Chemical Composition and Biological Activity of Essential Oil and Extract from the Seeds of *Tropaeolum majus* L. *var. altum*  

Running head: *Tropaeolum majus* L. *var. altum* Seeds – Chemistry and Biological Activity

Ivana Vrca¹*, Dina Ramić², Željana Fredotović³, Sonja Smole Možina², Ivica Blažević⁴ and Tea Bilušić¹

¹Department of Food Technology and Biotechnology, Faculty of Chemistry and Technology, University of Split, Ruđera Boškovića 35, 21 000 Split, Croatia  
²Department of Food Science and Technology, Biotechnical Faculty, University of Ljubljana, Jamnikarjeva ulica 101, 1000 Ljubljana, Slovenia  
³Department of Biology, Faculty of Science, University of Split, Ruđera Boškovića 33, 21000 Split, Croatia  
⁴Department of Organic Chemistry, Faculty of Chemistry and Technology, University of Split, Ruđera Boškovića 35, 21 000 Split, Croatia

Received: 31 January 2022  
Accepted: 28 September 2022

**SUMMARY**

*Research background.* Plant *Tropaeolum majus* L. belongs to the family Tropaeolaceae, and contains benzyl glucosinolate. The breakdown product of benzyl glucosinolate, benzyl isothiocyanate (BITC), exhibits various biological activities such as antiproliferative, antibacterial, and antiinflammatory activities. In order to optimize the content of biologically active volatile compounds in plant extract and essential oil, the use of appropriate extraction technique has a crucial role.

*Experimental approach.* The current study investigated the effect of two modern extraction methods (microwave-assisted distillation (MAD) and microwave hydrodiffusion and gravity (MHG)) on the chemical composition of volatile components present in essential oil and extract of *T. majus* L.
var. altum seeds. The biological activity of samples (essential oil, extract and pure compounds) was focused on the antiproliferative effect against different cancer cell lines: cervical cancer cell line (HeLa), human colon cancer cell line (HCT116), and human osteosarcoma cell line (U2OS), and the antibacterial activity which was evaluated against growth and adhesion to polystyrene surface of Staphylococcus aureus and Escherichia coli.

Results and conclusions. Essential oil and extract of T. majus seeds were isolated by two extraction techniques: MAD and MHG. BITC and benzyl cyanide (BCN) present in extract were identified by GC-MS. Essential oil of T. majus showed higher antiproliferative activity (IC₅₀<5 µg/mL) than T. majus extract (IC₅₀<27 µg/mL) against three cancer cell lines: HeLa, HCT116, and U2OS. BITC showed much higher inhibitory effect on all tested cells in contrast to BCN. Essential oil and extract of T. majus showed strong antimicrobial activity against S. aureus and E. coli.

Novelty and scientific contribution. This work represents the first comparative report on the antiproliferative activity of essential oil and extract of T. majus seeds, BITC and BCN against HeLa, HCT116, and U2OS cells as well as their antimicrobial activity against S. aureus and E. coli. This study demonstrated that essential oil of T. majus seeds exhibits stronger antiproliferative and antimicrobial activity than plant extract.

Keywords: Tropaeolum majus L.; benzyl isothiocyanate; benzyl cyanide; antiproliferative activity; antimicrobial activity

INTRODUCTION

Plant Tropaeolum majus L. belongs to the family Tropaeolaceae, and is known for its ornamental and medicinal uses (1). Thanks to its therapeutic properties, T. majus is also used in traditional medicine for the treatment of various diseases: externally as a disinfectant, for treatment of burns and diaper rash, and internally as a good remedy for the treatment of cancer, bronchitis, tuberculosis and asthma (2). It is also known for its antibacterial, antifungal, and antiviral activities, and is therefore used as pharmacological agent for the treatment of acute sinusitis, and urinary tract infections (3).

The leaves and seeds of T. majus contain fatty acids, flavonoids, tetracyclic triterpenes of cucurbitin and glucosinolates (GSLs) - benzyl glucosinolate and sinalbin (4). According to Bloem et al. (5), T. majus contains only one GSL–benzyl GSL (glucotropaeolin).

