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Bioactivity and phenolics profile of aqueous and ethyl acetate extracts of Satureja kitaibelii Wierzb. ex Heuff

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Article

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Abstract

This study aimed at investigating biological activity and chemical composition of Satureja kitaibelii Wierzb. ex Heuff. LC-PDA/MS analyses for the aqueous (A1-ste, leaves and flowers, A2-leves and flowers) and ethyl-acetate (E1-stem, leaves and flowers, E2-leaves and flowers) extracts obrtained by ultrasound-assisted extraction allowed the identification of thirty four compounds. Quantitative analysis revealed that the aqueous extract obtained from leaves and flowers was the richest in total phenolic acids (65.36 mg/g) and flavonoids (21.17 mg/g). Total polyphenol content was the highest in the aqueous extract obtained from leaves and flowers (27±2.4 mg Gallic Acid equivalents /g). The best antioxidant activity was observed for the same extract using the DPPH (SC50 20±10 µg/mL), ABTS (2.834±0.02 mg Ascorbic Acid/g), FRAP (1.922±0.03 mmol Fe2+/mg), and total reducing power tests (16.4±1.0 mg Ascorbic Acid/g). Both ethyl acetate extracts were the most active against strains of Bacillus cereus and Micrococcus flavus (MIC 1.70-1.99 mg/mL and 1.99-3.41 mg/mL, respectively). They were more efficient against Aspergillus ochraceus (MFC 0.86 mg/mL) and towards HeLa cell lines. All obtained results implied the good potential of investigated extracts to be used as an effective preservatives and functional ingredients in food products and dietary supplements.

Introduction

Satureja kitaibelii Wiersb. ex Heuff. is an annual, aromatic species that grows in eastern Serbia on warm limestone rocks also referred to as *S. montana* subsp. *kitaibelii* (Wierzb ex Heuff.) P.W. Ball or *S. montana* var. *kitaibelii* (Wierzb. ex Heuff.) Nyman[1]. This is one of the most popular species in Serbian traditional herbal medicine usually consumed as "rtanj tea"[2], and for the treatment of respiratory diseases, urinary complaints and digestive disorders, and externally for skin and mucous inflammation and as an aphrodisiac[3–5]. Essential oil analysis of *S. kitaibelii* and its antimicrobial activity was well documented[6–12], while data on the composition and bioactivity of *S. kitaibelii* are scarce. It was confirmed that aqueous-alcohol and ethanol extracts were rich in phenolic compounds and exhibited good antioxidant activity[13–15]. Methanol extract exhibited modest antimicrobial activity and antitumor activity against malignant cells[3].

Until now, there are no data about the chemical composition and biological activities of aqueous and ethyl acetate extracts of *S. kitaibelii*. The aim of the present study was to evaluate these extracts as potential source of valuable phytochemicals and beneficial activity, which might promote *S. kitaibelii* as a functional food ingredient or food additive. In that regard, we examined chemical composition, antioxidative, antimicrobial and cytotoxic activities of the ethyl acetate and aqueous extracts of the different aerial plant parts of *S. kitaibelii* using ultrasound assisted procedure.

Materials And Methods

Plant material, extraction and LC-PDA/MS analysis. Aerial parts of *Satureja kitaibelii* were collected in August 2015, on Rtanj Mountain N 43.752697 E 21.87479, situated in eastern Serbia. The herb was collected by botanist Slavica Grujic, following all the rules for collecting herbs (at that time, the Ministry of Environmental Protection did not issue permits for collecting this plant and this species was protected only a few years ago). The plant was identified by Slavica Grujić and a voucher specimen was deposited in the Herbarium of the Institute of Botany and Botanical Garden "Jevremovac", Faculty of Biology, University of Belgrade, Serbia (No. 17141, BEOU). Not all specimens of the species were collected but only individual specimens, in order to avoid the destruction of the species population. A total of 1 kg of fresh plant material was collected (300 g dried materials). The plant material was air-dried at room temperature in the shadow, deprived from wooden parts. The leaves and flower are separated from the stem. The air-dried material (10 g), containing stem, leaves and flowers (sample 1), or leaves and flowers (sample 2) was extracted with 100 mL distilled water or ethyl acetate (polarity of water and ethyl acetate are 1.000 and 0.228, respectively). Extraction procedure was described previously[15] and resulted in extracts E1 and E2 (obtained from samples 1 and 2, respectively, ethyl acetate), A1 and A2 (obtained from samples 1 and 2, respectively, aqueous). The obtained dried extracts were kept in the fridge at 4°C before use.

Qualitative and quantitative chromatographic analysis of the extracts was carried out exactly as it was described in Gopcevic et al[15]. The quantification of phenolic acids and flavonoids was performed by external calibration after regression and correlation analysis, based on the PDA chromatographic data. Rosmarinic acid and chlorogenic acid were used as standard compounds for the determination of phenolic acids, luteolin 7-*O*-glucoside for flavone glycosides, whereas flavone aglycons were quantified using

luteolin as the standard compound. All standard compounds used in LC-PDA/MS analysis of extracts were purchased from Carl Roth, except luteolin 7-*O*-diglucuronide which was previously isolated from the aerial parts of *Thymus pannonicus*[16].

The results of quantitative analysis are expressed as mean from three independent analysis ± standard deviation. Data on the measuring conditions and obtained calibration curves, as well as on the limits of detection (LOD) and quantitation (LOQ) are presented **in Supplementary Table S1.**

Supplementary Table S1. Calibration equations, correlation factors (R^2), limits of detection (LOD) and limits of quantification (LOQ) values for chromatographic analysis of standard compounds.

Total Phenolic Content. The total phenolic content (TPC) was determined using FC reagent[17], spectrophotometrically, and expressed as mg of gallic acid equivalents (GAE) per g of extract.

