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Research Article

ANTIMICROIAL ACTIVITY, CG/MS ANALYSIS AND CYTOTOXIC POTENTIAL of ESSENTIAL OIL of BERGAMOT (*Citrus bergamia* risso et poiteau) PEEL

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ABSTRACT: In this study, bergamut (*Citrus bergamia* Risso et Poiteau) essential oil (BEO) extracted from bergamut peel was examined for antimicrobial effects, volatile compounds, and cytotoxic properties against certain cancer cell lines. Disc diffusion and microdilution methods were orderly used to determine antimicrobial and the minimum inhibitory concentration (MIC) values of BEO. Cytotoxic activity was revealed using MTT assay. Among the six bacteria tested, only *Bacillus subtilis* and *Staphylococcus aureus* showed the highest antimicrobial activity, in comparision with erytromycin and ampicilline positive controls. BEO exhibited antimicrobial activity against *Candida albicans* as well. In addition, MIC value was only observed for *Escherichia coli* (8 mg/ml) and *C. albicans* (1.6 mg/ml). The GC/MS analyses showed the presence of 18 compounds in BEO. The main constituents were found to be linalool (46.58%), limonene (22.32%) and linalyl acetate (15.46%). BEO at 0.06% resulted in different cytotoxicities against MCF-7 (85%), A549 (78%) and HeLa (70%) cells.Jurkat cells were more resistant against BEO because only 38% cytotoxicity was observed at the same concentration of BEO. In conclusion, BEO from Turkey has potential source of active compounds for development of therapeutic and preventive strategies as well as cancer disease.

Keywords: *Citrus bergamia; essential oil; antimicrobial activity; GC/MS analysis; cytotoxic activity*

Özet: Bu çalışmada, bergamut kabuğundan ekstrakte edilen bergamut (*Citrus bergamia* Risso et Poiteau) esansiyel yağının (BEO), bazı kanser hücre hatlarına karşı antimikrobiyal etkileri, uçucu bileşikleri ve sitotoksik özellikleri açısından incelenmiştir. BEO'nun antimikrobiyal ve minimum inhibitör konsantrasyon (MİK) değerlerini belirlemek için disk difüzyon ve mikrodilüsyon yöntemleri sırayla kullanılmştır. Sitotoksik aktivite MTT tahlili kullanılarak ortaya çıkarılmıştır. Test edilen altı bakteri arasında yalnızca *Bacillus subtilis* ve *Staphylococcus aureus*, eritromisin ve ampisilin pozitif kontrolleriyle karşılaştırıldığında en yüksek antimikrobiyal aktiviteyi gösterdi. BEO, *Candida albicans*'a karşı da antimikrobiyal aktivite sergiledi. Ayrıca MİK değeri sadece *Escherichia coli* (8 mg/ml) ve *C. albicans* (1,6 mg/ml) için gözlenmiştir. GC/MS analizleri BEO'da 18 bileşiğin varlığını göstermiştir. Ana bileşenlerin linalool (%46,58), limonen (%22,32) ve linalil asetat (%15,46) olduğu belirlenmiştir. %0,06 BEO, MCF-7 (%85), A549 (%78) ve HeLa (%70) hücrelerine karşı farklı sitotoksisitelerle sonuçlanmıştır. Jurkat hücreleri BEO'ya karşı daha dirençli olduğu çünkü aynı BEO konsantrasyonunda yalnızca %38 sitotoksisite gözlendiği saptanmıştır. Sonuç olarak, Türkiye'den BEO, kanser hastalığının yanı sıra tedavi edici ve önleyici stratejilerin geliştirilmesi için potansiyel aktif bileşik kaynağına sahiptir.

Anahtar kelimeler: *Citrus bergamia; esansiyel yağ; antimikrobiyal aktivite; GC/MS analiz; sitotoksik aktivite*

INTRODUCTION

Current consumer trends and the increasing isolation of antibiotic resistant pathogens have led to a renewed scientific interest in plant derived compounds. In this content, especially Rutaceae, Alliaceae, Zingiberaceae and Malvaceae are known as an antimicrobial activity. Bergamot *(Citrus bergamia* Risso et Poiteau) is an aromatic herb belonging to Rutaceae. In Turkish folk medicine, the bergamot is a fruit with rudely pear-shaped. One of the main essential ingredients for perfume production is Bergamot oil and this oil is widely used in cosmetics such as aromatherapy, especially to reduce stress and anxiety [1, 2, 3, 4]. Antiinflammatory, anti-proliferative activities and analgesic effects were also demonstrated. This oil called as 'natural essence' and directly acquired from the cold-pressed peels of the fruit is a yellow-green liquid [3, 4].

