

Original Research Paper

# Chemical Composition and Antibacterial Activity of Subcritical CO<sub>2</sub> Extract of *Beta vulgaris* L.

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**Abstract:** The perpetual quest for novel antimicrobial agents is an ongoing endeavor within the scientific community. Contemporary investigations concerning members of the family *Amaranthaceae*, *Beta vulgaris*, have disclosed significant therapeutic properties in plants that were formerly exclusively employed as dietary resources. The roots and stems of red beets (*Beta vulgaris* L. var. *conditiva*) have been found to possess medicinal properties attributed to the presence of betalains and phenols. Furthermore, there exists an inadequacy of information regarding the biological activity pertaining to the seeds of this particular species. The aim of our research was to study the chemical composition of *Beta vulgaris* L. seeds and evaluate their antibacterial activity. Research objects: Subcritical CO<sub>2</sub> extracts of *Beta vulgaris* L. liquid POCO<sub>2</sub>. The component composition of the extract was determined on a Clarus 580 gas chromatograph (Perkin Elmer) with a Clarus-SQ mass spectrometric detector. Antibacterial activity was measured using strains recommended by the Republic of Kazakhstan's state pharmacopoeia. According to the results of the analysis of the chemical composition of the liquid CO<sub>2</sub> extract - 11 components. The dominant compounds in the extract of *Beta vulgaris* L. are phenol and creosol. The results of the determination of antibacterial activity make it possible to determine that the liquid CO<sub>2</sub> extract of beet seeds has a moderately pronounced potential for antibacterial and antifungal activity at a dosage of 25, 50, and 100 µg against microorganisms *S. aureus*, *B. subtilis*, *E. coli*, *P. vulgaris*, and yeast fungus *C. albicans*.

**Keywords:** Carbon Dioxide Seed Extract, Antibacterial Activity, *Beta vulgaris*, Component Composition, Biological Activity

## Introduction

The investigation of potential therapeutic properties inherent in various plant species has garnered the attention of researchers to explore naturally occurring bioactive compounds for the treatment of all illnesses known to people.

The scientific nomenclature of the vegetable commonly known as beet is *Beta vulgaris* L. The taxonomic classification of the species is the *Amaranthaceae* family, as documented in the reference. Judd and Stevens have consolidated *Amaranthaceae* and chenopodiaceous into a single family, namely, *Amaranthaceae*. This family encompasses a total of 1400 species, belonging to 105 genera (El-Mesallamy *et al.*, 2020).

*Beta vulgaris* L. originally grew in southern Europe as an upright annual or biennial plant with tuberous roots. *Beta vulgaris* L. is widely cultivated in Europe, America, and Asia. The plant and root of *Beta vulgaris* L. are used as a natural colorant in foods. It has been recommended by many reviews that it is not only used as a harmless natural food coloring but also plays a major role in reducing oxidative stress due to its ability to scavenge free radicals, oxidative stress is known to be a risk factor in chromosome damage (Sabirov *et al.*, 2023).

*Beta vulgaris* L. also contains various phytochemicals, including phenolic acids, carotenoids, folic acids, flavonoids, and minerals.

In the field of alternative medicine, red beets have established prominence for their purported efficacy in addressing afflictions of the blood, heart, liver, pancreas, digestive system, neurological system, and other prevalent ailments. Studies have shown that the consumption of red beets, which are rich in phenolic acids and have a high antioxidant capacity, also provides protection against aging-related disorders (Tomar and Yıldırım 2019).

Čanadanović-Brunet *et al.* (2011). demonstrated that red beetroot extracts exhibited antibacterial activity against gram-positive and gram-negative bacteria. Jacob and Shenbagaraman (2011) suggested that red beet containing betalains plays an important role as an antioxidant, antiviral, antimicrobial, hepatoprotective, and anticancer agent. Despite the fact that the composition and properties of the leaves and the root of *Beta vulgaris* L. are actively studied, there is a lack of information about the seeds of this species. Since seeds also have a nutritional and caloric value, which makes them necessary for the diet (Čanadanović-Brunet *et al.*, 2011; Jacob and Shenbagaraman, 2011).

