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Original Article

Rumex alveolatus plant essential oil: Constituent evaluation, antioxidant and antimicrobial traits

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ABSTRACT

The food industry considers plant derivatives as safe additives with strong antimicrobial, flavoring, and antioxidant attributes. The present research aims to evaluate the biochemical characteristics of essential oil substances in Rumex alveolatus plant. The plant's essential oil was extracted and obtained components were assessed through the gas chromatography-mass spectrometry and the Fourier transform infrared spectroscopy (FTIR). The total phenolic (TPC) and flavonoid (TFC) contents, antioxidant, cytotoxicity, antimicrobial attributes, and also essential oil affected on the pathogen morphology. The main chemical constituents of Rumex alveolatus essential oil contained β-santalol (15.88 %) and α-santalol (11.41 %) and FTIR identified their functional groups. The TPC and TFC were 102.12 ± 7.3 (mg gallic acid/ g dry weight) and 72.67 ± 4.78 (mg quercetin/g dry weight), respectively. The IC50 levels of essential oil were determined as 89.19 ± 2.3 by DPPH (2,2-diphenyl-1-picrylhydrazyl) inhibition and 101.07 ± 1.5 (µg/mL) through ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radicals, which calculated 38.49 (µg/mL) for survival of Caco-2 cancer cells. Based on the results of disk and well diffusion agars, minimum inhibitory concentration (MIC) and also minimum bactericidal concentration (MBC), Listeria monocytogenes (L. monocytogenes, 5 and 128 µg/mL) and Escherichia coli (64 and 512 µg/mL) were the most sensitive and resistant strains against essential oil, respectively. According to the scanning electron microscope images, the mentioned essential oil caused changes in the cell wall of Listeria monocytogenes. As a result, Rumex alveolatus essential oil is a promising antioxidant, anticancer, and antimicrobial constituent and is applied in food products.

1. Introduction

In recent years, awareness of people has improved about healthy consumption and tendency to produce natural and also safe products because they do not illustrate side effects on human (Kumar and Singh, 2020; Mishra et al., 2018; Orbán-Gyapai et al., 2017). One of the reliable alternatives to these additives is plants, especially their extracts and essential oils (Idris et al., 2017). The consumption of additives is reduced in the food industry when medicinal plants are employed with many effective constituents (Abdelhady and Badr 2016).

Rumex belonged to *Polygonaceae* family has 50 genera and about 1200 species, which 100 and 180 are *Rumex* and also *Polygonum* (seven-banded grass), respectively, which scattered in the northern hemisphere (Cebi et al., 2021). *Rumex* specie is derived from the Latin word dart and spear that refers to the leave shape (Naseri et al., 2019; Vasas et al., 2015). Rumex is identified as Torshak in Iran and has more than 24 species that grows in the western regions, Alborz and Zagros highlands (Naseri et al., 2019). Antioxidant, detoxification, anti-inflammatory, antimicrobial, anti-diarrheal, anti-tumor, anti-inflammatory, astringent or blood clotting, treatment of skin diseases, anti-bite, diuretic and antiviral features are mentioned among the therapeutic uses of *Rumex* family (Hundie et al., 2023). These characteristics of *Rumex* are attributed to anthraquinone glycosides (chrysophenol and emodin), leucoanthocyanins, saponins, flavonoids (isorinthin, vitexin, orintin and isovitexin), tannins, glycosides and alkaloids (Naseri et al., 2019; Vasas et al., 2015). Polyphenols (phenolic acids and flavonoids) in the form of an aromatic ring with hydroxyl groups and alpha-tocopherol are the main antioxidants of this genus, which donate a hydrogen atom or an electron to free radical and inhibition derived from oxygen (Abonyi et al., 2018). Flavonoids inhibit enzymes that are responsible for superoxide anion

production such as xanthine oxidase and protein kinase C (Behbahani et al., 2017).