GSLs are specialized plant metabolites found in the botanical order Brassicales, in which the Brassicaceae represents the largest family (6). GSLs are present in all parts of T. majus, especially in leaves, flowers and seeds (7). Intact GSLs are not biologically active compared to their breakdown
products, especially isothiocyanates (ITCs), which are reported to be very active against a wide range of organisms (7), including microrganisms such as bacteria. GSLs are hydrolyzed by enzyme myrosinase contributing to the formation of various degradation products (ITCs, thiocyanates, nitriles) that depend on physiological conditions such as pH and the presence of certain cofactors like epithiospecifier protein (ESP) (8,9). ITCs are formed at neutral pH, while at acidic pH nitriles are dominant products (10). Solvent-free extraction methods such as microwave-assisted distillation (MAD), and microwave hydrodiffusion and gravity (MHG) are excellent replacements to conventional extraction methods in order to obtain isolates rich in volatile compounds (9). These new extraction methods are much faster, easier, enviromently friendly and enable extraction of biologically active compounds with decreased energy (9). ITCs can potentially be used to prevent various cancers such as lung, liver, breast and, colon cancers (11). Due to their ability to cause growth arrest and cell death selectively in cancer cells, cancer prevention with dietary ITCs is ready for clinical translational research (12). T. majus has been reported to possess anticancer activity (13,14), which can be explained by the presence of benzyl isothiocyanate (BITC) – a degradation product of benzyl GSL (1,15–17). BITC has shown excellent antiproliferative activity against human colon cancer cell line HT-29 (17). According to Xie et al.(18), the BITC treatment leads to suppression of tumor growth, inhibition of cellular proliferation and increased apoptosis in the breast tumors. However, the data on the anticancer activity of T. majus volatile sulphur compounds called "essential oil" and extract obtained by microwave technique is lacking. Moreover, ITCs are volatile compounds that have inhibitory effects on various pathogenic microorganisms at low concentrations, making them desirable antimicrobial agents (19). One of the important biological activities of T. majus is antimicrobial activity, and is attributed to the presence of BITC (20). Antimicrobial activity of BITC has been investigated (21–23). Contrary, the research on the antimicrobial activity of T. majus essential oil and extract is scarce. Bazylko et al. (20) reported that aqueous and hydroethanolic extracts obtained from air-dried and freeze-dried T. majus showed no antimicrobial activity against Staphylococcus aureus, Bacillus subtilis, Micrococcus luteus, Escherichia coli, Pseudomonas aeruginosa and Bordetella bronchiseptica. Main explanation is probably a low content of BITC in the extracts. There are also some reports that aromatic ITCs, such as BITC, have stronger antibacterial activity in comparison to aliphatic ones against plant pathogenic bacteria, foodborne pathogens and spoilage bacteria, and methicillin-resistant S. aureus (21). Dias et al. (23) reported that among all tested ITCs against 15 isolates of methicillin-resistant S. aureus, the BITC was the most efficient. Bacterial microorganisms can adhere to the surface and make a bacterial biofilm emerged from bacterial cells and surrounding extracellular polymeric substances (24). Bacteria in the planktonic state are less resistant to usual
antibacterial compounds and substances compared to bacteria in biofilms (24), which is why it is important to identify new natural components with high antibacterial and biofilm efficacy. The aims of this study were: to obtain EO and extract of T. majus with two modern extraction techniques: MAD and MHG, to determine volatile components in prepared samples by gas chromatography-mass spectrometry (GC-MS), to examine antiproliferative activity of EO, extract, pure compounds (BITC and benzyl cyanide (BCN)) against cervical cancer cell line (HeLa), human colon cancer cell line (HCT116) and human osteosarcoma cell line (U2OS), and to determine the antibacterial activity of prepared samples and pure compounds against growth and adhesion to polystyrene surface of S. aureus and E. coli.

MATERIALS AND METHODS

Plant material and reagents

The seeds of T. majus L. var. altum were purchased from Marcon d.o.o. (Novi Marof, Croatia). Before the extraction of volatile compounds, the seeds of T. majus were milled to a fine powder using a coffee grinding machine. Afterward, the ground seeds were soaked in distilled water directly before MAD technique, and approximately 1 h before MHG extraction technique. BITC, BCN and benzaldehyde were commercially purchased from Sigma-Aldrich, Merck KgaA, USA. All other used chemicals and reagents were of analytical grade.

Microwave-assisted isolation

Isolation of EO and extract of T. majus was made by MAD and MHG extraction techniques, respectively, using an ETHOS X device (Milestone, Italy) and applying microwave power of 500 W as desribed by Vrca et al. (1,9). The EO was isolated from T. majus seeds (50 g). The temperature inside the microwave oven was ca. 98 °C, and the extraction time was set to 30 min. After MAD, EO of T. majus, collected in the pentane trap, was dried with anhydrous sodium sulfate in order to remove any residual water. Extract of T. majus seeds (50 g) was isolated by setting to the "flavor" and time to 15 min. The extract of T. majus seeds was collected in a glass beaker at the bottom of the apparatus, filtrated and extracted with dichloromethane from water extract. The volatile isolates were stored in vials at -20 °C until further analysis.