In the present study, we obtained the liquid CO<sub>2</sub> extract from the seed of *Beta vulgaris* L. in subcritical conditions, explored component composition depending on the solvent, and established its antimicrobial activity against pathogenic microorganisms for the first time. The results of this study make it possible to use the CO<sub>2</sub> extract of *Beta vulgaris* L. to develop antibacterial drugs and cosmetic products for medical practice.

## Materials and Methods

### Research Objects

Subcritical CO<sub>2</sub> *Beta vulgaris* L. extract: Liquid POCO<sub>2</sub>. CO<sub>2</sub>-extract was obtained on CO<sub>2</sub>-extract from air-dry seeds at a pressure of 69-76 atm and a temperature of 18-21°C (Sisengalieva *et al.*, 2015).

### Determination by Chromato-Mass Spectrometry

The determination was carried out by chromatography-mass spectrometry on a Clarus 580 gas chromatograph (perkinelmer). Detector mass spectrometric detector Clarus-SQ 8 with NIST database-300.000 compounds (the table shows the Kovac indices on a semi-polar column).

### Method of Determining the Component Composition

The component composition of the extract was determined on a Clarus 580 gas chromatograph (perkinelmer) equipped with a Clarus-SQ 8 mass spectrometric detector. The sensitivity of the instrument is determined by the signal-to-noise ratio and is 800: 1 (actually 2000: 1).

Chromatographic conditions: Capillary column Restek®-5Sil MS 0.25 mm ×30 m ×0.25 μm, sample

volume: 1.0 μL, carrier gas: Flow rate of carrier gas: 1 mL/min, flow division 1:25, t of the column: 40°C, temperature rising of 2°C/min up to 280°C, t of evaporator-280°C, mass spectrometric detector: T-2400C, EI+ = 70 eB, scanning time: From 4-120 min, ion scanning mode: 39-500 m/z. The percentage of components was calculated automatically based on the peak areas of the total ion chromatogram. The components were identified by mass spectra and retention times using the NIST library and compared with the retention indices of normal alkanes.

### Study of Antimicrobial Activity

Determination of the antibacterial and antifungal activity of the test samples was carried out using the agar diffusion method with test cultures: *Staphylococcus aureus* ATCC 6538; *Bacillus subtilis* ATCC 6633; *Escherichia coli* ATCC 25922; *Proteus vulgaris* ATCC 6896, *Pseudomonas aeruginosa* ATCC 27853 and candida albicans ATCC 10231, *Streptococcus pyogenes* ATCC 19615.

These cultures were sown by the lawn method on the following nutrient media: Chistovich's medium (yolk-salt agar), nutrient agar, Endo's medium, and Saburo's medium. Then petri dishes with inoculations were incubated for a day for bacteria at 36±1°C and for fungi at -28°C (Donato *et al.*, 2020).

The antimicrobial activity of the samples was assessed by the diameter of the growth inhibition zones of the test strains. Zone diameters less than 10 mm and continuous growth in the petri dish were assessed as the absence of antimicrobial activity, 10-15 mm weak activity, 15-20 mm moderately pronounced activity, and more than 20 mm pronounced.

### Determination of the Minimum Inhibitory (MIC) and Bactericidal (MBC) Concentration Against Bacteria

To determine the antibacterial activity of the CO<sub>2</sub> extract of *Beta vulgaris* L., we used the modified method described in Raafat *et al.* (2019). Duplicate dilutions of the compound were prepared in sterile 96-well round-bottom plates in BCH containing 0.15 M TES-ACES-MES-Na buffer with pH ranging from 5.50-8.00 in increments of 0.25. Then, a bacterial suspension in the same medium was added to the wells of the plate to a final concentration of 2.5×10<sup>5</sup> cfu/mL. After incubation at 37°C for 24 h on a shaker (220 rpm), the MIC CO<sub>2</sub> extract of *Beta vulgaris* L. was determined by the absence of culture growth in the wells containing the minimum concentration of the compound. MBC was considered to be the minimum compound concentration at which the number of live cells decreased by 99.9% or more after 24 h of incubation compared with the baseline level. This study was performed using a stat fax 2600 enzyme immunoassay analyzer at a wavelength of 450. Live cells were counted by inoculating an aliquot of solution from

the plate onto a solid culture medium as a control (guidance determination of the sensitivity of microorganisms to antimicrobial drugs).