In past researches, antimicrobial activity of *Rumex alveolatus* extract (Korkorian and Mohammadi-Sichani, 2017), protective effect of hydroalcoholic extract on liver damage caused by CCl4 in mice (Naseri et al., 2019) and chemical and also anti-bacterial features for methanolic extract (Noshad, 2021) had been evaluated. The *Rumex alveolatus* essential oil contains 85 to 99 % and 1 to 15 % volatile and also non components that are mixtures of terpenes, terpenoids, aromatic and aliphatic constituents, which are characterized by their low molecular weight, respectively (Kumar and Singh 2020). Terpenes are composed of several 5-carbon units and monoterpenes (C10) and sesquiterpenes (C15) are detected as the main terpenes (Larayetan et al., 2017). In the present study, *Rumex alveolatus* essential oil was isolated using distillation method; then, compounds, total phenol and flavonoid contents, antioxidant function, antimicrobial activity and cytotoxicity were identified that have not been done so far and this knowledge is of great importance in novel medicine fields.

2. Materials and Methods

2.1 Materials

The 2,2-diphenyl-1-picrylhydrazyl (DDPH) and 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) were obtained from Sigma-Aldrich. CA. The strains of *Listeria monocytogenes* (*L. monocytogenes*) ATCC 23074 were prepared from National Center of Biological and Genetic Resources in Iran as lyophilized forms.

2.2 Essential oil extraction

The leaf part of *Rumex alveolatus* plant was obtained from a local market and identified by Tehran University of Research Sciences and Faculty of Food Science and Engineering, Iran. In the next step, distillation technique with water was applied by a Clevenger aparatus (Borosil, India) to extract (Cebi et al., 2021).

2.3 Chemical composition analysis of essential oil

The 0.1 µL volume of sample was diluted in hexane (10:100 ratio) and injected into a Thermo Fischer capillary gas chromatograph, which was directly coupled to a mass spectrometer system (model GC ULTRA S/N 20062969; Polaris QS/N 210729). An HP-5MS non-polar fused silica capillary column was applied with dimensions of 60 m × 0.32 mm × 0.25 µm film thickness. The heating rate was set at 2 °C/min from an initial temperature to final 260 °C for obtaining complete extraction of *Rumex alveolatus* components (Bendif et al., 2018).

2.4 Fourier transform infrared spectroscopy (FTIR)

First, sample of essential oil was dried by a freeze dryer and functional groups was analyzed using in structures an FTIR spectrophotometer (Perkin Elmer, USA) in 400 to 4000 cm^{-1} range (Hundie et al., 2023).

2.5 Antioxidant attribute

Initially, 3.9 mL DPPH stock (0.004 g in 100 mL methanol) was prepared and transferred to tube for examining. Afterwards, 0.1 mL each extract was added to stock solution and put in darkness about 30 min; then, absorbance was expressed at 517 nm (Kumar and Singh, 2020). In the first step, reaction solution (7 mM ABTS and 2.4 mM potassium persulfate) was placed in a dark incubation during 14 h at 24 °C; after that, diluted with ethanol to absorb at 734 nm (Kumar and Singh, 2020).

2.6 Cytotoxicity assay

The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) component was used to determine cytotoxicity of essential oil against Caco-2 line, cell number IBRC C10097, National Center for Genetic and Biological Resources in Iran. The cell viability curve was drawn by control cells and cytotoxicity was detected in terms of IC_{50} (Quradha et al., 2019).

2.7 Investigation of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The strains of *L. monocytogenes*, *Bacillus cereus* (*B. cereus*), *Salmonella typhimurium* (*S. typhimurium*), *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*), *Shigella dysentery* (*Sh. dysentery*) and *Staphylococcus epidermidis* (*S. epidermidis*) were obtained from National Center of Biological and Genetic Resources in Iran as lyophilized bacteria.

Dilution method was applied to determine MIC and MBC values; for this purpose, successive dilutions of each essential oils were prepared between 3.75 and 600 µg/mL and 100 µL culture of microorganisms were added to all 96-well microtiter plate. A concentration equivalent to 1 McFarland standard (3×10^8 CFU/mL) was added and microplates were placed in an incubator at 37 °C. After 24 h, the lowest essential oil concentration that had no microbial growth and turbidity was measured as MIC. The 5 µL was taken from microplate wells, transferred to culture medium without essential oil and kept in an incubator at 37 °C for 24 h to determine MBC and concentration that prevented microorganism growth was calculated as MBC.

2.8 Influence of microorganism morphology under essential oil using scanning electron microscope (SEM) images

The microorganism culture of essential oil concentration, centrifuging at 5000 g about 5 min, washing microorganism by 0.1 M phosphate buffer (pH 7), filtration using apolycarbonate, fixing pure microorganism in 2.5 % glutaraldehyde solution, storing

during 2 h at 4 °C, soaking several times by distilled water, drying sample with different ethanol solutions for 10 min, covering sample using gold and finally examining treatments were investigated through SEM (Zeiss (LEO) 1450 VP model, Germany) image (Miao et al., 2019).