GC-MS analysis

System gas chromatographic used for quantification contained gas chromatograph, model 8890 GC, equipped with an automatic liquid injector, model 7693A, and tandem mass spectrometer (MS/MS), model 7000D GC/TQ (Agilent Inc., Santa Clara, CA, USA). The volatile isolates were
analyzed on a non-polar HP-5MS UI column (dimensions: 30 m length, inner diameter 0.25 mm and stationary phase layer thickness 0.25 μm, Agilent Inc., Santa Clara, CA, USA). The column temperature program was adjusted at 60 °C for the first 3 min and then heated to 246 °C at 10 °C/min, and maintained for 3 min isothermally. Helium was the carrier gas and the flow rate was 1 mL/min. The inlet temperature was 250 °C and the volume of the injected sample was 1 μL. Additional conditions were: ionization energy was 70 eV; transferline temperature was 280 °C; ion source temperature was 230 °C; the temperature of the quadrupoles was set at 150 °C, while the flow of nitrogen through the collision cell was 1.5 mL/min. By comparison of their retention indices the individual peaks were identified (relative to C₆-C₄₀ n-alkanes for HP-5MS UI) and mass spectra with those of authentic samples, as well as by computer matching against the Wiley 9N08 MS (Wiley, New York, NY, USA) (25) and NIST17 (Gaithersburg, MD, USA) mass spectral databases (26).

**Antiproliferative activity**

The antiproliferative activity of *T. majus* EO and extract, as well as pure compounds BITC and BCN was determined on HeLa, HCT116 and U2OS cancer cell lines kindly given by Prof. Janoš Terzić (the School of Medicine, University of Split) as described by Fredotović *et al.* (27). The antiproliferative activity was determined using the MTS-based CellTiter 96® Aqueous Assay (Promega). Cells were grown in a CO₂ incubator at 37 °C and 5 % CO₂ until they reached 80 % confluency. Afterwards, cells were counted using the automatic handheld cell counter (Merck), and 0.5×10⁴ cells/well were seeded in 96-well plates and treated with serially diluted extracts. Cells were further grown for 48 h, after which 20 μL of MTS tetrazolium reagent (Promega) was added to each well. The absorbance was measured at 490 nm using a 96-well plate reader (Bio-Tek, EL808) after 3 h of incubation at 37 °C and 5 % CO₂. Solvent control was measured and incorporated into the obtained results. Experiments were carried out in four replicates for each concentration and IC₅₀ values were calculated from three independent experiments using GraFit 6 data analysis software (Erithacus, East Grinstead, UK) (28).

**Bacterial strains and growth conditions**

Gram positive *Staphylococcus aureus* ATCC 25923 and Gram negative *Escherichia coli* ATCC 11229, which are part of the collection of microorganisms of the Laboratory for Food Microbiology of the Department of Food Science and Technology, Biotechnical Faculty, University of Ljubljana, were used for antibacterial testing. Those bacteria were stored at -80 °C in tryptic soy broth (Biolife, Milan, Italy) together with 15 % glycerol (Kemika, Zagreb, Croatia), revitalised on Mueller-Hinton (MH) agar (BioMérieux, Marcy-l'Étoile, France) and incubated at 37 °C, aerobically for 24 h.
Standardised inocula with a cell concentration $10^5$ colony-forming units (CFU)/mL were prepared in MH broth (Oxoid, Hampshire, UK) for antibacterial assays.

**Antibacterial susceptibility**

Microdilution method was used to determine the minimal inhibitory concentrations (MICs). Briefly, the EO, extract and pure compounds (BITC and BCN) were dissolved and diluted in absolute ethanol (Merck, Darmstadt, Germany). 2-fold serial dilutions of the EO, extract and pure compounds were performed in a 96-well microtiter plate to achieve concentrations from 2 mg/mL to 0.06 mg/mL with final volume 50 µL. 50 µL of prepared inoculum ($10^5$ CFU/mL) was added to each well and mixed. 10 µL 2-p-iodophenyl-3-p-nitrophenyl-5-tetrazolium chloride (INT, Sigma Aldrich, St. Louis, MO, USA) was added after incubation and was used as indicator for bacterial metabolic activity (29). The lowest concentration at which bacterial growth was not detected as reduction of INT to red formazan was MIC. To avoid the effect on the growth of the selected bacteria, the ethanol concentration in the assay never exceeded 1 %. To determine minimal bactericidal concentrations (MBCs), 5 µL of cultures, collected from each well of 96-well microtiter plate and were inoculated on MH agar, afterwards cultures were incubated aerobically for 24 h at 37 °C. MBC was determined as the concentration where bacteria did not grow.

