μg/mL for methanolic extracts of *A. flavum*. In contrast, another study reported values ranging between 64.34 and 243.34 μg/mL for different extracts of the same species (Curcic et al., 2015).

Superoxide radical is one of the major biological sources of reactive oxygen species. Although superoxide anion is classified as a weak oxidant, it gives rise to the generation of reactive and dangerous hydroxyl radicals and singlet oxygen, both contributing to oxidative stress (Saeed et al., 2012). Our results showed that *A. flavum* flowers and stem extracts had an IC_{50} ranging from 206.04 to 424.14 μg/mL, AFSF having the most potent superoxide radical scavenging activity (Table 2 and SM1). Remarkably, superoxide radical inhibitory activity correlated with the content of antioxidant compounds such as polyphenols and flavonoids. These results align with the previous ones, where Štajner et al. (2008) reported good superoxide radical scavenging potential for extracts obtained from *A. roseum* and *A. subhirsutum*.

The lipid peroxidation inhibitory activity exerted by *Allium* extracts confirms the results mentioned above. As depicted in Table 2, AFFF extracts exerted the most potent lipid peroxidation inhibitory activity (IC_{50} = 75.01 μg/mL). Previous studies demonstrate that *Allium* extracts can also inhibit lipide peroxidation *in vivo*. In a recent study, Rankovic et al. (2021) demonstrated that increasing doses of *A. ursinum* methanol extract beneficially affect cardiac ischemia/reperfusion injury (I/R), especially on lipid peroxidation. The lipid peroxidation inhibitory activity exerted by *Allium* extracts confirms the results above. Previous studies demonstrate that *Allium* extracts can also inhibit lipide peroxidation *in vivo*. In a recent study, Rankovic et al. (2021) demonstrated

Table 2
In vitro antioxidant activity and enzyme inhibitory potential of *A. flavum* extracts.

	mg Trolox eq./g extract			IC_{50}, μg/mL				
	DPPH	**FRAP**	**TEAC**	**$O_2^{\bullet-}$ scav. act.**	**TBARS**	**OxHLIA**		**α-Glu.**	**Lipase**
						60 min Δ*t*	**120 min Δ*t***		
AFFF	43.23 ± 0.69^{c}	6.99 ± 0.13^{d}	40.01 ± 0.24^{c}	324.80 ± 57.70^{a}	75.01 ± 3.61^{d}	6.86 ± 0.28^{d}	13.29 ± 0.33^{d}	1231.15 ± 52.56^{b}	4904.52 ± 1058.18
AFSF	43.67 ± 0.91^{c}	6.70 ± 0.02^{c}	42.74 ± 1.55^{c}	206.04 ± 18.50^{b}	195.78 ± 0.56^{b}	111.97 ± 6.08^{a}	231.12 ± 8.23^{a}	1254.91 ± 43.17^{b}	nd
AFFL	8.50 ± 0.20^{a}	1.97 ± 0.04^{a}	11.70 ± 0.28^{a}	424.14 ± 96.29^{a}	265.81 ± 1.94^{a}	39.73 ± 0.72^{b}	65.39 ± 1.35^{b}	966.20 ± 40.99^{c}	nd
AFFP	16.24 ± 0.33^{b}	2.79 ± 0.08^{b}	18.42 ± 1.12^{b}	320.15 ± 131.11^{a}	170.94 ± 1.86^{c}	15.08 ± 0.49^{c}	21.05 ± 0.47^{c}	3812.98 ± 102.69^{a}	nd
Standard compound	–	–	–	0.053 ± 0.002*	11.12 ± 0.25*	21.80 ± 0.25*	43.51 ± 0.82*	229.68 ± 30.52*	72.52 ± 48.72*