2.9 Statistical analysis

The data were evaluated using Minitab software (version 16) via one-way analysis of variance ANOVA and Duncan test at confidence level of 95 % ($p < 0.05$) was applied to determine differences between means and experiments were performed at three replications. Also, results expressed as means ± SD.

3. Results

3.1 Screening chemical components of essential oil using gas chromatography-mass spectrometry

In the present research, 33 compounds were found, which included 98.62 % of total volatile components with identified peaks and essential oil was rich in monoterpenoids and oxygenated forms (Table 1). The main chemical substances were detected containing β-santalol (15.88 %), α-santalol (11.41 %), α-hemiolene (9.75 %), hemlitol (7.74 %), decosanoic acid (6.23 %), nonan (5.51 %), 4-vinyl guaiacol (4.56 %), linalool oxide (4.12 %), squalene (3.85 %), trimethylcyclohexane (3.56 %), palmitic acid ethyl ester (3.11 %), chosinol (3.21 %), tridecane (2.24 %) and E-caryophyllin (1.93 %), as indicated in Table 1 and Figure. I. The most of identified constituents were oxygenated sequiterpenes that these substances had better biological performance compared to non-oxygenated ones. The decosanoic acid (6.23 %), trimethylcyclohexane (3.56 %), palmitic acid ethyl ester (3.11 %), 4-methyloctane (1.7 %), 1,2-benzene dicarboxylic acid and bi(2-methylpropyl) ester (1.46 %) were found as methyl esters in this plant.

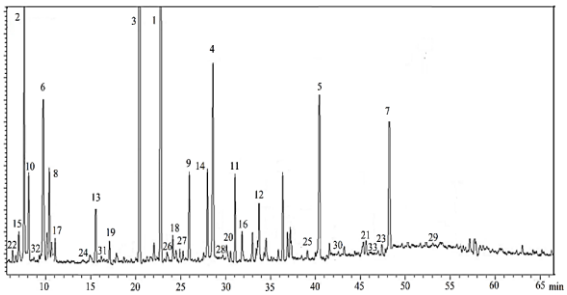

Fig 1. Chromatogram of *Rumex alveolatus* essential oil gas chromatography-mass spectrometry

3.2 Assessment of functional groups and bonds in essential oil compounds

FTIR test is applied to identify functional groups and bonds in various compound structures and spectrum of Rumex alveolatus essential oil is presented in Figure. II. The 3430 cm^{-1} peak was corresponded to stretching fluctuations of hydroxyl group (O-H) which was present in alcoholic functional groups and carboxylic acids. The essential oil including a set of hydroxyl, carboxyl, carbonyl and phenol ring functional groups and also spectrum peaks in present study indicated compounds.

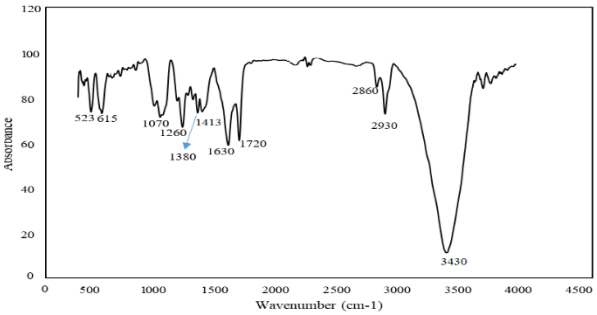

Fig 2. Fourier transform infrared spectroscopy spectrum of *Rumex alveolatus* essential oil

3.3 Antioxidant evaluation

According to the inhibition results, IC_{50} values of DPPH, vitamin C and TBHQ samples were 89.19 ± 2.3, 76.23 ± 1.2 and 71.88 ± 3.1 (µg/mL), respectively (Figure. III). IC_{50} levels were calculated for ABTS (101.07±1.5 µg/mL), vitamin C (87.15±1.2 µg/mL) and TBHQ (82.09±4.1 µg/mL). The IC_{50} of essential oil sample in both procedures was extremely close to vitamin C and TBHQ, which was a promising result. Inhibition percentages of DPPH and ABTS radicals were determined from 6.21 to 75.32 % and 5.11 to 69.34 % in essential oils (concentration 10 to 500 µg/mL), respectively. There was a significant difference between the percentages of radical inhibition in all concentrations ($p < 0.05$). Generally, the inhibition level of reference antioxidants was higher; however, a slight difference was found between them.