**Bacterial growth kinetics**

The EO, extract and pure compounds, BITC and BCN, were added to 5 mL of growth medium to give final concentrations for *S. aureus* ATCC 25923 from 0.25 mg/mL to 0.031 mg/mL and for *E. coli* ATCC 11229 from 1 mg/mL to 0.125 mg/mL. Furthermore, BITC and BCN, were tested together in ratio 1:2 and 1:1 against *S. aureus* ATCC 25923 and *E. coli* ATCC 11229 regarding to the results obtained for MIC. Cultures (*S. aureus* or *E. coli*) without the addition of plant preparations were used as a positive control. For negative control, MH broth with or without the addition of plant preparations in different concentrations was used and after measurements was deducted from the obtained results. Inocula were prepared as described above. 100 µL of prepared cultures and negative controls, with or without the addition of plant preparations, were added to 96-well microtiter plates (Nunc 266 120 polystyrene plates; Nunc, Denmark). The absorbance was measured at 600 nm using Multiskan reader (ThermoScientific Waltham, MA, USA) every 30 min over 24 h at 37 °C to gain growth curves.

**Antiadhesion assay**

The adhesion of *S. aureus* ATCC 25923 and *E. coli* ATCC 11229 was analysed under treatments with EO, extract, BITC and BCN. Inocula were prepared as described above and treated
Chemical composition of EO and extract of T. majus seeds

The volatile components from benzyl GSL obtained after MHG from T. majus seeds were identified by GC-MS. Analysis showed that main volatile compounds in extract of T. majus were BCN and BITC (37.00 %, and 54.35 %, respectively) (Table 1). Vrca et al. (1) reported that BITC was the main component in EO (97.81 %), while BCN was presented in low concentration (0.80 %) after 30 min treatment and application of 500 W microwave power using MAD. The higher amount of BCN in the extract after MHG can be explained by the presence of ESP. The interaction of ESP with enzyme myrosinase diverts the reaction toward the production of epithionitriles or nitriles depending on the glucosinolate structure (33). Given that ESP is thermally sensitive, it is known that its activity reduces significantly at the temperature of 50 °C or higher (34), which is the main reason for the low presence of BCN in EO of T. majus. On the other side, soaking in water 1 h before MHG technique enabled formation of BCN, and explains its high percentage in the extract of T. majus seeds.

Table 1.

According to Wielanek et al. (35), after in vitro hydrolysis of benzyl GSL in hairy root cultures of T. majus, degradation products were BITC, BCN, and benzyl thiocyanate (BTC). BTC was not detected in the analyzed samples. The enzymatic formation of organic thiocyanates is believed to

Statistical analysis

Experiments were carried out in triplicate as three or more independent experiments. The data are expressed as means ± standard deviation (SD), with analysis using Origin 2018 (OriginLab, Northampton, USA) (31). IBM SPSS Statistics 23 (Statsoft Inc., Tulsa, USA) was used to perform statistical analysis (32). The Kolmogorov-Smirnov test of normality was used to determine distribution of data and statistical significances were determined using T-tests for two independent means. Data were significant at p-value <0.05.

RESULTS AND DISCUSSION

Chemical composition of EO and extract of T. majus seeds

The volatile components from benzyl GSL obtained after MHG from T. majus seeds were identified by GC-MS. Analysis showed that main volatile compounds in extract of T. majus were BCN and BITC (37.00 %, and 54.35 %, respectively) (Table 1). Vrca et al. (1) reported that BITC was the main component in EO (97.81 %), while BCN was presented in low concentration (0.80 %) after 30 min treatment and application of 500 W microwave power using MAD. The higher amount of BCN in the extract after MHG can be explained by the presence of ESP. The interaction of ESP with enzyme myrosinase diverts the reaction toward the production of epithionitriles or nitriles depending on the glucosinolate structure (33). Given that ESP is thermally sensitive, it is known that its activity reduces significantly at the temperature of 50 °C or higher (34), which is the main reason for the low presence of BCN in EO of T. majus. On the other side, soaking in water 1 h before MHG technique enabled formation of BCN, and explains its high percentage in the extract of T. majus seeds.

Table 1.