Antioxidant Activity. Four different methods were used to evaluate antioxidant activity. The ferric-reducing antioxidant potential method (FRAP), with reading at 593 nm, according to[18] and results expressed as μmol Fe^{2+} equivalents per g of an extract. Scavenging effect on 1, 1-diphenyl-2-picrylhydrazil free radical (DPPH) determined according to[19] was measured at 517 nm the results were presented as SC_{50} values (μg/mL). The ABTS method was performed according to[20]. The scavenge activity results were presented as equivalents of ascorbic acid (AA) per g of an extract. The total reducing capacity (TRC) of extracts was evaluated through the development of Perl's Prussian blue color according to[21]. The results were expressed as mg AA/g. All absorbance readings were performed on UV-2600 UV-VIS spectrophotometer (Shimadzu, Japan). All analyses were done in triplicate.

Antimicrobial activity. The antibacterial and antifungal activity was evaluated using a microdilution method as previously described in[22, 23] towards eight bacteria and eight fungi (**in Supplementary Table S2.**).

Supplementary Table S2. Bacterial and fungal strains used for the antimicrobial effects examination of aqueous and ethyl acetate extracts of *Satureja kitaibelii.*

Streptomycin and ampicillin were used as positive controls for antibacterial, while bifonazole and ketoconazole were used for antifungal activity.

Cytotoxic activity. For the *in vitro* evaluation of the cytotoxic activity of tested extracts, we used: normal human embryonic lung fibroblast (MRC-5), human cervical adenocarcinoma (HeLa), human breast adenocarcinoma (MCF-7) and human prostate adenocarcinoma from bone metastasis (PC-3) cell lines obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). For the evaluation of cytotoxicity of tested extracts we used the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide - MTT), described previously in[24]. Each experiment with every cell line was performed in triplicate.

Statistical Analysis. All the results are presented as mean ± standard deviations of three determinations. Statistical analyses were performed using the Student's t-test and one-way analysis of variance. Correlation between tested activities of various extracts was established using regression analysis at a 95% significance level ($p \leq 0.05$). IC_{50} values were calculated by nonlinear regression analysis from the sigmoid dose-response inhibition curves. Statistica 6.0 software (2001, Stat Soft, Tulsa, OK, USA) was used to evaluate the $p < 0.05$ level (regarded as significant) and $p < 0.01$ (very significant).

All methods were performed in accordance with the relevant guidelines and regulations.

Results And Discussion

Phytochemical analysis

LC-PDA/MS analysis of aqueous and ethyl acetate extracts of stem, leaves and flowers (A1 and E1, respectively) and leaves and flowers (A2 and E2, respectively) of *S. kitaibelii* revealed the presence of phenolic acids and flavonoids, as well as jasmonic acid derivatives and a diterpene. The obtained chromatographic and spectral data, *i.e.* retention times (R_t) on UV (350 nm) and MS chromatograms, λ_{max} (nm), ESI-MS data (*m/z*), peak assignments, as well as the occurrence and content of the compounds in the extracts, are presented in **Table 1** and chromatograms of the extracts are shown on **Supplementary Figure 1.**

Table 1. Results of qualitative and quantitative LC-PDA/MS analysis of aqueous and ethyl acetate extracts of stems, leaves and flowers (A1 and E1, respectively) and leaves and flowers (A2 and E2, respectively) of *S. kitaibelii*

Supplementary Figure 1. Chromatograms of aqueous and ethyl acetate extracts of stems, leaves and flowers (A1 and E1, respectively) and leaves and flowers (A2 and E2, respectively) of *S. kitaibelii* recorded at 350 nm. The numbering of the constituents corresponds to those in **Table 1.**

Qualitative composition of the extracts

Fourteen of 34 identified compounds belonged to phenolic acids, all being derivatives of hydroxycinnamic acid (HCA), including simple acids, depsides of quinic acid and, especially, HCA oligomers. Among the latest the most abundant were dimeric rosmarinic acid (**21**) and hexameric clinopodic acid O (**25**) (**Supplementary Table 1**). In addition to these two constituents, two trimers (**7** and **19**), two tetramers (**22** and **24**), as well as two more hexamers of HCA (**27** and **30**), were detected in the extracts. The majority (14) of eighteen identified flavonoids, were flavones, *i.e.* aglycons luteolin (**29**), apigenin (**32**), and genkwanin (**34**), as well as glycosides of luteolin (**5**, **8**, **10**, **11**, **13**, and **16**) and apigenin (**6** and **14**) and their methylated derivatives (**17**, **23**, and **26**) (**Table 1**). The MS data obtained in the analysis of extracts and available standards, indicated that the sugar moieties of glycosides were either monosaccharide (hexose or hexuronic acid) or disaccharide units (composed of two hexuronic acids or deoxyhexose and hexose). In addition, among luteolin glycosides, three were identified as those acylated with HCAs (**10**, **11**, **16**) based on their UV and MS spectral characteristics[25-27]. In the current study we detected the presence of one flavonol glycoside (**9**), two flavanone aglycons (**28**, **31**), two hexosyl jasmonic acid derivatives (**2**, **20**) and one diterpene (**33**) in the *S. kitaibelii* extracts, as well.

Figure 1. shows the distribution of the identified constituents between the extracts. Nine compounds were common to all extracts: 12-hydroxyjasmonic acid 12-O-hexoside (**2**), caffeic acid (**3**), luteolin 7-*O*-diglucuronide (**5**), salvianolic acid K/isomer (**7**), luteolin 7-*O*-glucuronide (**13**), rosmarinic acid (**21**), Me-apigenin deoxyhexosyl-hexoside (**23**), clinopodic acid *O* (**25**) and genkwanin (**34**). By sharing 18 common compounds, ethyl acetate extracts E1 and E2 were the most similar regarding qualitative composition compared to other extracts, emphasizing the selectivity of the extracting solvent, while aqueous extracts, shared 13 compounds.

Quantitative composition of the extracts

Quantitative chromatographic analysis revealed that extract A2 was the richest among the assayed extracts regarding the content of two major groups of metabolites, *i.e.* phenolic acids and flavonoids (**Table 1., Supplementary Figure 1.**), while other extracts contained substantially lower amounts of these specialized metabolites. The lowest amount of phenolic acids was observed in extract A1, with HCA oligomers present only in trace amounts, while E2 was the poorest regarding flavonoids.