3.4 Cytotoxicity investigation

The cytotoxicity results of different concentrations for essential oils are given in Figure. IV. Cell survivals at 0, 10, 25, 50, 100 and 200 (mg/mL) levels were 98.52, 91.06, 65.28, 49.15, 30.09 and 14.21 %, respectively; and IC_{50} value was measured as 38.49 (µg/mL). The survival of cancer cells had declined by adding extract and there was a significant difference between all tested concentrations ($p < 0.05$). Antioxidant components decrease the oxidative stress using absorbing free radicals and malignancy burden in body cells.

3.5 Antibacterial properties

The MIC and MIB results of Rumex alveolatus essential oil are presented against pathogenic bacteria in Table 2. According to obtained results, the order of MIC and MBC for studied pathogens was as follows: E. coli > S. typhimurium > B. cereus > S. dysentery = S. epidermidis = S. aureus > L. monocytogenes and E. coli > S. dysentery = S. epidermidis > S. typhimurium = S. aureus = B. cereus > L. monocytogenes.

Table 1. Compositions of *Rumex alveolatus* essential oil identified through gas chromatography-mass spectrometry

Code	Compound	Retention time (min)	GC%	Code	Compound	Retention time (min)	GC%
1	β-Santalol	23.6	15.88	18	1,2-Benzene dicarboxylic acid, *bis* (2-methylpropyl) ester	24.3	1.46
2	α-Santalol	6.42	11.41	19	Tridecene	16.2	1.22
3	α-Humulene	28.56	9.75	20	γ-Cadinene	29.91	1.04
4	Hemellitol	20.34	7.74	21	β-Cholestane	46.23	0.91
5	Docosanoic acid	40.34	6.23	22	Ethyl-benzene	4.8	0.83
6	Nonane	8.10	5.51	23	α-Cholestane	47.5	0.80
7	4-Vinylguaiacol	48.34	4.56	24	α -Copaene	15.45	0.75
8	Linalool oxide	10.51	4.12	25	Phytol	39.5	0.70
9	Squalene	25.1	3.85	26	1(2H)-Naphthalene,3,4-dihydro-4,5,6-trimethyl	23.15	0.64
10	Trimethylcyclohexane	7.4	3.56	27	p-Mentha-1,3-diene or	24.45	0.61
11	Palmitic acid ethyl ester	31.2	3.11	28	2,6-Diisopropylnaphthalene	29.43	0.55
12	Khusinol	33.9	2.61	29	Stigmastane	53.19	0.51
13	Tridecene	15.8	2.24	30	Octadecene	42.95	0.42
14	E-caryophyllene	28.26	1.93	31	β-Cyclocitral	16.35	0.35
15	4-Methyl-octane	5.95	1.71	32	p-Xylene	8.43	0.25
16	Palmitic acid methyl ester	31.9	1.65	33	Eicosene	46.29	0.21
17	Pentadecanone	11.02	1.51		Total		98.62

Table 2. Minimum inhibitory concentration and minimum bactericidal concentration of *Rumex alveolatus* for pathogenic bacteria

Bacteria	MIC (µg/mL)	MBC (µg/mL)
L. monocytogenes	5	64
B. cereus	32	128
S. typhimurium	64	128
S. aureus	16	128
E. coli	128	512
Sh.dysenteriae	16	256
S. epidermidis	16	256