According to Wielanek et al. (35), after in vitro hydrolysis of benzyl GSL in hairy root cultures of T. majus, degradation products were BITC, BCN, and benzyl thiocyanate (BTC). BTC was not detected in the analyzed samples. The enzymatic formation of organic thiocyanates is believed to

with EO, extract, BITC and BCN at concentrations MIC, ½ MIC and ¼ MIC. Treated inocula (200 µL) were then transferred to 96-well polystyrene microtitre plates (Nunc 266 120 polystyrene plates; Nunc, Denmark) and incubated at 37 °C, aerobically for 24 h. To remove non-adherent cells, each well in microtiter plate was rinsed three times with phosphate-buffered saline (PBS) (Oxoid, Hampshire, UK), afterwards 200 µL of PBS was added to each well and sonicated for 10 min (28 kHz, 300 W; IskraPlo, Šentjernej, Slovenia). CFU/mL was the measure for the adhesion of cells as previously described by Šikić Pogačar et al. (30). The untreated culture was used as the negative control.
require thiocyanate-forming protein (TFP). However, recent report by Todorovska-Rašić and Radulović (36) suggests they could be formed via metabolic routes that do not involve TFP.

Antiproliferative activity

The antiproliferative activity of EO and extract of *T. majus*, as well as pure compounds BITC and BCN was determined for the first time on HeLa, HCT116, and U2OS cancer cell lines using MTS cell proliferation assay, as far as authors know (Fig. 1). Cancer cells were treated for 48 h, and the results were expressed as IC$_{50}$ values (50 % cell growth inhibitory concentrations). EO of *T. majus* showed better effect on all three cancer cell lines: HeLa, HCT116 and U2OS (IC$_{50}$ 4.91 µg/mL, 1.49 µg/mL, and 4.53 µg/mL respectively) compared to extract of *T. majus* (IC$_{50}$ 26.93 µg/mL, 11.79 µg/mL, and 22.09 µg/mL respectively). BITC showed much higher inhibitory effect on all tested cells (IC$_{50}$ 1.39 µg/mL, 0.85 µg/mL, and 1.27 µg/mL) in contrast to BCN (IC$_{50}$ 26.29 µg/mL, 22.82 µg/mL and 24.7 µg/mL).

BITC has been reported to exhibit antitumor properties in various types of carcinoma (11), which is consistent with the fact that BITC has anticancer activity. At concentrations of BITC >10 μM (1.49 µg/mL), the survival rate of 4T1-Luc murine mammary carcinoma cells was <50 % using MTT assay (11). Previous studies on *in vitro* cell culture have revealed that BITC shows anticancer activity by inducing apoptosis and G2/M cell cycle arrest in different cancer cells (breast cancer, lung cancer, pancreatic cancer and leukemia cells) at concentrations between 2 to 100 µmol/L (0.30 µg/mL-14.92 µg/mL) (15). These results are consistent with ours on HeLa, HCT116, and U2OS cancer cell lines where IC$_{50}$ was <2 µg/mL for pure compound BITC, and IC$_{50}$ was <5 µg/mL for EO of *T. majus*. Results of MTT assay showed that BITC (5–20 µmol/L) (0.75 µg/mL-2.98 µg/mL) reduced the number of viable TRAMP-C2 prostate cancer cells and DU145 human prostate cancer cells (5–10 µmol/L) (0.75 µg/mL-1.49 µg/mL) in a dose-dependent manner (15). According to Veeranki et al. (16), BITC inhibits osteosarcoma which is consistent with our results obtained against human osteosarcoma cell line (U2OS). Huong et al. (37) also demonstrated that ITCs have high antiproliferative activity against human cervical cancer cell lines (HEp-2 and KB) using the MTS assay. Huong et al. (37) reported a significant decrease in cell viability by 2-phenylethyl ITC (PEITC), which makes PEITC a potent growth inhibitor of cervical cancer cells.

![Fig. 1.](image)

Nastruzzi et al. (38) reported that ITC from benzyl glucosinolate appears to be the most active compound of all tested degradation products from various GSLs against human K562 erythroleukemic cell line, doing a 50 % cell growth inhibition at concentrations in the 1-6 μM (0.15–0.90 µg/mL) range. Nitrile from benzyl GSL showed lower antiproliferative activity against human K562 erythroleukemic...
cell line in comparison to BITC (38), which is consistent to our results where IC$_{50}$ was <27 µg/mL for BCN. According to criteria used to categorize the activity against the tested cell lines that was based on IC$_{50}$ values (39), our results suggest highly active antiproliferative activity of EO of *T. majus* and pure compound BITC on all three tested cancer cell lines. Extract of *T. majus* also showed highly active antiproliferative activity on HCT116 cancer cell line. Extract of *T. majus* showed moderately active antiproliferative activity on other tested cancer cell lines (HeLa and U2OS). BCN showed moderately active antiproliferative activity on all tested cancer cell lines. BITC can successfully manifest anticancer effects at concentrations (*in vitro*) or doses (*in vivo*) that are non-toxic to normal tissues (40). The results presented in our study show that the degradation product of benzyl GSL, in our case BITC, has high biological activity and it could be used as a new anticancer agent for the prevention of various types of cancer.