In earlier, the antimicrobial activity of flavonoids extracted from bergamot peel which is a spin-off of essential oil industry was examined towards gram-negative bacteria, gram-positive bacteria and the yeast *Saccharomyces cerevisiae*. The ethanolic fractions of bergamot were determined to be active on all gram-negative bacteria tested, and their antimicrobial capacity was reported to increase after enzymatic deglycosylation [4].

Nowadays, discovery of new plant extract with high anticancer and antimicrobial activities has being gained so much attention. Because the level and type of secondary metabolites from the same plant species may change depending on the environmental factors, the aim of this present study was to identify the chemical composition and antimicrobial activity of BEOcollected from Turkey against both clinical and food borne microorganisms and the cytotoxic potential of BEO against certain cancer cell lines.

MATERİALS AND METHODS

Collection of plant material

Samples of *C. bergamia* et Poiteauwas collected from farmers in Muğla, Turkey and identified by Professor Dr Aykut Güvensen (Department of Biology/Botany, Faculty of Science, Ege University, İzmir).

Essential oil extraction

Forty grams of air-dried aerial parts of each plant were hydrodistilled using a Clevenger-type apparatus for 3 h. Oil samples were dried over anhydrous sodium sulphate and kept at a low temperature and then analysis were carried out. For GC analysis, the essential oil samples were dissolved (2% v/v) in n-hexane.

Microorganisms and culture conditions

Six bacteria strains, three Gram-positive bacteria strains, including *Staphylococcus aureus* ATCC 6538P, *Bacillus subtilis* ATCC 6633 and *Bacillus cereus* CCM 99, three Gram-negative bacteria strains, including *Escherichia coli* ATCC 29998, *Salmonella typhimurium* CCM 3819, *Pseudomonas aeruginosa* ATCC 27853, and also a yeast *Candida albicans* ATCC 10259, were used for determining the antimicrobial activities of bergamot peel essential oil (BEO). The bacteria species were cultured in Mueller- Hinton Broth (Merck) overnight at 37 °C. *C. albicans* was incubated in Sabouraud Dextrose Broth (Oxoid) for 48 h at 30 °C.

Determination of antimicrobial activity by disc diffusion method

Antimicrobial activity of the bergamot peel essential oil (BEO) was determined by the paper disc diffusion method [5]. Shortly, sterile, 6 mm diameter filter paper discs (Schleicher and Schüll, No. 2668, Germany) were made suck with 30 µl of extract (1.0 mg/disc). The test organisms were added to the agar plates. After incubation, the sterile discs impregnated with the different extracts were placed on the agar plates. Incubation conditions were at 30±0.1 °C for 48 h for the bacterial plates, and at 25±0.1 °C for 72 h for the yeast plates. Erythromycin discs (Oxoid, 10 µg/disc), ampicilline discs (Oxoid, 10U/disc) and nystatin discs (Oxoid, 30 µg/disc) were served as a positive control for detecting the sensitivity of the strains tested. After incubation, all plates were examined in terms of observing the growth inhibition zones measured in mm of their diameters. All tests were carried out in duplicate and repeated two times under sterile conditions.

Determination of minimum inhibitory concentration (MIC)

The MIC was detected for antimicrobial activity of BEO. The lowest concentration that inhibited growth following incubation was considered MIC. The microdilution assay was made according to expressed in the CLSI standards with some modifications. In summary, The bacterial (5×10^6 cfu/mL) or yeast (5×10^5 cfu/mL) suspensions in Mueller–Hinton broth or Sabouraud Dextrose Broth, respectively was added to each well. The different final conconcentrations of BEO (400, 200, 100, 50, 25, 12.5, 6.25, 3.12, 1.56 and 0.78 mg/mL) was used for this assay. Each 96-well microplates prepared for all bacteria strains and *C. albicans* was incubated at 37 °C for 18-24 h and at 30 °C for 48 h, respectively. The lowest concentration that inhibited growth following incubation (no visible growth) was considered MIC.