In the extract A2, the most abundant compounds were clinopodic acid O, rosmarinic acid and luteolin 7-*O*-diglucuronide (35.627 mg/g, 15.046 mg/g and 7.955 mg/g, respectively), with the amounts which were at the same time the highest in comparison to their concentrations in the other extracts. Rosmarinic acid and clinopodic acid O, together with Me-apigenin deoxyhexosyl-hexoside, were the dominant constituents of the extract E2 (5.719 mg/g, 1.164 mg/g, and 1.258 mg/g, respectively). In the extract A1 luteolin 7-*O*-diglucuronide, *p*-coumaric acid, and luteolin 7-*O*-glucuronide dominated over other compounds (2.569 mg/g, 1.223 mg/g and 0.778 mg/g, respectively), while E1 contained 3,5-dicaffeoylquinic acid, rosmarinic acid, and chlorogenic acid as the major constituents (1.822 mg/g, 1.755 mg/g and 1.317 mg/g, respectively). Moreover, the abundance of every single caffeoylquinic acid was the highest in the extract E1.

TPC and Antioxidant activity

Differences between extracts in their total phenol content are noticed due to different polarity of extracting solvents and using different plant parts and results are summarized in **Table 2**.

Table 2 Total phenols contents of aqueous and ethyl acetate extracts of stems, leaves and flowers (A1 and E1, respectively) and leaves and flowers (A2 and E2, respectively) of *S. kitaibelii* and comparison with relevant studies

The TPC has been determined in aqueous and ethyl acetate extracts of *S. kitaibelii* for the first time in this work. The highest content of total phenols was observed in A2, i.e. aqueous extract obtained from leaves and flowers (274 mg GA/g). The TPC was higher in the both aqueous extracts (164 and 274 mg GA/g) compared to the TPC of both ethyl acetate extracts (44.5 and 51.5 mg GA/g).

Free radical scavenging capacity and reducing power of the tested extracts was measured by DPPH, ABTS, FRAP and TRP assays and results are shown in Table 3.

Table 3 Free radical scavenging activity and reducing power of aqueous and ethyl acetate extracts of stem, leaves and flowers (A1 and E1, respectively) and leaves and flowers (A2 and E2, respectively) of *S. kitaibelii.* BHA and L-ascorbic acid were used as standard antioxidants.

Antimicrobial activity of extracts of S. kitaibelii

Results of antimicrobial activity of investigated extracts, presented as a minimal inhibitory concentration, for both bacteria and fungi, are shown in Figure 2 a, b, c and d.

MIC values for bacteria varied from 1.70 to 22.73 mg/mL, while MBC ranged from 3.41 to 45.45 mg/mL (data not shown). Ethyl-acetate and aqueous extract obtained from stem, leaves and flowers (A1 and E1) were more effective against the majority of tested bacteria than extracts made of leaves and flowers (A2 and E2). The ethyl acetate extracts (E1 and E2) exhibited lower MICs compared to the aqueous indicating that antibacterial properties of extracts also depended on solvent polarity which was also confirmed by[13]. Particularly sensitive to ethyl acetate extracts E1 and E2 were *B. cereus* (MIC 1.70 and 1.99 mg/mL, respectively) and *M. flavus* (MIC 3.41 and 1.99 mg/mL, respectively) among G^+ positive bacteria. The most affected among tested G^- bacteria were *E. cloacae* by ethyl acetate extract of aerial parts (E1) and *E. coli* by ethyl acetate extract of leaves and flowers (E2). However, *E. coli* was the most resistant on the remaining examined extracts (A1, A2 and E1).

Cytotoxic activity of S. kitaibelii extracts

The *in vitro* cytotoxic activity of investigated extracts is presented as IC_{50} values in Table 4.

Table 4 Cytotoxic a ctivity (IC_{50} values) of aqueous and ethyl acetate extracts of stems, leaves and flowers (A1 and E1, respectively) and leaves and flowers (A2 and E2, respectively) of *S. kitaibelii* against human cancer cell lines.

The obtained data suggest that extracts of *S. kitaibelii* showed tumor-selective cytotoxic activity depending on the cell line type and extracting agent. Both ethyl acetate extracts showed stronger cytotoxic effects towards HeLa, PC-3 and MCF-7 cancer cell lines, as well as towards non-malignant cell line, compared to aqueous extracts. HeLa cell line was the most sensitive in the presence of ethyl acetate extracts, PC-3 and healthy MRC-5 cells were moderately sensitive, whereas quite poor effect was observed towards MCF-7 cell line. Aqueous extracts A1 and A2 exhibited low cytotoxic activity only towards MCF-7 cell line (**Table 4**).

Discussion

In the current study, phytochemical analysis by LC-PDA/MS was performed in order to investigate the bioactive compounds present in aqueous and ethyl acetate extracts of *Satureja kitaibelii*. LC/PDA-MS analysis revealed presence of numerous compounds some of which identified for the first time. Derivatives of HCA are common for plants belonging to the family Lamiaceae. Rosmarinic acid, is often one of the major polyphenol constituents in aerial parts polar extracts of these plants, including plants of the genus *Satureja*[28-32]. Besides rosmarinic acid, Lamiaceae species produce higher HCA oligomers which contain up to 8 monomer units[33,27]. In several studies trimeric[27,34] and tetrameric oligomers[35-37] were detected in the leaf or herb extracts of *Satureja* species, including previously analyzed ethanol extracts of *S. kitaibelii*[15]. On the other hand, to the best of our knowledge, clinopodic acids O and K, *i.e.* HCA hexamers, were reported only in the extracts of *S. biflora*, *S. hortensis* L. and previously analyzed ethanol extracts of *S. kitaibelii*[27,15,38], in addition to a few other Lamiaceae species[39,40]. In previously analyzed polar extracts of savory species, in addition to various flavone glycosides, a limited number of flavonol and flavanone glycosides was found which is in accordance with the currently analyzed aqueous extracts of *S. kitaibelii*. The presence of mono- and diglucuronides, glucosides, diglucosides and rutinosides of luteolin, apigenin, and their hydroxylated and methylated derivatives was confirmed in a number of studies[37, 42, 43, 34]. On the other hand, to the best of our knowledge, the presence of acylated glycosides, such as those detected in the present work, was rarely reported in plants of the genus *Satureja*[27]. The extraction conditions, as well as species specific composition, might influence such behavior.