**Antibacterial activity**

Table 2 shows results of antibacterial activity of pure compounds (BITC and BCN), EO after MAD, and extract after MHG. According to presented results, *S. aureus* was more sensitive to the above-mentioned samples in comparison to *E. coli*. EO and extract had the same effect against *S. aureus* with MIC 0.0625 mg/mL and MBC 0.125 mg/mL, while the effect of BITC and BCN was two-times weaker. EO and extract were effective also against *E. coli*, with MIC 0.25 mg/mL and MBC 0.5 mg/mL. BITC had MIC 0.5 mg/mL against *E. coli*, while MBC was 1 mg/mL. BCN had two-times weaker effect against *E. coli* in comparison to BITC.

The results presented in our study show that the degradation product of benzyl GSL, in our case BITC, has high biological activity and it could be used as a new anticancer agent for the prevention of various types of cancer.

**Table 2**

ITCs are considered to be the most interesting compounds that are obtained by degradation of glucosinolates and are known as significant growth inhibitors of Gram negative and Gram positive pathogenic bacteria (24). Among all tested ITCs (allyl isothiocyanate, BITC, PEITC) against more isolates of methicillin-resistant *S. aureus*, the BITC was the most effective with a MIC varying from 2.9 to 110 µg/mL (23), which is in line with our results. The higher efficiency of BITC against 15 isolates of methicillin-resistant *S. aureus* compared to some other ITCs (PEITC, and allyl isothiocyanate) can be attributed to the aromatic ring and short carbon chain against 15 isolates of methicillin-resistant *S. aureus* (23). According to Ko *et al.* (21), MIC and MBC of BITC were four-fold higher from our obtained results (MIC=(0.500±0.000) mg/mL, and MBC>1000 mg/mL). Kaiser *et al.* (24) reported that mean MIC for BITC for all tested *Pseudomonas aeruginosa* isolates was (2145±249) µg/mL. Consequently, degradation products of benzyl GSL (especially BITC) are promising alternative antibacterial agents.
Effect of volatile compounds present in *T. majus* seeds on bacterial growth

To evaluate the effect on the bacterial growth, pure compounds (BITC and BCN), EO and extract from *T. majus* seeds were used. *S. aureus* was exposed to plant preparations for 24 h in concentrations in the interval from 0.25 mg/mL to 0.031 mg/mL. As can be seen in Fig. 2a, BITC and BCN in concentration of 0.25 mg/mL completely inhibited *S. aureus* growth, while the effect of EO and extract was even more pronounced. BITC had a bactericidal effect on the growth of *E. coli* at the concentration of 1 mg/mL, while BCN had no bactericidal effect at this concentration (Fig. 2b). EO and the extract had a two-fold lower bactericidal concentration compared to BITC (Fig. 2b), again indicating that the combination of these two pure components is momentous for the antibacterial properties.

Fig. 2.

EO and extract both inhibited growth of *S. aureus* in concentration of 0.125 mg/mL. This stronger effect can be due to combination of pure compounds BITC and BCN which are an integral part of these preparations. To confirm this hypothesis, BITC and BCN were tested together in different ratios against *S. aureus*. When the ratio of BITC and BCN was 1:1 the effect was stronger in comparison when the ratio was 1:2 (Figs. 3a and 3b). When the concentration of BITC and BCN corresponded to their MICs, their effect was bactericidal, such as for EO and extract. When the concentration of BITC in combination was two-fold lower, there was no bactericidal effect, but inhibitory effect was observed. Even in lower concentrations in both combinations inhibitory effects were observed. These results indicate that BITC is the main compound that determines the antibacterial properties. This can be noticed also by observation of *S. aureus* growth curves while exposed to EO, where even lower concentrations inhibited *S. aureus* growth. In comparison to EO, the inhibitory effect of extract on the growth of *S. aureus* was weaker, which can be explained by lower percentage of BITC in extract.

In order to obtain the same effect of *T. majus* preparations on the growth of *E. coli* as was observed with *S. aureus*, higher concentrations (ranged from 1 mg/mL to 0.125 mg/mL) had to be used. It is known that Gram negative bacteria are more resistant to treatments compared to Gram positive bacteria, due to the composition of the outer membrane (41). Numerous studies have shown that disease resistance to a combination of compounds is less likely than to a single active component (42), which is also supported by our data on *S. aureus* and *E. coli*. Even lower concentrations of plant preparations had an inhibitory effect (Fig. 3b).