The results are expressed as average ± standard deviation of three parallel measurements. Statistical differences were assessed by one-way ANOVA, followed by Tukey's HSD post hoc test ($\alpha = 0.05$). In each column, different lower-case letters within the same column indicate significant differences between extracts. Asterisks (*) within the same column indicates statistical differences between standard compound and different extracts according to Student's *t*-test ($\alpha = 0.05$). Standard compound used in $O_2^{\bullet-}$ scavenging activity test was gallic acid; in TBARS and OxHLIA assays it was Trolox; while in α-glucosidase and pancreatic lipase inhibition activity tests it was acarbose and orlistat, respectively. AFFL = *A. flavum* flowers from Lăpuștești; AFFP = *A. flavum* flowers from Piatra-Secuiului; AFFF = *A. flavum* flowers from Făget; AFSF = *A. flavum* stems from Făget; nd = not detected.

that increasing doses of *A. ursinum* methanol extract beneficially affect cardiac ischemia/reperfusion injury (I/R), especially on lipid peroxidation.

Oxidative hemolysis inhibition assay (OxHLIA) assay is based on inhibiting free radical-induced membrane damage in erythrocytes by antioxidants. Our study reveals that, remarkably, the AFFF extract is more potent in inhibiting free radical damage, compared to a standard reference, both at 60 min and 120 min (IC_{50}: 6.86 μg/mL and 13.29 μg/mL compared to Trolox: 21.80 μg/mL and 43.51 μg/mL, at 60 min and 120 min, respectively) (Table 2). The AFFP extract also stood out in this bioassay, especially for longer reaction times.

3.3. Enzyme inhibition potential of the different A. flavum extracts

Regarding the enzyme inhibition potential, previous studies showed that *A. stylosum* extract is capable of inhibiting AChE enzyme (Emir & Emir, 2021), while the extract from *A. cepa* is very active in improving memory and learning via AChE inhibition and antioxidant activity in the mice brain (Kaur et al., 2020). Moreover, a recent study by Rocchetti et al. (2022) highlighted that among 9 *Allium* species studied, *A. cappadocicum* bulbs showed the highest inhibition against acetylcholinesterase. In this context, we aimed to assess whether *A. flavum* extracts can also inhibit AChE. To our knowledge, this is the first study assessing the AChE inhibitory activities of extracts from *A. flavum.* Modified Ellman's method was performed for AChE inhibitory activities of samples (data not shown). However, the results showed no detectable AChE inhibitory activity exerted by *A. flavum* extracts. This can be caused, in part, by the polarity of the compounds extracted. Further studies are needed to reveal whether non-polar extracts from *A. flavum* can inhibit AChE.

Tyrosinase is a metalloprotein and it is classified among the type 3 copper enzyme family. Its basic biological mechanism is based on the hydroxylation of monophenols to *o*-diphenols (monophenolase or cresolase activity) together with the oxidation of o-diphenols to *o*-quinones (diphenolase or catecholase activity). Besides, the polymerization of these products leads to melanin formation (Nokinsee et al., 2015). The inhibition of tyrosinase represents an efficient strategy for decreasing melanogenesis and skin hyperpigmentation (Ferro et al., 2018). A study by Jeong et al. (2017) showed that fermented onions extract effectively suppressed melanin production by inhibiting tyrosinase expression in B16F10 melanoma cells (Jeong et al., 2017). Moreover, a compound (quercetin 4′-*O*-β-D-glucopyranoside) extracted from the skins of red onion showed remarkable tyrosinase inhibitory activity in a study by Arung et al. (2011). In this context, we aimed to assess the anti-tyrosinase potential of *A. flavum* extracts. However, no tyrosinase inhibitory activity could be detected using the mushroom tyrosinase assay previously described (data not shown).

As far as the α-glucosidase inhibitors are concerned, they are well-known oral anti-diabetic agents, preventing carbohydrate digestion and then reducing the impact of carbohydrate hydrolysis on blood sugar. In a recent study, Masood et al. (2023) reported that the ethanolic extract of onion peel inhibited the α-glucosidase to a higher degree than the ethanolic bulb extract. Additionally, a study by Kim et al. (2010) reported that the α-glucosidase inhibitory activity of the onion extracts was correlated to the polyphenolic profile and antioxidant activity of the extracts tested. In this study, we tested whether the *A. flavum* extracts follow the pattern found in similar studies employing species from the same genus. The results are summarized in Table 2. The extract obtained from flowers of *A. flavum* species collected from Lăpușești exerted the most potent inhibitory activity against α-glucosidase (Table 2 and SM1).