Several more observations could be made on the distribution of compounds in respect to the starting plant material and solvent used for the preparation of the extracts. Except for luteolin glucuronides **5** and **13**, that were detected in all extracts (although in ethyl acetate extracts in low amounts), other detected flavonoid hexuronides (**6**, **10**, **11**, **16**) were found exclusively in aqueous extracts, which corresponds to their high hydrophilicity. In that regard, rosmanol/isomer (**33**) and all flavonoid aglycons (**28**, **29**, **31**, **32** and **34**), *i.e.* compounds of lower hydrophilicity, were common for both ethyl acetate extracts regardless of the starting plant material. It is also worthy of noting that, except clinopodic acid *O* that was present in all extracts, all four other HCA tetramers and hexamers were detected only in extracts A2 and E2. On the other hand, caffeoylquinic acids were detected in A1, E1, or both. Our results indicate that HCA oligomers are dominantly located in the leaves and flowers of *S. kitaibelii*, whereas caffeoylquinic acids are distributed mainly in the stems. However, especially high content of rosmarinic acid was previously determined in flowers of *Glechoma hederacea* L. (Lamiaceae) in comparison to leaves and stems. At the same time, the highest amount of chlorogenic acid was found in the leaves of this herb (even though its distribution varied very slightly)[44]. All this imply that the proportion of leaves, flowers and stems in the starting material played an important role in the process of extraction in the current study.

Our results indicate that the composition of extracts is highly influenced by both, starting plant material and extracting agent. This is to some extent in contrast with the previous findings that the contents of rosmarinic acid and clinopodic acid O in the ethanol extracts of leaves+flowers or stems+leaves+flowers of *S. kitaibelii* were much more dependent on the application of ultrasound during the extraction than on the starting plant material[15]. However, the solubility and extractability of metabolites present in *S. kitaibelii* in ethanol, which was used in the previous study, and water and ethyl acetate are much different and thus substantially control the process of extraction.

Our measured TPC values (from both aqueous and ethyl acetate extracts) were comparable with some previously obtained[14,15,45] (Table 2), but higher than the values for EtOAc and n-ButOH extracts of aerial parts in[13].

The results of both assays for free radical scavenging ability for the investigated extracts of *S. kitaibelii* clearly indicate the influence of used solvents on this activity. Namely, aqueous extracts showed higher antioxidant activity than ethyl acetate ones, which is well correlated with determined TPC. The difference in the activity of the aqueous and ethyl acetate extracts is a consequence of the different composition of obtained extracts in terms of the components that are extracted depending on the different properties of the extractants (Table 1). The aqueous extracts, especially from leaves and flowers (A2), were the most powerful in the neutralization of DPPH free radicals (IC_{50} 20 μg/mL), which is in accordance with the most favorable composition of this extract in comparison to other investigated extracts in respect to radical scavenging features of the present compounds. Ethyl acetate extracts of *S. kitaibelii* expressed the lower values as compared to aqueous (IC_{50} 280-320 μg/mL). Those data are in agreement with the reported results of[46], where *S. kitabelii* hot and cold aqueous extracts showed IC_{50} between 9.75-27.92 μg/mL.

Lopez-Cobo et al.[14] presented results of DPPH activity of 116.36 μg/mL for 70% methanol extract. Ethanol extracts of *S. kitaibelii* aerial parts showed higher SC_{50} values - 102.24 and 106.94 μg/mL[15]. Aqueous extracts obtained with ultrasonic extraction with gradual increase in temperature show IC_{50}value 0.087 mg/mL[45]. The scavenging effect of both aqueous extracts of *S. kitaibelii* on the ABTS was also more pronounced comparing to the ethyl acetate. Aqueous extracts from leaves and flowers showed the strongest free ABTS radicals scavenging ability (2.834 mg AA/g), these values are comparable to those obtained by[45]. According to our knowledge, there is no comparable data on this activity of *S. kitaibelii* extracts.

Likewise, aqueous extracts shown better results on Fe^{3+} reducing activity, measured by the FRAP assay but they are significantly lower than values obtained in[46]: 221.74 and 271.88 μmol Fe/g). The highest reducing capacity showed aqueous extract of leaf and flower (16.4 mg AA/g). TRP assay confirmed results obtained from FRAP. There are no literature data to comparing these values.

In the examined extracts, HCA oligomers were identified in considerable amounts, especially rosmarinic acid. Rosmarinic acid shows a good antioxidant activity: value obtained from FRAP was 16.54 mmol Fe^{2+}/g and from DPPH assay SC_{50} 3.90 μg/mL[16].

In a limited number of studies the activity of HCA oligomers was examined mainly *in vitro*, and less frequently *in vivo*. Good antioxidant activity was found for several higher oligomers[47, 48], as well as the ability to inhibit hyaluronidase[49, 50], the ability to inhibit metalloproteinase[51], the ability to inhibit lipoxygenase[48], antimicrobial activity[52] etc. Antioxidant activity of another phenolic acid present especially in A2, caffeic acid (CA) could be related to its iron-chelating property through formation of iron-CA complexes

inhibiting Fenton-induced oxidative damage by preventing the formation of free hydroxyl radicals[53]. Furthermore, good antioxidant activity was confirmed for majority of other constituents. For example, chlorogenic acid (CGA) the most abundant isomer among caffeoylquinic acid in E1 extract of *S. kitaibelii*, is an important and biologically active polyphenol, playing several important and therapeutic roles such as free radicals scavenger, antioxidant activity, anti-microbial, and many others[54-57]. Flavonoid luteolin and its glycosides possess a variety of pharmacological activities, including anti-oxidant, anti-inflammatory, antimicrobial and anticancer activities[58, 59].

Although the antimicrobial activity of essential oil of different *Satureja* species is well-known, the reports about the antimicrobial activity of extracts are limited. The antibacterial effects of *S. kitaibelii* petroleum ether, chloroform, ethyl acetate and *n*-butanol extracts were examined and obtained MIC values which ranged from 10 to >100 mg/mL[13].