Similar to *S. aureus*, the combinations of BITC and BCN in the ratio 1:1 and 1:2 showed that BITC is the main compound that induces higher antibacterial activity of *T. majus* plant preparations.
In the combination corresponding to MICs, BITC and BCN had bactericidal activity (Fig. 3c and 3d). When the concentration of BITC in the combination was two-times lower compared to BCN, the inhibitory effect was weaker (Figs. 3c and 3d).

Fig. 3.

**Effect of volatile compounds present in T. majus seeds on bacterial adhesion to polystyrene surface**

Plant preparations obtained from the seeds of *T. majus*, i.e. EO and extract, and a pure compounds BITC and BCN were tested for their activity against the adhesion of *S. aureus* and *E. coli* to the polystyrene surface. Concentrations MIC, ½ MIC and ¼ MIC were tested. It is important to use such low concentrations of plant preparations to affect not only bacterial growth, but also properties important for bacterial phenotypes such as biofilm formation, virulence, quorum sensing, pathogenicity (43). Moreover, the use of such low concentrations may reduce the risk of developing bacterial resistance (44). ITCs are desirable compounds because of their ability to prevent biofilm formation as well as good efficacy on established biofilms (24). As can be seen in Fig. 4, all preparations at MIC concentration significantly reduced the adhesion of *S. aureus* and *E. coli* to the polystyrene surface (p <0.05). BITC and EO significantly reduced the adhesion of *S. aureus* and *E. coli* to polystyrene surface (p <0.05), also at concentration ½ MIC. The lowest concentration did not have anti-adhesion effect. The similar results for BITC and EO can be explained by the chemical composition of EO, which consists mainly of BITC. Kaiser *et al.* (24) reported that even low ITC concentrations could inhibit biofilm formation and disturb biofilm viability, which is consistent to our obtained results. BITC had the most pronounced anti-adhesion effect against *S. aureus* and *E. coli*. BITC reduced the adhesion of *S. aureus* at MIC and ½ MIC concentration by 99.99 % and 99.9 %, corresponding to a reduction of 4 and 3 log$_{10}$ CFU/mL, respectively. Similar observations were made for *E. coli*, where BITC at the MIC concentration reduced the adhesion by 99.99 %, which corresponded to reduction of 4 log$_{10}$ CFU/mL. All other preparations in concentration MIC reduced the adhesion of both bacteria for more than 90 %, which is more than 1 log$_{10}$ CFU/mL. This is in line with the recommendations suggested by the European Food Safety Authority (45). From obtained results, BITC is promising natural compound with strong activity against the adhesion of *S. aureus* and *E. coli* to the polystyrene surface.

Fig. 4.

**CONCLUSIONS**

Two volatile compounds were found in EO and extract of *T. majus*: BITC and BCN. EO and extract enriched with BITC showed highly active and moderately active antiproliferative activity
against three cancer cell lines: HeLa, HCT116, and U2OS as well as high antimicrobial activity against S. aureus and E. coli. BITC showed much higher inhibitory effect on all tested cells in contrast to BCN. Studies on the biological activities of ITCs such as antiproliferative and antimicrobial activities, are of great importance as they have the potential to target different types of carcinomas or to extend the shelf life of food as natural preservatives in the food industry. One of the goals for the future may be the encapsulation of the T. majus plant extract so that a higher amount of BITC is biologically accessible and available.

ACKNOWLEDGEMENTS

The authors are also grateful for the scientific-research equipment funded by EU grant “Functional integration of the University of Split, PMF-ST, PF-ST and KTF-ST through the development of the scientific and research infrastructure” (KK.01.1.1.02.0018).

FUNDING

This research has been entirely supported by the Croatian Science Foundation in the framework of the project "Plants as a source of bioactive sulfur compounds and their ability to hyperaccumulate metals", HRZZ-IP-2016-06-1316, and partly by Slovenian Research Agency (ARRS Research Programme P4-0116).

DECLARATION OF COMPETING INTEREST

The authors declare not to have any conflict of interest.

AUTHORS' CONTRIBUTION

Ivana Vrca designed the experiments, interpreted the results, and wrote the manuscript. Dina Ramić participated in an implementation of the experiments, investigation, processing data, and manuscript writing. Željana Fredotović performed formal analysis and investigation. Sonja Smole Možina helped in the methodology, research, and revision of the manuscript. Ivica Blažević contributed to the research, funding acquisition, and revision of the manuscript. Tea Bilušić participated in the supervision, and revision of the manuscript. All authors have read the manuscript and approved publication of all its contents.