GC/MS analysis

For GC/MS analysis, the steam-distilled components were used. A gas chromatograph (HP 6890) accoutred with a HP- PTV and a 0.32 m x 0.60 m HP-Innowax capillary column (0.5µm coating) was used for the GC analysis. GC/MS analysis was carried out on a HP-5973 mass selective detector coupled with a 6890 gas chromatograph, equipped with a HP 6890 gas chromatograph, equipped with HP-1capillary column. From an initial temperature of 60 °C to a final temperature of 250 °C at 15 °C/min, the column temperature was programmed. Helium (14.1 ml/min) was used as the carrier gas. Individually compound identification was carried out by comparing mass spectra with data from literature and by comparing their retention time (Rt) relative to a C8-C32 n-alkanes mixture[6]. Wiley 7n.l GC/MS library and ARGEFAR GC/MS library created with authentic samples were used in the computerized inquiry.

Cell lines and culture conditions

A549 (lung adenocarcinoma), HeLa (cervix adenocarcinoma), MCF-7 (breast adenocarcinoma), Jurkat (acute T cell leukemia) and BEAS-2B (non-cancerous bronchial epithelium) human cell lines were obtained from American Type Culture Collection (ATCC). All cell lines were cultured in Roswell Park Memorial Institute (RPMI)-1640 Medium (Biochrom, Germany) with heat inactivated 10% fetal bovine serum (FBS) (Biochrom, Germany), 100 U/mL penicillin and 100 µg/mL streptomycin (Biochrom, Germany). The cells were maintained in a humidified incubator with 95% air and 5% CO_2 at 37 °C.When the cells reached 70–80% confluence, they were passaged or used for analysis. Additionally, the trypan blue (Applichem, USA) dye exclusion was used to determine number of viable cells in our cell suspensions.

Determination of cytototoxicity by MTT assay

The cytototoxic effects of BEO on A549, HeLa, Jurkat, MCF-7 and BEAS-2B and cell lines were determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5- dipenyltetrazolium bromide] (Applichem, USA) assay [7]. The mitochondrial dehydrogenase in metabolically active living cells are able to reduce yellow soluble MTT into insoluble blue formazan product. So, the amount of formazan formed reveals the cell viability. In this assay, the 200 µl of exponentially growing cells were plated at $4x10^3$cells/well in 96-well microplates (Greiner, Germany) as triplicate and incubated for 24 hours in 5% CO_2 incubator at 37 °C. Then, the cells were treated with the bergamot essential oil at eight

different serial concentrations from 1% to 0.01% (v/v) prepared in cell growth medium and incubated for additional 24 hours. Afterwards, the medium in each well was replaced with 100 µl of fresh growth medium and 10 µl of MTT (5 mg/ml) in phosphate buffer solution (PBS) was added into each well. After 4 hours incubation, the medium was removed and replaced with 100 µl of DMSO (Applichem, USA) to dissolve the formazan crystals. Lastly, the absorbance of reduced MTT was measured at 540 nm on a microplate reader (Thermo Scientific, Multiscan FC, USA). Eachindependent experiments was repeated three times and untreated cells served as control. The percentage of cytotoxicity was calculated using the formula shown below:

cytotoxicity %= [(Mean Abs of control - Mean Abs of treated cells) / Mean Abs of control] x 100

Statistical analysis

All data obtained from cytotoxicity experiments were analyzed using GraphPad Prism 7.0 (GraphPad Software, Inc., San Diego, CA). Significance was reported as $****P<0.0001$ and $***P\leq0.001$ compared to control. The IC_{50} value (causes a 50% cell death) was also calculated using Graph Pad Prism 7.00.

FINDINGS

Antimicrobial activity and minimum inhibitory concentration of BEO

The antimicrobial activity ofBEOwas studied by disc diffusion method. When BEO was tested against six bacteria and one fungus, various levels of antimicrobial activity were observed (Tab. 1). Among the bacteria, *B.subtilis* showed the best antimicrobial activity with 37 mm inhibition zone. Erithromycin and ampicilline were tested as positive control. Based on the results presented in Tab. 2, only *E.coli* showed antimicrobial activity with 8 µg/mL MIC value.