The effects of *S. kitaibelii* methanol extract examined in[3] and found significantly lower MICs (0.32 to 1.25 mg/mL) and MBCs (2.50 to 5.00 mg/mL) than in the current study. The antibacterial activity of subcritical water extract of *S. kitaibeli* was estimated in[60] and obtained MIC values that ranged from 1.04 to >33.3 mg/mL. Unlike in[60], the results presented herein indicated no selectivity of *S. kitaibelii* extracts among G^- and G^+ bacteria which is congruent with the results of other researchers[3]. The MICs for fungi were from 0.86 to 16.06 mg/mL, while MFCs were between 1.72 and 32.12 mg/mL (data not shown). Similar to antibacterial activity, ethyl acetate extract of all aerial parts (E1) was more efficient in preventing fungal growth, especially against *A. ochraceus* (MIC/MFC 0.86/1.72 mg/mL). Unlike antibacterial activity, the aqueous extracts were more efficient in inhibiting fungal growth, particularly *A. versicolor*, *A. niger* and *P. verrucosum* var *cyclopium*. The least potent antifungal agent was the ethyl acetate extract of leaves and flowers (E2), especially on *T. viride* (MFC/MIC 32.12/16.06 mg/mL). The antifungal properties of those *S. kitaibelii* extracts were not examined so far. However, *S. kitaibelii* methanol extract inhibited the growth of 6 micromycetes (MIC varied from 0.16 to 1.25 mg/mL) and two *Candida* species (MIC varied from 0.32 to 0.62 mg/mL)[3]. Anticandidial activity of *S. kitaibelii* aqueous extract was later confirmed in[60]. Moreover, *S. cerevisiae* and *Aspergillus brasiliensis* were also sensitive to *S. kitaibelii* aqueous extract[60].

The antimicrobial properties of *S. kitaibelii* methanol extract were associated to the presence of rosmarinic acid in[3], which is contradictory to our results since its content was the highest in the low active aqueous extract of aerial parts as well as to previously report modest antimicrobial activity of this compound[16]. On the other hand, it was suggested in[13] that other non-phenolic compounds could contribute to the antimicrobial activity of *S. kitaibelii* extracts.

Our results indicate that examined *S. kitaibelii* extracts are rich in phenolics which particular influence to antimicrobial response of extracts should be more studied, as well as synergistic effects among them. Thus the presented results could contribute in further examination of antimicrobial activities of *S. kitaibelii* extracts, particularly because they were insufficiently studied so far.

In our study both types of extracts contained phenolic compounds which previously showed cytotoxic activity in different assays. Several studies have shown that extracts rich in certain phenolic carboxylic acids and flavonoids exhibit cytotoxic activity against the different cell lines both malignant and healthy, by various mechanisms[37, 61-64, 53]. In the study[64] it has been revealed the potential molecular mechanisms of phenolic acids and flavonoids and the great promising effects of phenolic acids and flavonoids against breast, colon, lung and prostate cancers. On the other hand, extracts investigated in the present study were all abundant with phenolic compounds, but sensitivity of the tested cell lines (except MCF-7) differed markedly depending on the extracting agent used for extract preparation. What is more, the extract which was the most abundant with phenolic compounds (A2) wasn't the most active. The reason for this might be in some differences in the composition which could not be detected with the applied methods, and that non-phenolic constituents are responsible for good cytotoxic activity of ethyl acetate extracts. Furthermore, polarity of compounds present in ethyl acetate extracts might be beneficial for better availability for the tested cell lines. In congruence with our results, less polar extracts, especially ethyl acetate extract, of *Origanum majorana* showed more pronounced cytotoxicity towards MDA-MB-231 and HT-29 cell lines (IC_{50} 30.90±1.39 and 50.11±1.44 µg/ml) in comparison to aqueous extract (IC_{50} 69.18±3.10 and 177.82±4.07 µg/ml)[65].

In accordance with our results on the cytotoxicity of ethyl acetate extracts, in a previous study, methanol extract of *S. kitaibellii* exhibited moderate activity against estrogen-dependant (MDA-MB-361) and estrogen-nondependant (MDA-MB-453) breast cancer, colon cancer (LS174), and HeLa cell lines, as well as strong cytotoxic activity against human malignant melanoma cells (Fem-x), which was partly correlated with tested activity of rosmarinic acid[3]. On the other hand, low cytotoxic potential of aqueous extracts of

related species, *S. subspicata* and *S. horvatii*, was noted previously in human lymphocytes *in vitro*[31], as well as of *S. montana* aqueous and aqueous ethanol extracts with IC_{50} values above 600 μg/ml against Caco-2, TR146 and HeLa cell lines[36]. Moreover, aqueous and DMSO extracts of *S. subspicata* and *S. horvatii* downregulated pro-apoptotic and upregulated anti-apoptotic genes of the Bcl-2, showing anti-apoptotic activity, which authors linked with previously shown similar effects of rosmarinic acid. Similar anti-apoptotic effects, i.e. increased Bcl-2/Bax ratio, was observed for methanol extract of *S. hortensis*, which was characterized by high amount of rosmarinic acis[66]. These data, together with previously confirmed low genotoxic potential of aqueous extracts of *Satureja* plants and rosmarinic acid as their dominant compound[31], fit well with safe usage of *S. kitaibelii* aqueous extracts, but this certainly needs to be better justified in future.

Conclusion

The results of the current study highlighted the aqueous and ethyl acetate extracts of different aerial parts of *Satureja kitaibelii* Wierzb. ex Heuff. endemic plant to be a reach source of active compounds some of which are extracted and detected for the first time. Aqueous extract of leaves and flowers was the richest source of two major groups of metabolities, i.e. phenolic acids and flavonoids. Dominant constituents, which could be considered as chemical markers, were hydroxycinnamic acid oligomers, rosmarinic acid and clinopodic acid O. All examined extracts possessed considerable antioxidant activity, which was confirmed by four different assays. Regarding antibacterial activity, the best results showed both ethyl acetate extracts towards *Bacillus cereus* and *Micrococcus flavus*, whereas in case of other tested bacteria all investigated extracts exhibited moderate to weak activity. In the presence of ethyl acetate extract of all aerial parts the most sensitive fungus was *Aspergillus ochraceus*, while all other fungal strains were moderately susceptible to all tested extracts. Ethyl acetate extract were cytotoxic against all tested cell lines, including healthy ones. On the other hand, aqueous extracts exhibited weak activity only towards MCF-7 cells. All obtained results implied the good potential of investigated, especially aqueous, extracts of *S. kitaibelii* to be used as a source of new pharmaceuticals and healthcare products as well as an effective preservatives and functional ingredients in food products, dietary supplements and other pharmaceutical products.