The determination of the Minimum Inhibitory Concentrations (MIC) of the CO<sub>2</sub> extract of *Beta vulgaris* L. with respect to fungi was performed by the method of double serial dilution in Muller-Hinton broth (Kurkin *et al.*, 2018). To evaluate the antifungal activity of the CO<sub>2</sub> extract of *Beta vulgaris* L. against yeast-like fungi, double dilutions of the compound were prepared in Sabouraud medium (pH 5.5-5.7) in 96-well plates and then a daily suspension of fungal cells was added to a final cell count of 2×10<sup>5</sup> cfu/mL. After 48 h of incubation at a temperature of 28°C, the MIC CO<sub>2</sub> extract of *Beta vulgaris* L. was determined by the complete absence of culture growth in the wells containing the minimum concentration of the compound. Inhibition of hyphal formation in the yeast fungus *C. albicans* was evaluated by counting the number of yeast-like cells in 10 different fields of view and the number of mycelial structures was counted in the respective fields of view. The proportion of mycelial structures was calculated in relation to the number of yeast-like cells in the experiment. The proportion of mycelial structures in the control variant without the addition of CO<sub>2</sub> extract of *Beta vulgaris* L. was assumed to be 100%.

All substances were first dissolved in 0.05 mL of dimethyl sulfoxide and adjusted to the desired concentration with 0.9% sodium chloride solution. Doses of substances above 1000 µg/mL were not considered due to their low solubility and potentially low efficacy (Kurkin *et al.*, 2018).

The bacterial dose was determined using the McFarland turbidity standard and administered in a volume of 0.1 mL. The number of bacterial cells introduced was monitored by seeding the inoculum onto a dense culture medium and then counting the number of colony-forming units (cfu/ml). Incubation was performed at 37°C for 24 h. After incubation, observation of the cultures determined the minimum bacteriostatic (inhibitory) Concentration (MIC), i.e., the lowest effective dose corresponding to the absence of visible growth or causing a significant decrease in culture growth compared to continuous growth in the control, when another 0.1 mL was seeded onto the solid medium. The Minimum Bactericidal Concentration (MBC) was determined as the

dose of the compound in the sample at which no growth was observed after further inoculation on a dense culture medium. Incubation was performed at 37°C for 24 h.

### Statistical Analysis

Data analysis was processed using an IBM SPSS Statistics 22 software package. Processing operations have included the calculation of arithmetic Mean values (M), standard errors of the arithmetic mean (m), Confidence Intervals (CI), and standard deviation for variables with normal distributions. Differences between the groups with normal distribution were found by means of parametric statistical methods and the student's t-test for averages of the two groups and determine their differences.

## Results

According to the results of the analysis of the chemical composition of the liquid CO<sub>2</sub> extract, 11 components were identified (Table 1 and Fig. 1). The dominant components of the extract are phenol and creosol. The sample contains phenol -0.4 and cresol -0.3% of all volatile substances.

### Antimicrobial Activity

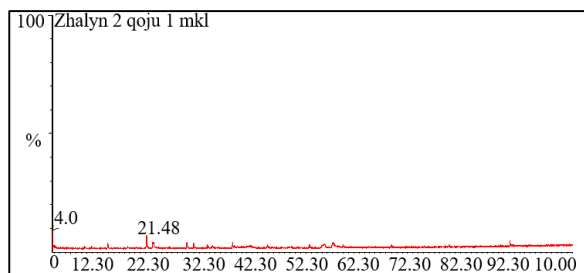
When the efficacy of the subcritical CO<sub>2</sub> extract from the seeds of *Beta vulgaris* L. at a concentration of 25 µg/mL was studied, the diameters of the growth inhibition zones were as follows: *S. aureus* - 12±0.52, *B. subtilis*-18±0.28 mm, *E. coli*-15±0.46 mm, *P. vulgaris*-15±0.18 mm, *C. albicans*-12±0.15\* mm.