Table. 1. Antimicrobial activity of bergamot essential oil (beo) by the disc diffusion method

Microorganisms	BEO (Zone of Inhibition) (mm)	Antibiotics*		
		E	A	N
S.aureus	33	21	13	NT
P.aeuruginosa	9	17	23	NT
E.coli	10	-	-	NT
B.subtilis	37	31	23	NT
B.cereus	27	33	31	NT
S.typhimurium	13	7	17	NT
C.albicans	10	NT	NT	24

*E: Erithromycin A: Ampicilline N: Nystatin NT: Not tested (-): No inhibition

Table. 2. MIC values of bergamot essential oil (beo) erithromycinand nystatin against test microorganisms.

Microorganisms	BEO (MIC) (mg/mL)	Antibiotics*		
		E	A	N
S.aureus	-	3.2	1.6	NT
P.aeuruginosa	-	0.8	0.8	NT
E.coli	8	1.6	1.6	NT
B.subtilis	-	0.4	0.8	NT
B.cereus	-	0.8	0.8	NT
S.typhimurium	-	3.2	5.6	NT
C.albicans	1.6	NT	NT	0.4

*E: Erithromycin A: Ampicillin N: Nystatin NT: Not tested (-): No effect

GC/MS Analysis of BEO

The GC/MS analyses allowed 18 compounds to be determined; the main constituents of the essential oil of bergamot (*C. bergamia* Risso et Poiteau) were linalool 46.58%, limonene 22.32%, linalyl acetate 15.46%. The GC-MS results were given Tab. 3.

Table. 3. Volatile components of the essential oil of bergamot (*Citrus bergamia* Risso et Poiteau) peel (GC-MS analysis).

Component[a]	Area (%)	Retention[b]
Linalool	46.58	5.54
Limonen	22.32	6.95
Linalylacetate	15.46	7.94
Alpha Terpineol	3.42	8.87
Gamma Terpinen	3.06	9.56
Neryl Acetate	1.11	10.00
Beta Myrcene	0.87	10.05
Geranial	0.66	11.09
Nerol	0.61	10.02
Trans € Beta Ocymene	0.48	23.29
Neral	0.47	23.32
Alpha Pinen	0.36	23.69
Alpha Terpinelon	0.28	24.00
Trans alpha bergamotene	0.21	24.55
Cis(€ Beta Ocymene)	0.23	24.82
Beta Pinen	0.17	25.42
Geranial Acetate	2.00	26.66
Trans Geranial	1.69	27.97

[a] Components listed in order of elution from a HP-1capillary column
[b] Retention time (as min)