Additionally, in this work we evaluated an enzyme widely associated to obesity issues, namely pancreatic lipase. Obesity represents a worldwide major health issue, caused by the imbalance between energy intake and expenditure. Accordingly, among the known therapies against obesity, inhibiting pancreatic lipase is one of the main studied mechanisms. Therefore, the inhibition of lipase into the digestive system is considered an effective way to prevent the progress of obesity (Rusu et al., 2020). Few studies concerning *Allium* species' inhibitory potential on pancreatic lipase were published recently. Among them, a recent study reported that Tropea red onion (*A. cepa* var. Tropea) has significant inhibitory activity *in vitro* against pancreatic lipase in a dose-dependent manner. Besides this finding, Wang et al. (2019) reported that the aqueous and methanolic extracts of *A. mongolicum* exerted an activity in inhibiting lipase of 179.48 mg/mL and 275.57 mg/mL, respectively. In this study, we aimed to test the inhibition potential of *A. flavum* against pancreatic lipase, employing the aforementioned assay. Noteworthy, the extract from the flowers of *A. flavum* collected from Făget, which previously showed the highest content in total phenolic compounds, was the only extract with a relatively good inhibition capacity against pancreatic lipase (IC_{50} = 4904.52 μg/mL, Table 2, SM1). This finding demonstrates that the phenolic compounds found in *A. flavum* are good candidates for lipase inhibition agents.

3.4. Pearson's correlations

Pearson's correlation coefficients (r) were then checked to discover those polyphenols better correlating with the bioactivities measured. Generally, the correlogram obtained (Fig. 3) showed that flavones, flavan-3-ols, flavonols, phenolic acids, and other phenolic compounds exhibited a greater number (n = 4) of significant correlations ($p < 0.05$, SM1) with the bioactivities examined. Interestingly, the carotenoid content of this specific *Allium* species was correlated with lipase inhibitory activity. This result is confirmed by a study by Matsumoto et al. (2010), demonstrating that marine carotenoids can inhibit triglyceride absorption in lymph duct-cannulated rats. Remarkably, although the carotenoid content did not strongly correlate with antioxidant activity measured by conventional *in vitro* antioxidant assays (DPPH, FRAP, and TEAC), it was correlated with lipid peroxidation inhibitory activity (TBARS) as well with oxidative hemolysis inhibitory activity (OxHLIA). Moreover, the lipase inhibitory activity was also correlated with content in organosulphur compounds, a class specific to *Allium* species. This finding confirms the results of a previous study which hypothesized and demonstrated *in silico* that organosulfur compounds from *Allium* species have lipase inhibitory potential (Nickavar, 2022).

4. Conclusions

A. flavum is one of the wild-growing species of *Allium* genus, which is not yet comprehensively studied for its health benefits. The current study aimed to study the phytochemical characteristics and biological effects exerted by hydroalcoholic extracts of *A. flavum* flowers and stems collected from three different areas in Romania. The extracts showed high antioxidant activity, correlated with the content of phenolics and flavonoids. Besides, extracts showed good inhibitory activity against α-glucosidase, while only one showed modest lipase inhibition. The extracts studied did not exert any AChE or tyrosinase inhibitory activity. The AFFF extract had the highest polyphenol content and showed the strongest antioxidant capacity in various *in vitro* assays (DPPH, FRAP, TEAC) and cell-based assays (OxHLIA, TBARS). All extracts demonstrated potential α-glucosidase inhibition, but only the AFFF extract showed anti-lipase inhibitory activity. Metabolomics analysis revealed that flavonoids (including dihydrochalcones, flavanones, flavones, isoflavonoids, flavan-3-ols, and flavonols), phenolic acids (such as hydroxycinnamics, hydroxybenzoics, hydroxyphenylacetics, and hydroxyphenylpropanoics), other phenolics (including lower molecular weight compounds), carotenoids, and organosulfur compounds (consisting of 37 compounds, such as alliin derivatives and disulfides) represent the most abundant compounds in analyzed extracts, highlighting their potential to represent a novel source of nutraceuticals.