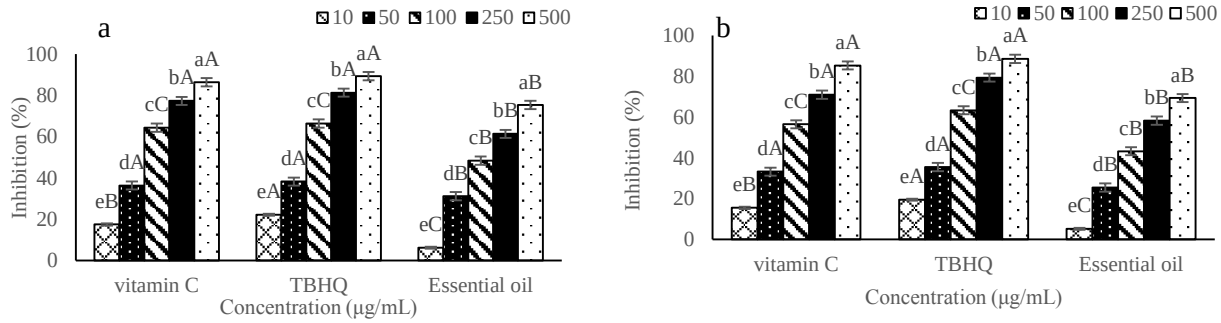

Fig 3. The graph related to inhibition percentage of DPPH (a) and ABTS (b) radicals by *Rumex alveolatus* essential oil, vitamin C and TBHQ Letters [a-e] indicate a significant difference between different concentrations of an antioxidant and letters [A-C] indicate a significant difference between essential oil, vitamin C and TBHQ at a fixed concentration.

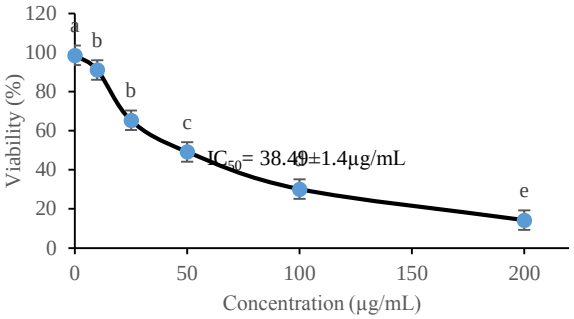

Fig 4. The toxicity effect of various concentrations for *Rumex alveolatus* essential oil on CaCo-2 cell line viability
Letters [a-e] indicate a significant difference between various concentrations for *Rumex alveolatus* essential oil.

As can be deduced from Table 2, MBC of E. coli was further towards to other bacteria with a value of more than 512 µg/mL and > L. monocytogenes was the lowest in 64 µg/mL level. MIC values of E. coli (5 µg/mL) and L. monocytogenes (128 µg/mL) were the minimum and maximum, respectively. According to comparison results obtained from DDA and WDA methods with MIC and also MBC assays, L. monocytogenes was the most sensitive and E. coli was the highest resistant strain against essential oil.

3.6 Antibacterial effect of Rumex alveolatus essential oil on microorganism morphology

The structure of L. monocytogenes is naturally double coccobacillus and treated sample with essential oil causes changes into cell wall to wrinkle and indent, fold, membrane tear and also leakage for intracellular components to outside; finally, lysis is done (Figure. Va) and bacterial inhibition is illustrated (Figure. Vb).
Figure. V

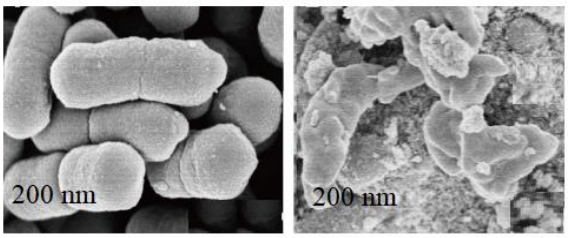

Fig 5. Scanning electron microscope images of *L. monocytogenes* bacteria (a) before and (b) after treatment with *Rumex alveolatus* essential oil

4. Discussion

Nowadays, 268 substances such as 56 quinones, 57 flavonoids, 25 tannins, 6 stilbenes, 22 naphthalene, 6 terpenes, 3 diterpene alkaloids, 14 lignans and 79 other types have been isolated and identified from 29 *Rumex* species (Naseri et al., 2019; Vasas et al., 2015). In general, these constituents are the major factors of antimicrobial, antioxidant and anticancer attributes in *Rumex* plant and the reason for difference in their amount and type is due to various species, harvesting time, growing place, shelf life, nutrients, light, water and extract processes (Li et al., 2022). According to the present results, α-santhalol (29.63 %) and β-santhalol (25.60 %) were the oxygenated compounds in *Rumex Crispus* essential oil (Hundie et al., 2023).