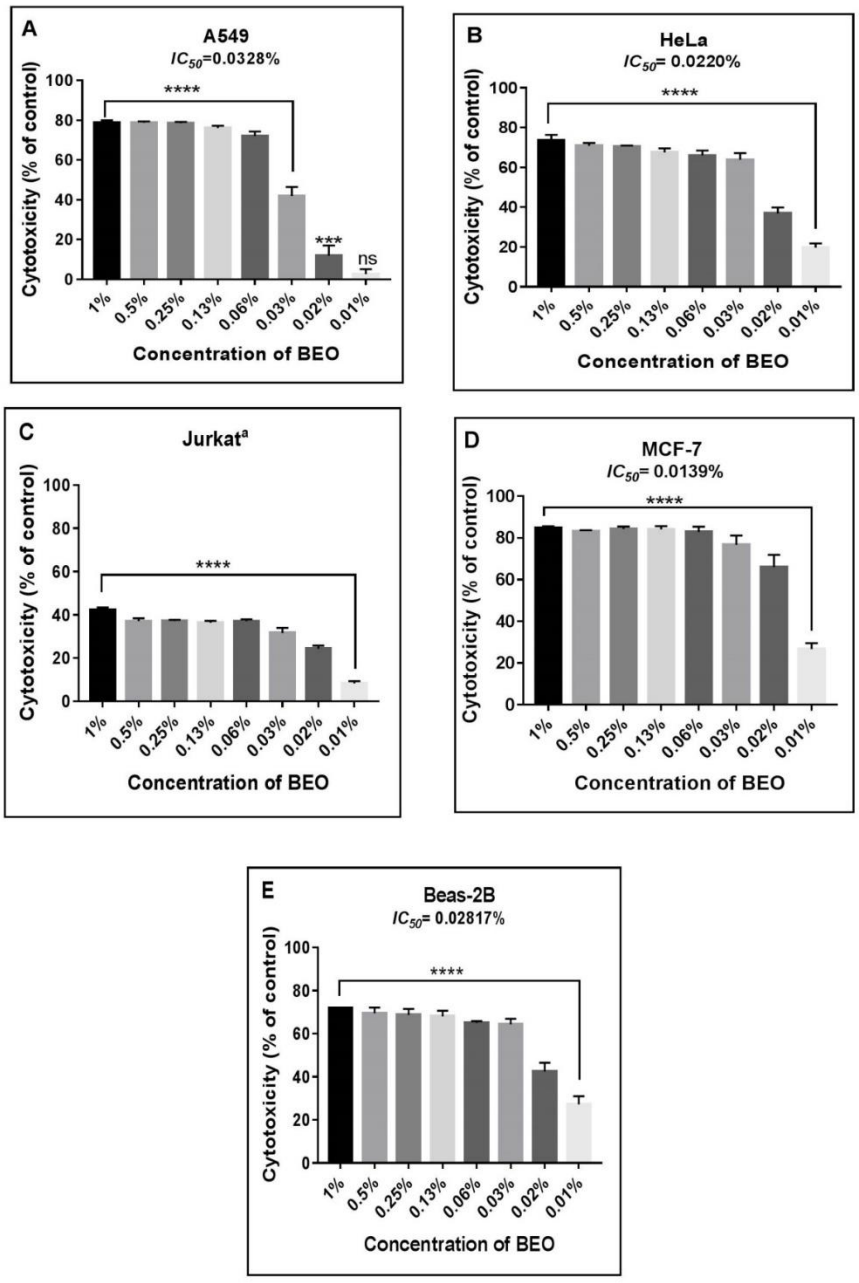
Cytotoxic activity of BEO on different human cancer cell lines

This present study examined the cytotoxic activity of BEO against different cancer cell lines. Serial dilutions of BEO from 1% to 0.06% caused 79% to 72% cytotoxicity against A549 cells (Fig. 1A). However, level of cytotoxicity of BEO at lower

concentrations of 0.03%, 0.02% and 0.01% decreased to 42%, 12% and 0.25% in A549 cells, respectively (Fig. 1A). Cytotoxicity of BEO from 1% to 0.03% concentrations changed between 74% and 64% in HeLa cells whereas BEO at 0.02% and 0.01% concentrations resulted in 37% and 17% cytotoxicity (Fig. 1B). Although cytotoxicity of each concentration of BEO tested on Jurkat cell was statistically significant, they caused cytotoxicity less than 45% (Fig. 1C). Among the all cell lines tested, MCF-7 cells were the most sensitive cells to BEO because they exhibited 85% cyoyoxicity in the presence of BEO at 1% and 0.06% concentrations (Fig. 1D). In addition to cancer cell lines, the cytotoxicity of BEO was also determined on normal bronchial epithelium cell line (BEAS-2B) and found that BEO caused cytotoxicity of BEAS-2B similar to that observed on HeLa cells (Fig. 1E).

BEO exhibited cytotoxicity on the all cancer cell lines tested. Among the cell lines, Jurkat was the most resistant cells because it exhibited 42% cytotoxocicity against BEO at 1% whereas other cell lines showed more than 70% cytotoxicity. In addition, MCF-7 was found to be most sensitive cell line because 85% cytotoxicity was observed on these cells. Most importantly, selective cytotoxicity of BEO was detected between MCF-7 and BEAS-2B cells. For example, BEO at 0.02% resulted in 70% cytotoxicity on MCF-7 whereas it caused 40% cytotoxicity on BEAS-2B cells. In other words, IC_{50} values of MCF-7 and BEAS-2B cells were 0.013% and 0.028%, repectively.