The diameters of the inhibitory zones at a dose of 50 µg /mL of the subcritical CO<sub>2</sub> extract from the seeds of *Beta vulgaris* L. were given as follows: *S. aureus*-17±0.88, *B. Subtilis*-18±0.05, *E. coli*-18±0.20, *P. vulgaris*-18±0.24, *C. albicans*-18±0.14 mm.

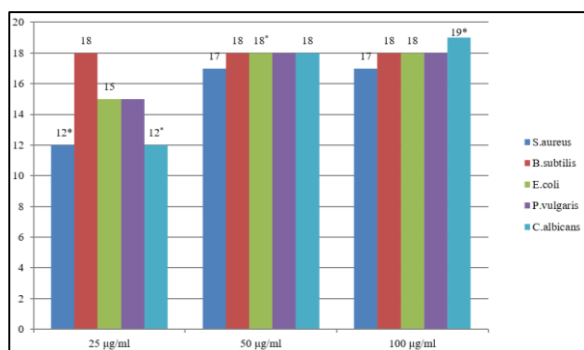
The subcritical CO<sub>2</sub> extract from the seeds of *Beta vulgaris* L. at a dose of 100 µg/mL did not differ in activity from the previous substance at a dosage of 50 µg/mL subcritical seed extraction, whose growth inhibition zone diameter was: *S. aureus*-17±0.12, *B. subtilis* -18±0.15, *E. coli* -18±0.11, *P. vulgaris*-18±0.27, *C. albicans* -19±0.23 mm (Fig. 2).

**Table 1:** Component composition of the liquid CO<sub>2</sub> extract of *Beta vulgaris* L. seeds

№	RT	RI lit	RII	Component	Variability	Area %
1	14.195	9800±4	982	Phenol	893	0.4
2	21.485	1090±3	1080	Phenol, 2-methoxy-	817	1.0
3	22.644	1029 iu	1095	1H-Pyrrole, 2,5-dihydro-1-nitroso-	690	0.4
4	22.802			Unknown 1		0.6
5	29.013	1169±3	1165	Phenol, 4-ethyl-	860	0.6
6	30.264	1193±3	1179	Creosol	858	0.3
7	32.917	1207±8	1209	1,4:3,6-Dianhydro-α-d-glucopyranose	690	0.2
8	37.557	1282±4	1265	Phenol, 4-ethyl-2-methoxy-	860	0.4
9	44.036	1355±5	1343	Phenol, 2,6-dimethoxy-	760	0.3
10	54.837			Unknown 2		0.3
11	56.290			Unknown 3		1.0



**Fig. 1:** Chromatogram of liquid CO<sub>2</sub> extract of *Beta vulgaris* L. seeds



**Fig. 2:** The diameters of the growth inhibition zones of *Beta vulgaris* L. seeds

**Table 2:** Minimal Inhibitory (MIC) and Bactericidal (MBC) concentration against bacteria of subcritical CO<sub>2</sub> extract from the seeds of *Beta vulgaris* L.

Strains/samples	MIC mg/mL	MBC mg/mL	Chloramphenicol mg/mL	Nistatin mg/mL
<i>S. aureus</i>	2,5	5,0	1,25	-
<i>B. subtilis</i>	1,25	2,5	1,25	-
<i>E. coli</i>	2,5	5,0	1,25	-
<i>P. vulgaris</i>	2,5	5,0	1,25	-
<i>C. albicans</i>	5,0	5,0	-	1,25