Declarations

Data Availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Author contributions

KG: designed research work, interpreted and analyzed the data, reviewed, edited the manuscript draw the figures/tables. SG: conducted experiments on antioxidant activity, wrote the paper, reviewed and edited the manuscript. JA: conducted experiments on qualitative and quantitative chromatographic analysis using liquid chromatography with UV/VIS and MS detection, wrote the paper, draw the tables/figures, reviewed the manuscript. ADž: conducted experiments on antioxidant activity. IV: conducted experiments on antimicrobial activity, wrote the tables and figures. LIŽ: prepared extracts, wrote the paper. AM: prepared extracts, wrote the paper, draw the figures/tables. JM: prepared extracts, conducted experiments on antioxidant activity. MS: conducted experiments on antimicrobial activity. AĐ: conducted experiments on cytotoxic activity, draw the tables. All authors approved the final version of the manuscript.

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Competing Interests

None

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Tables

Table 1. Results of qualitative and quantitative LC-PDA/MS analysis of aqueous and ethyl acetate extracts of stems, leaves and flowers (A1 and E1, respectively) and leaves and flowers (A2 and E2, respectively) of *S. kitaibelii*

Peak#	R_t (UV)	R_t (MS)	Assignement	Spectral data		Content (mg/g of dry extract)				Reference
				λ_{max} (nm)	ESI-MS data (m/z)	A1	A2	E1	E2	
Jasmonic acid derivatives										
2	9.92	10.01	12-Hydroxyjasmonic acid 12-O-hexoside	228	387.2 [M-H]⁻	d.n.d.	d.n.d.	d.n.d.	d.n.d.	Pacifico et al., 2015[L]; Moreira et al., 2020[S]
20	15.48	15.59	12-O-(Caffeoylhexosyl)-jasmonate	230, 294, 326	549.2 [M-H]⁻	n.d.	d n.d.	d.n.d.	d.n.d.	Pacifico et al., 2015[L];
Phenolic acids										
			Simple HCA							
3	11.02	11.17	Caffeic acid * [b]	296, 326	179.0 [M-H]⁻	tr.	2.354 ± 0.050	0.184 ± 0.007	0.453 ± 0.019	Moreira et al., 2020[S]; Tsimogiannis et al., 2017[S]
12	13.72	13.75	*p*-Coumaric acid * [b]	312	163.0 [M-H]⁻	**1.223 ± 0.078**	n.d.	n.d.	n.d.	Boroja et al., 2018[S]; Čakar et al., 2018[S]
			Caffeoylquinic acids							
1	9.49	9.61	Chlorogenic acid * [a]	298, 326	353.1 [M-H]⁻, 191.0	0.127 ± 0.025	n.d.	**1.317 ± 0.005**	n.d.	Boroja et al., 2018[S]; López-Cobo et al., 2015[S]
4	11.48	11.60	Cynarin * [a]	298, 324	515.1 [M-H]⁻, 353.1	n.d.	n.d.	0.535 ± 0.001	n.d.	López-Cobo et al., 2015[S]
15	14.38	14.50	Dicaffeoylquinic acid isomer [a]	n/a	515.1 [M-H]⁻, 353.1	n.d.	n.d.	0.682 ± 0.022	n.d.	López-Cobo et al., 2015[S]
18	15.16	15.27	3,5-Dicaffeoylquinic acid * [a]	296, 328	515.0 [M-H]⁻, 353.0	0.333 ± 0.360	n.d.	**1.822 ± 0.016**	n.d.	López-Cobo et al., 2015[S]
			HCA dimers							
21	15.90	16.01	Rosmarinic acid * [b]	294, 328	359.0 [M-H]⁻, 197.1, 161.0	tr..	**15.046 ± 2.545**	**1.755 ± 0.039**	**5.719 ± 1.045**	Damašius et al., 2014[S]; Moghadam et al., 2015[S]; Boroja et al., 2018[S]; Gomes et al., 2020[S]
			HCA trimers							
7	12.96	13.08	Salvianolic acid K/isomer [b]	284	555.1 [M-H]⁻, 357.0	tr.	0.210 ± 0.163	tr.	tr.	Celano et al., 2017[L]

Peak#	R_t (UV)	R_t (MS)	Assignement	Spectral data		Content (mg/g of dry extract)				Reference
				λ_{max} (nm)	ESI-MS data (m/z)	A1	A2	E1	E2	
19	15.24	15.35	Salvianolic acid A/isomer [b]	288, 330	493.1 [M-H]⁻, 331.1	tr.	2.305 ± 0.439	n.d.	n.d.	Tsimogiannis et al., 2017[S]; Moreira et al., 2020[S]; Gomes et al., 2020[S]
			HCA tetramers							
22	16.69	16.74	Clinopodic acid I [b]	286, 330	717.2 [M-H]⁻, 519.1, 357.0	n.d.	2.077 ± 0.809	n.d.	tr.	Moghadam et al., 2015[S]
24	17.01	17.12	Salvianoilc acid E/L isomer [b]	286, 330	717.1 [M-H]⁻, 519.1, 339.0	n.d.	2.886 ± 0.175	n.d.	tr.	Chen et al., 2011[L]; Celano et al., 2017[L]
			HCA hexamers							
25	17.79	17.90	Clinopodic acid O [b]	286, 330	1075.2 [M-H]⁻, 519.0, 339.0	tr.	**35.627 ± 2.533**	0.233 ± 0.007	**1.164 ± 0.205**	Moghadam et al., 2015[S]
27	18.44	18.55	Clinopodic acid K [b]	286, 330	1075.2 [M-H]⁻, 357.0	n.d.	3.137 ± 0.116	n.d.	tr.	Moghadam et al., 2015[S]
30	19.30	19.33	Hydroxycinnamic acid hexamer [b]	286, 330	1075.1 [M-H]⁻, 555.1, 519.1, 357.1	n.d.	1.721 ± 0.384	n.d.	tr.	Celano et al., 2017[L]; Pacifico et al., 2015[L]
Total phenolic acids						*1.68*	*65.36*	*6.53*	*7.34*	
Flavonoids										
			Flavonoid glycosides							
5	11.56	11.63	Luteolin 7-*O*-diglucuronide * [c]	254, 266, 348	637.1 [M-H]⁻, 351.0, 285.0	**2.569 ± 0.089**	**7.955 ± 0.699**	tr.	tr.	Gomes et al., 2020[S]; Marczak et al., 2010
6	12.77	12.91	Apigenin dihexuronide [b]	268, 334	621.1[M-H]⁻, 351.0, 269.0	0.091 ± 0.005	tr.	n.d.	n.d.	Marczak et al., 2010
8	13.18	13.25	Luteolin 7-*O*-rutinoside * [b]	254, 268, 348	593.2 [M-H]⁻, 285.0	tr.	tr.	0.048 ± 0.036	tr.	Tsimogiannis et al., 2017[S];
9	13.21	13.34	Isoquercitrin * [b]	256, 266, 354	463.1 [M-H]⁻	n.d.	n.d.	n.d.	0.252 ± 0.051	Boroja et al., 2018[S];