The several resulted peaks proved the presence of phenolic, carboxyl, benzene ring, propyl, aldehyde, cyclohexene, terpenoids (monoterpenes, sesquiterpenes and oxygenated derivatives) and phenylpropanoids (Noshad, 2021). The peaks related to symmetric and asymmetric stretching fluctuations of C-H alkyl group (nCH) had represented in 2860 to 2930 cm^{-1} range (Albayrak et al., 2022). The peaks at 1720 and 1630 cm^{-1} were attributed to C=O in aldehyde groups and C=C stretching vibrations of aromatic ring, respectively (Cebi et al., 2021).

Some studies compared different methods such as DPPH and ABTS, which were based on free radical removal using antioxidants (Kengne et al., 2021; Naseri et al., 2019). It had been reported that applied technique for measurement of antioxidant activity influenced on the obtained result (Salama et al., 2022). The antioxidant results of *Rumex Alveolatus* extract demonstrated that extract was capable to inhibiting 69.8 % DPPH and 80.59 % ABTS radicals (Noshad, 2021). The antioxidant assays against DPPH and ABTS free radicals indicated a significant inhibitory potential of *Rumex hastatus* essential oil with 3.71 and 6.29 (µg/mL) IC_{50} values, respectively; however, this level against both free radicals was lower from 0.1 $µg/mL^{-1}$ for ascorbic acid (Albayrak et al., 2022).

The toxicity of cancer cells was related to polyphenol and bioactive components leading to anti-mutagenic features (Quradha et al., 2019). Some previous research illustrated that a relationship was detected between cytotoxicity and antioxidant activity (Idris et al., 2017). The anticancer results of *Rumex nersus* illustrated that a significant activity was detected using crude methanolic extract against MCF-7 cell line with an IC_{50} equal to 20.51 (µg/mL). The IC_{50} results against MDA-MB-231 cell line illustrated that crude extract was 25.17 (µg/mL) level (Quradha et al., 2019). The IC_{50} value was found to be 501.4 (µg/mL) for *Rumex vesicaris* extract at 0 to 1000 (µg/mL) concentrations towards to HepG2 cell line (Salama et al., 2022).

The results exhibited that essential oil had strong antibacterial activity against different species. Similar to the present results, different parts of *Rumex* species were investigated as important factors in inhibiting bacteria such as *S. epidermidis*, *S. aureus*, Methicillin-resistant *S. aureus* MRSA, *B. subtilis*, *Moraxella catarrhalis*, *Streptococcus pyogenes*, *Streptococcus pneumoniae* and *E. coli* with inhibition zones > 15 mm (Orbán-Gyapai et al., 2017). In previous study, three *Rumex abscisicus* extracts were active towards to all tested

microorganisms, which MIC and MBC values ranged from 32 to 256 µg/mL. The highest activity was observed with crude extract (MIC equal to 32 µg/mL) against *Clostridium neoformans* (Kengne et al., 2021). Several tests on different plant parts of various *Rumex* species had confirmed their strong antibacterial functions against Gram-positive and negative bacteria. Various factors affected antimicrobial behavior of essential oils, which included type and amount of antimicrobial substances (phenolics, quinones, beta-carotene, flavonoids and tannins), microorganisms, essential oils and their actions and also mechanism (Behbahani et al., 2017; Miao et al., 2019). Generally, phenolic constituents are the most important antimicrobials in plant extracts, usually their mechanisms are based on enzymatic inhibition of oxidized compounds or blocking sulfhydryl groups in proteins (Dholwani et al., 2008). Gram-negative bacteria have a complex cell membrane structure based on lipopolysaccharide (two layers of phospholipids), but Gram-positive bacteria indicated monolayered mucopeptide in structure (Behbahani et al., 2017; Raut and Karuppayil, 2014).

The effect of shikonin component was indicated *L. monocytogenes* ATCC 19115 cells that bacterial biofilms were thick, heterogeneous and strong in control sample; however, integrity, three-dimensional structure, numbers attached to surface and single cells after treatment gradually decreased (Li et al., 2022). One of the defense mechanisms for *L. monocytogenes* was motility and film formation controlled through genes and essential oil probably reduced the transcription of flagella gene (flaA), which indicated an important factor in adhesion (Miao et al., 2019). The essential oil stimulates the depolarization of mitochondrial membranes by decreasing wall potential in eukaryotic cells (Kumar and Singh, 2020; Mishra et al., 2018; Orbán-Gyapai et al., 2017).

5. Conclusion

As a result, essential oil of this plant has necessary potential to act an important role in food and applies as a capsule (supplement) due to various secondary and biological metabolites in structure.

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