**Table 3:** Antibacterial activity of subcritical CO<sub>2</sub> extract from the seeds of *Beta vulgaris* L.

Subcritical CO <sub>2</sub> extraction of <i>Beta vulgaris</i> L.	1000 mcg/mL	500 mcg/mL	250 mcg/mL	125 mcg/mL	62,5 mcg/mL	31,25 mcg/mL
<i>S. aureus</i> ATCC 6538	No growth	No growth	No growth	181±11,3 CFU/mL	Solid growth	Solid growth
<i>St. Pyogenes</i> ATCC 19615	No growth	209±11.6 CFU/mL	Solid growth	Solid growth	Solid growth	Solid growth
<i>B. subtilis</i> ATCC 6633	No growth	No growth	No growth	No growth	No growth	101±10,3 CFU/mL
<i>E. coli</i> ATCC 25522	No growth	No growth	181±11,3 CFU/mL	Solid growth	Solid growth	Solid growth
<i>P. vulgaris</i> ATCC 25522	No growth	No growth	181±11,3 CFU/mL	Solid growth	Solid growth	Solid growth
<i>P. aeruginosa</i> ATCC 27853	Solid growth	Solid growth	Solid growth	Solid growth	Solid growth	Solid growth
<i>C. albicans</i> ATCC 10231	No growth	No growth	111±10,6 CFU/mL	Solid growth	Solid growth	Solid growth

\* - not active at the studied concentration (1000 µg/mL) - continuous growth

MIC is considered the main indicator for determining the antimicrobial activity of antibacterial compounds (Table 2).

The sensitivity of subcritical CO<sub>2</sub> extract from the seeds of *Beta vulgaris* L. for museum test strains at a dose of 25 µg/mL showed weak activity against *S.aureus*, *C. albicans*, moderately pronounced activity against *B.subtilis*, *E.coli*, *P.vulgaris*. At a 50 µg/mL dose, 100 µg/mL showed moderately pronounced activity against all gram-positive and gram-negative bacteria.

When studying the effect of the substances on the daily culture of *S. aureus* ATCC 6538 introduced into the wells of the tablet as a suspension in the medium at a concentration of 2.5×10<sup>5</sup> cfu/mL, the result of subcritical CO<sub>2</sub> extract from the seeds of *Beta vulgaris* L. was taken into account, which showed more pronounced activity against the gram-positive test flora. The sample showed activity at a dose of 125 µg/mL and caused growth retardation of the test culture up to 181±11.3 cfu/mL.

Subcritical CO<sub>2</sub> extract from the seeds of *Beta vulgaris* L. against *St. pyogenes* IC<sub>50</sub> = 500 µg/mL showed inhibitory activity up to 209±11.6 CFU/mL. At the other dosages, it did not show an overwhelming effect but showed a continuous increase.

It showed excellent activity against *B. subtilis* at all doses except IC<sub>50</sub> = 31.25 µg/mL up to 101±10.3 CFU/mL.

The results of subcritical CO<sub>2</sub> extract from the seeds of *Beta vulgaris* L., against *E. coli*, *P. vulgaris*, and *C. albicans* also showed positive results at maximum doses of IC<sub>50</sub> = 1000 µg/ml. At doses of IC<sub>50</sub> = 250 µg/mL, it showed inhibitory activity up to 181±11.3 CFU/mL against *E. coli* and *P. vulgaris*, 111±10.6 CFU/mL against *C. albicans*. When tested for antimicrobial activity, the antibacterial activity is naturally less pronounced in gram-negative microorganisms (500 and 1000 µg/mL), which is probably related to the properties of the bacterial cell wall. *P. aeruginosa* didn't show bacteriostatic activity (Table 3).

## Discussion

According to the results of the analysis of the chemical composition of the liquid CO<sub>2</sub> extract, 11 components were identified (Table 1 and Fig. 1). The sample contains phenol - 0.4% and cresol - 0.3% of all volatile substances, 2-methoxy, 1H-Pyrrole, 2,5-dihydro-1-nitroso-, Phenol, 4-ethyl-, 1,4:3,6-Dianhydro- $\alpha$ -d-glucopyranose, Phenol, 4-ethyl-2-methoxy-, Phenol, 2,6-dimethoxy-, are phenol category. The dominant components of the extract are phenol and cresol.