Peak#	R_t (UV)	R_t (MS)	Assignement	Spectral data		Content (mg/g of dry extract)				Reference
				λ_{max} (nm)	ESI-MS data (m/z)	A1	A2	E1	E2	
10	13.23	13.33	Luteolin caffeoyl-dihexuronide [b]	252, 270sh, 338	799.1 [M-H]⁻, 513.1, 285.0	0.674 ± 0.800	5.732 ± 0.401	n.d.	n.d.	Moghadam et al., 2015; Marczak et al., 2010; Stochmal and Oleszek, 2007
11	13.43	13.55	Luteolin sinapoyl-dihexuronide [b]	270, 336	843.2 [M-H]⁻, 557.2, 285.0	n.d.	2.312 ± 0.331	n.d.	n.d.	Marczak et al., 2010; Stochmal and Oleszek, 2007
13	13.95	14.06	Luteolin 7-*O*-glucuronide * [b]	254, 266, 348	461.0 [M-H]⁻, 285.0	**0.778 ± 0.087**	3.886 ± 0.607	0.053 ± 0.005	0.136 ± 0.051	Moghadam et al., 2015[S]; Celano et al., 2017[L]
14	14.25	14.35	Apigenin deoxyhexosyl-hexoside [b]	268, 332	577.2 [M-H]⁻, 269.0	n.d.	tr.	tr.	tr.	Hossain et al., 2010[L]
16	14.43	14.56	Luteolin *p*-coumaroyl-dihexuronide [b]	270, 326	783.1 [M-H]⁻, 285.0	0.229 ± 0.049	1.288 ± 0.100	n.d.	n.d.	Marczak et al., 2010; Stochmal and Oleszek, 2007
17	14.75	14.86	Diosmin * [b]	n/a	607.1 [M-H]⁻, 299.0, 284.0	tr.	n.d.	tr.	tr.	Celano et al., 2017[L]
23	16.77	16.90	Me-apigenin deoxyhexosyl-hexoside [b]	268, 332	591 [M-H]⁻, 283, 268	0.038 ± 0.007	2.384 ± 0.664	1.178 ± 0.123	**1.258 ± 0.370**	López-Cobo et al., 2015[S]; Šimunović et al., 2020[S]; Marin et al., 2001[S]
26	18.25	18.36	Me-apigenin hexoside [b]	332	445.0 [M-H]⁻, 283.0, 268.0	tr.	n.d.	n.d.	n.d.	Park et al., 2019[L]
			Flavonoid aglycons							
28	18.71	18.94	Eriodyctiol * [d]	288	287.1 [M-H]⁻	n.d.	n.d.	tr.	tr.	Tsimogiannis et al., 2017[S];
29	19.26	19.38	Luteolin * [d]	348	285.0 [M-H]⁻	tr.	n.d.	0.105 ± 0.005	tr.	Tsimogiannis et al., 2017[S]; Boroja et al., 2018[S]
31	21.33	21.39	Naringenin * [d]	288	271.0 [M-H]⁻	n.d.	n.d.	tr.	tr.	Tsimogiannis et al., 2017[S]; Boroja et al., 2018[S]; Čakar et al., 2018[S]

Peak#	R_t (UV)	R_t (MS)	Assignement	Spectral data		Content (mg/g of dry extract)				Reference
				λ_{max} (nm)	ESI-MS data (m/z)	A1	A2	E1	E2	
32	21.61	21.72	Apigenin * [d]	336	269.0 [M-H]⁻	n.d.	n.d.	0.127 ± 0.007	tr.	López-Cobo et al., 2015[S]; Boroja et al., 2018[S]
34	26.02	26.13	Genkwanin * [d]	334	283.0 [M-H]⁻, 268.0	tr.	n.d.	0.291 ± 0.008	tr.	Hossain et al., 2010[L]
Total flavonoids						*4.06*	*21.17*	*6.53*	*1.65*	
Diterpenes										
33	24.35	24.47	Rosmanol / isomer	280, 328	345.1 [M-H]⁻, 283.0	n.d.	n.d.	d.n.d.	d.n.d.	Celano et al., 2017[L]; Hossain et al., 2010[L]

HCA, hydroxycinnamic acid; n/a, not available; n.d., not detected; d.n.d., detected, but not determined; tr., trace.

*, constituent identified by comparison with the standard compound; [S], previously detected in a plant belonging to the genus *Satureja*; [L], previously detected in a plant belonging to the family Lamiaceae.

[a], expressed as chlorogenic acid equivalents; [b], expressed as rosmarinic acid equivalents; [c], expressed as luteolin 7-*O*-glucoside equivalents; [d], expressed as luteolin equivalents. Major constituents in extracts are marked in bold figures.