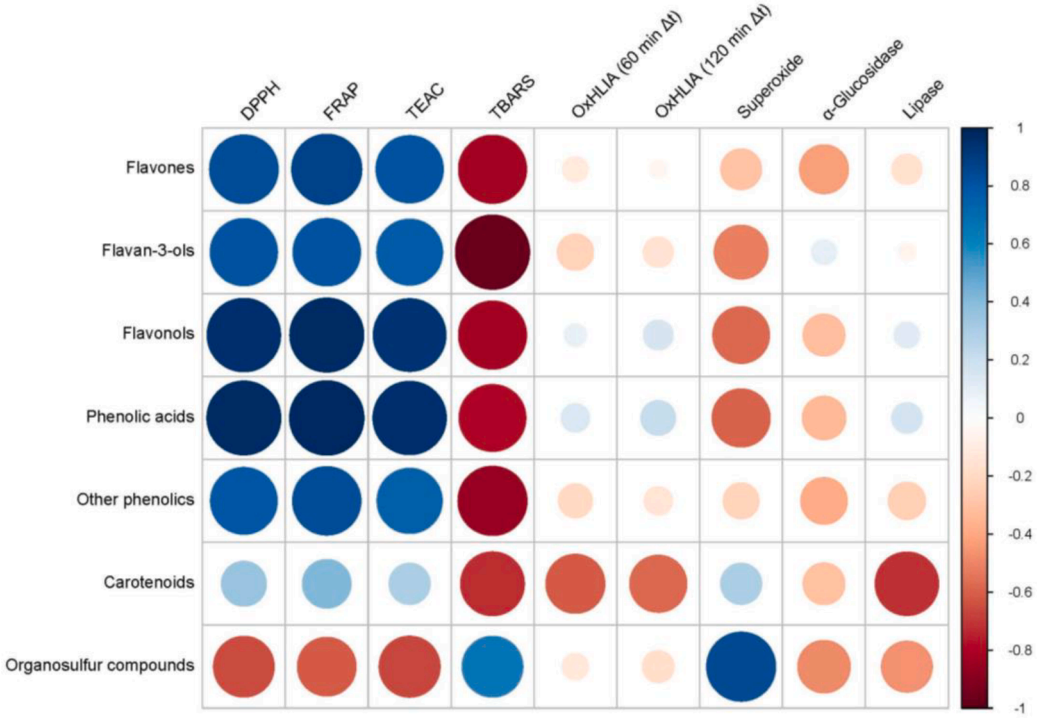

Fig. 3. Correlation diagram considering the annotated phenolic classes and the corresponding bioactivity values.

CRediT authorship contribution statement

Cadmiel Moldovan: Conceptualization, Methodology, Validation, Formal analysis, Data curation, Writing – original draft, Writing – review & editing. **Oleg Frumuzachi:** Methodology, Validation, Formal analysis, Data curation, Writing – original draft, Writing – review & editing. **Mihai Babotă:** Formal analysis, Data curation, Writing – original draft, Writing – review & editing. **José Pinela:** Methodology, Validation, Formal analysis, Writing – review & editing. **Lillian Barros:** Methodology, Validation, Supervision, Resources, Writing – review & editing. **Gabriele Rocchetti:** Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Víctor López:** Supervision, Writing – review & editing. **Luigi Lucini:** Supervision, Writing – review & editing. **Gianina Crișan:** Supervision, Writing – review & editing. **Andrei Mocan:** Conceptualization, Methodology, Validation, Supervision, Resources, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data (The excel file contains 1) Annotated and structurally confirmed phytochemical compounds; 2) Total polyphenolic and flavonoid contents of *A. flavum* extracts; 3) Superoxide radical scavenging (**A**), α-glucosidase (**B**), and pancreatic lipase (**C**) inhibition activity exerted by different *Allium flavum* extracts; 4) Pearson's correlation coefficients related to Figure 3.) to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.136503.

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