The overall effect of the extract is determined not only by the constituents predominant in the primary composition but also by the constituents present in small amounts, which affect the biological activity by either blocking or enhancing the effect of the individual substances.

The obtained results showed that subcritical CO<sub>2</sub> extraction of *Beta vulgaris* L. seed extract in most cases at doses of 25-50 µg showed a moderate effect on some gram-negative and gram-positive bacteria used in the experiment.

These results are in agreement with previous cytotoxicity studies, which reported that the extract from the seeds of CO<sub>2</sub> red beet at a concentration of 10 mg/mL had a cytotoxicity of 68% (Konkabayeva *et al.*, 2021).

MIC is considered the main indicator for determining the antimicrobial activity of antibacterial compounds. Low MIC values indicate that less active ingredient is required to inhibit the growth of a microorganism, which means that drugs with a low MIC value are effective against microbes and have a low threshold of toxicity. Currently, several electronic MIC databases have been developed where MIC parameters are important in diagnostic laboratories to determine the resistance of microorganisms to antibacterial compounds and to monitor the activity of new antimicrobial agents. Thus, the MIC index helps in therapy and prevents the development of drug-resistant strains of microorganisms (Kowalska-Krochmal and Dudek-Wicher, 2021).

The determination of MIC showed that the CO<sub>2</sub> extract from beet seeds had different indicators with respect to gram-positive and gram-negative bacteria. gram-positive bacteria such as *S. aureus* and *B. subtilis* showed good antimicrobial potential and the doses of minimum

inhibitory concentrations are lower than, for example, gram-negative *E. coli* and *P. vulgaris*, *P. aeruginosa*, an opportunistic pathogen, proved resistant at all doses tested in this study. Such differences in the sensitivity of microorganisms to antimicrobial agents can be explained by the composition of their cell walls and inherited genes on plasmids, among other factors (Ahmadi *et al.*, 2020). Infections with *P. vulgaris* and HL are among the most difficult to treat with conventional antibiotics, but in this study, it was found that some doses against *P. vulgaris* showed comparable efficacy to the control drug.

In general, gram-positive bacteria are more sensitive to herbal antibiotics than gram-negative bacteria, which has been confirmed by several studies (Farhadi *et al.*, 2019; Khalil *et al.*, 2018). This can be explained by the fact that gram-negative bacteria have a central resistance to a variety of antimicrobial agents, which is probably due to the hydrophobic surface of their outer membrane, which forms a barrier against toxic agents and is rich in lipopolysaccharide molecules. Due to porin proteins, small hydrophilic molecules can freely pass through the outer membrane (Arulmozhi *et al.*, 2018).

## Conclusion

Thus, a study of the component composition of CO<sub>2</sub>-extracts from *Beta vulgaris* L. seeds was carried out, according to the results of which 11 of the liquid extract components were identified. The dominant components are phenol and cresol.

In conclusion, antibacterial activity, Minimum Inhibitory Concentration (MIC), and Bactericidal Concentration (MBC) have been studied for the first time, due to studies on the possibility of antibacterial activity, antifungal activity with the establishment of a bacteriostatic dose, and IC<sub>50</sub>.

Thus, the results of the screening allow us to isolate the subcritical CO<sub>2</sub> extract from the seeds of *Beta vulgaris* L., which has a moderate potential for antibacterial and antifungal activity against the microorganisms *S. aureus*, *B. subtilis*, *E. coli*, *P. vulgaris* and the yeast *C. albicans* at doses of 25, 50 and 100 µg.

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## Author's Contributions

**Aidana Yerubay, Saule Akhmetova and Aiman Konkabayeva:** Participated in all experiments,

coordinated the data analysis and contributed to the written of the manuscript.

**Bibigul Rakhimova, Sara Baiguzhina and Aliya Baiduisenova:** Designed the research planed and organized the study.

**Igor Kalymanov:** Analysis, data collection, data interpretation, final approval.

## Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and that no ethical issues are involved.

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