ORCID ID

I. Vrca https://orcid.org/0000-0001-9762-4757
D. Ramić https://orcid.org/0000-0002-4929-3962
REFERENCES


https://doi.org/10.29329/ijiaar.2019.194.11

https://doi.org/10.1016/j.phymed.2016.02.025


https://doi.org/10.1016/j.jfca.2020.103483

doi: 10.4236/abc.2014.42022

https://doi.org/10.1007/s13277-015-3391-5


29. Klančnik A, Piskernik S, Jeršek B, Možina SS. Evaluation of diffusion and dilution methods to
https://doi.org/10.1016/j.mimet.2010.02.004

https://doi:10.1002/jsfa.7391

31. OriginLab: Northampton, USA. Available from:

32. IBM SPSS Statistics: Statsoft Inc., Tulsa, USA. Available from:

https://doi.org/10.1074/jbc.M807500200


https://doi.org/10.2298/FUPCT2002077T

https://pubs.acs.org/doi/full/10.1021/jf2006358

https://pubs.acs.org/doi/full/10.1021/jf000191p

https://doi.org/10.1080/13880209.2016.1223146


Table 1. Chemical composition of volatile compounds in T. majus extract after microwave-assisted isolation

<table>
<thead>
<tr>
<th>Volatile compounds</th>
<th>RI</th>
<th>MHG (φ/%)</th>
<th>MAD* (φ/%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzaldehyde</td>
<td>962</td>
<td>4.42</td>
<td>-</td>
</tr>
<tr>
<td>BCN</td>
<td>1144</td>
<td>37.00</td>
<td>0.80</td>
</tr>
<tr>
<td>BITC</td>
<td>1369</td>
<td>54.35</td>
<td>97.81</td>
</tr>
<tr>
<td>Total (φ/%)</td>
<td></td>
<td>95.77</td>
<td>98.61</td>
</tr>
</tbody>
</table>

* Vrca et al. (1); RI, retention indices determined on a HP-5MS UI capillary column
Table 2. Minimal inhibitory and bactericidal concentrations (mg/mL) against *S. aureus* ATCC 25923 and *E. coli* ATCC 11229 for pure compounds (BITC and BCN), *T. majus* EO after MAD, and *T. majus* extract after MHG

<table>
<thead>
<tr>
<th>Pure compounds and plant preparations</th>
<th><em>S. aureus</em> ATCC 25923</th>
<th><em>E. coli</em> ATCC 11229</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC (mg/mL)</td>
<td>MBC (mg/mL)</td>
</tr>
<tr>
<td>BITC</td>
<td>0.125</td>
<td>0.25</td>
</tr>
<tr>
<td>BCN</td>
<td>0.125</td>
<td>0.25</td>
</tr>
<tr>
<td>EO after MAD</td>
<td>0.0625</td>
<td>0.125</td>
</tr>
<tr>
<td>Extract after MHG</td>
<td>0.0625</td>
<td>0.125</td>
</tr>
</tbody>
</table>
Fig. 1. Antiproliferative activity of *T. majus* EO after MAD and *T. majus* extract after MHG (a), and antiproliferative activity of BITC and benzyl cyanide BCN (b) on HeLa, HCT116 and U2OS determined by MTS cell proliferation assay. The results are expressed as means of three independent experiments with SD values (presented as error bars). For statistical analyzes were used the two-way ANOVA method and Sidak's multiple comparisons test. Statistically significant differences are labeled as **** $p < 0.0001$
Fig. 2. Effects of pure compounds (BITC and BCN), EO and extract of T. majus in different concentrations (mg/mL) on the growth of S. aureus (a) and E. coli (b). Cultures were aerobically incubated for 24 h at 37 °C. Negative controls were deducted from the obtained results. Average values of $A_{600\text{nm}} \pm \text{SD}$ are shown.

Fig. 3. Effects of BITC and BCN in different ratios (1:1 and 1:2; mg/mL), determined regarding MIC values, on the growth of S. aureus (a,b) and E. coli (c,d). Cultures were aerobically incubated for 24 h at 37 °C. Negative controls were deducted from the obtained results. Average values of $A_{600\text{nm}} \pm \text{SD}$ are shown.
Fig. 4. Effects of pure compounds (BITC and BCN), EO and extract of *T. majus* in concentration MIC, ½ MIC and ¼ MIC on the adhesion to polystyrene surface of *S. aureus* (a) and *E. coli* (b). The results are expressed as means ± SD, *p*-value <0.05.