Table 2. Total phenols contents of aqueous and ethyl acetate extracts of stems, leaves and flowers (A1 and E1, respectively) and leaves and flowers (A2 and E2, respectively) of *S. kitaibelii* and comparison with relevant studies

Total phenols (mg GA/g)

References	Species	Extracts				Extraction medium
		A1	A2	E1	E2	
This study	*S. kitaibelii* Wiersb. ex Heuff.	**164±1.80**	**274±2.4**	**44.5±2**	**51.5±1.2**	Water EtOAc
Trifonova et al., 2021	*S. kitaibelii* Wierzb. ex Heuff. (flowering stems)	160.10 mg GA/g				Water
Lopez-Cobo et al., 2015	*S. montana* subsp. *kitaibelii* (Wierzb. ex Heuff.) P.W. Ball	25.82 mg GA/g				Aqueous MeOH
Gopčević et al., 2019	*S. kitaibelii* Wiersb. ex Heuff.	105.06±11.32-158.54±14.15 mg GA/g (stems, leaves and flowers) 158.85±15.02 – 195.95±21.35 mg GA/g (leaves and flowers)				EtOH
Ćetković et al., 2007	*S. montana* L. subsp. *kitaibelii* (aerial parts)	969.43 mg GA/g 1358.14 mg GA/g,				EtOAc *n*-ButOH
Moreira et al., 2020	*S.montana* L. (leaves)	12-24 mg GA/g				Water
Serrano et al., 2011	*S. montana* L.	164.2 mg GA/g, 187.5 mg GA/g				Cold water Hot water
Zeković et al., 2017	*S. montana* L.	29.5-74.4 mg GA/g				Water-EtOH
Vladić et al., 2014	*S. montana* L	39-72 mg GA/g				Water- EtOH (20-80%)
Tsimogiannis et al., 2017	*S. thymbra* L. (leaves)	249.5 ± 0.9 mg GA/g 154.0 ± 11.9 mg GA/g 289.3 ± 6.2 mg GA/g				Water EtOAc EtOH
Choulitoudi et al., 2016	*S. thymbra* (leaves)	154.0 mg GA/g				EtOAc

Abbreviations: EtOH-Ethanol; MeOH-Methanol; EtOAc-Ethyl acetate; Polarity index: Water - 1; Methanol –0.762; Ethanol –0.654, Ethyl Acetate –0.440.

Table 3. Free radical scavenging activity and reducing power of aqueous and ethyl acetate extracts of stem, leaves and flowers (A1 and E1, respectively) and leaves and flowers (A2 and E2, respectively) of *S. kitaibelii.* BHA and L-ascorbic acid were used as standard antioxidants.

Extract	DPPH SC_{50} (µg/mL)	ABTS (mg AA/g)	FRAP (µmol Fe^{+2}/mg)	TRP (mg AA/g)
A1	35±10	2.105±0.05	1.547±0.02	10.5±0.4
A2	20±10	2.834±0.02	1.922±0.03	16.4±1.0
E1	320±30	0.449±0.01	0.257±0.01	7.7±0.4
E2	280±20	0.467±0.19	0.430±0.02	7.19±0.5
BHA	5.43±0.02	/	1.83±0.41	10.97±0.29
L-ascorbic acid	3.74±0.12	/	6.30±0.22	/

Table 4. Cytotoxic activity (IC_{50} values) of aqueous and ethyl acetate extracts of stems, leaves and flowers (A1 and E1, respectively) and leaves and flowers (A2 and E2, respectively) of *S. kitaibelii* against human cancer cell lines.

	HeLa	MCF-7	PC-3	MRC-5
	IC_{50} (µg/ml)			
A1	>200	188±5,97	>200	>200
A2	>200	182,3±6,06	>200	>200
E1	22,26±0,23	168±5,39	74,41±7,9	72,5±5,06
E2	20,42±1,93	155,2±12,36	58,31±1,1	60,92±4,39

IC_{50} values (µg/mL) are expressed as the mean ± SD determined from the results of MTT assay in three independent experiments.

Figures

Figure 1

Distribution of the identified constituents (**1**-**34**) in aqueous and ethyl acetate extracts of stems, leaves and flowers (A1 and E1, respectively) and leaves and flowers (A2 and E2, respectively) of *S. kitaibelii*. The numbering of the constituents corresponds to those in Table 2. Chlorogenic acid (**1**), 12-Hydroxyjasmonic acid 12-O-hexoside (**2**), Caffeic acid (**3**), Cynarin (**4**), Luteolin 7-*O*-diglucuronide (**5**), Apigenin dihexuronide (**6**), Salvianolic acid K/isomer (**7**), Luteolin 7-*O*-rutinoside (**8**), Isoquercitrin (**9**), Luteolin caffeoyl-dihexuronide (**10**), Luteolin sinapoyl-dihexuronide (**11**), *p*-Coumaric acid (**12**), Luteolin 7-*O*-glucuronide (**13**), Apigenin deoxyhexosyl-hexoside (**14**), Dicaffeoylquinic acid isomer (**15**), Luteolin *p*-coumaroyl-dihexuronide (**16**), Diosmin (**17**), 3,5-Dicaffeoylquinic acid (**18**), Salvianolic acid A/isomer (**19**), 12-O-(Caffeoylhexosyl)-jasmonate (**20**), Rosmarinic acid (**21**), Clinopodic acid I (**22**), Me-apigenin deoxyhexosyl-hexoside (**23**), Salvianolic acid E/L isomer (**24**), Clinopodic acid O (**25**), Me-apigenin hexoside (**26**), Clinopodic acid K (**27**), Eriodyctiol (**28**), Luteolin (**29**), Hydroxycinnamic acid hexamer (**30**), Naringenin (**31**), Apigenin (**32**), Rosmanol /isomer (**33**), Genkwanin (**34**).

Figure 2

Antibacterial (a, b) and antifungal activity (c, d) of aqueous and ethyl acetate extracts of stems, leaves and flowers (A1 and E1, respectively) and leaves and flowers (A2 and E2, respectively) of *S. kitaibelli*. MIC – minimal inhibitory concentration, (mg/mL); STR – streptomycin; AMP – ampicillin; BIF – bifonazole; KET – ketoconazole. Detailed data on all microorganisms are given in Supplementary Table 2.

Supplementary Files

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