Figure. 1. Cytotoxicity of BEOon different cancer cell lines.Cells were plated onto 96-well plate and treated or untreated (control) with different concentrations of BEO for 24 h. Cytotoxicity was determined based on MTT assay. Data represents mean value of three independent experiments. Statistical analysis was performed with One-Way ANOVA, Tukey's multiple comparison post hoc test using Graph Pad Prism 7.00. Significance was reported as ****$P<0.0001$ and ***$P\leq0.001$ compared to control and ns = non-significant ($P>$ 0.05). The IC_{50} value was estimated using Graph Pad Prism 7.00 and [a]=IC_{50} value was not calculated because no more than 50% of cytotoxic activity was observed.



DISCUSSION

The antibacterial and antifungal properties of BEO have been shown against *Escherichia coli* O157, *Campylobacter jejuni*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus* and dermatophytes [8, 9]. BEO possess in vitro activity against Candida species and this property of BEO indicates its potential function in the local therapy of Candida illnesses[10]. A study was conducted to examine the in vitro efficacy of oils and vapours of bergamot and its constituents against common foodborne pathogens, and it was revealed that the most effective antibacterial component was

linalool[8,9]. In a previous study, a dose-dependent important inhibitory effect of chitosan-based films comprising BEO at 0.5, 1, 2, and 3% w/w on the growth of *Penicillium italicum* was demonstrated [10].

Similar to our results, Kirbaşlar et al. [2009] reported that all bergamot peel oils were more active against *S. aureus* among the tested microorganisms[6]. Also, it is observed that bergamot peel oils are effective on all bacteria and fungi. Quirino et al. [2016] hat distilled bergamot extract showed the highest antimicrobial activity, especially when compared with essential oil, against drug-resistant Gram-negative bacteria such as *Klebsiella pneumoniae*, *Stenotrophomonas maltophilia*, *E. coli*, *Acinetobacter baumannii*, which were the public health issue in worldwide because of their ability to cause hospital-acquired infections [11]. Gabriele et al. (2017) [12] reported that the most sensitive Gram negative microorganisms were *E. coli* ATCC 25922 and *S. typhimurium* ATCC 14028 with MIC values of 500 µg/mL in their study which they determined the antibacterial activity of Bergamot extract on potentially pathogenic bacteria. However, they stated that *Enterobacter aerogenes* ATCC 13048 was inhibited at 1000 µg/mL. Also, they reported that compared to two Gram-positive bacteria, *S. aureus* ATCC 25923 with MIC values of 500 µg/mL was more susceptible than Enterococcus faecalis ATCC 29212 with MIC values of 700 µg/mL. Gram-positive and Gram-negative strains were found to be similar in terms of the inhibitory effect. Furneri et al. (2012) reported that *C. bergamia* essential oil inhibited *Mycoplasma hominis* with MIC50 values of 0.5% and 1% for MIC90, and *Mycoplasma pneumoniae* with MIC value of 0.5%, *Mycoplasma fermentans* with a MIC value of 1% (v/v) [13].

Extensive research has been conducted to determine the chemical composition of BEO

[14,15]. BEO including different bioactive molecules with beneficial properties for health consists of a volatile (93-96% of total) and a non-volatile (4-7% of total) fraction. Monoterpene and sesquiterpene hydrocarbons, and their oxygenated derivatives, along with aliphatic aldehydes, alcohols, and esters are firstly represented[14,15]. A furocoumarins-free essential oil has been made for perfumery and cosmetic uses because of photo-toxicity induced by 5-MOP. The distinctive flavour of *Citrus* oils is chiefly furnished by linalool, citral and linalyl acetate. In addition, the limonene and pinene with not much flavouring are required to be eliminated for improving the shelf life of the products as they are relatively unstable compounds when exposed to heat and light[16].

Bergamot contains another terpene linalool which is significant in defining the intensity of the fragrance and may exhibit any significant antimicrobial activity. Citral and linalool defined as active antimicrobial compounds in *Pelargonium* EOs were stated to possess antimicrobial activities towards both *B. cereus* and *S. aureus* [17,18]. (Lis-Balchin et al. 1998) [17], Marotta et al. (2016) [18] and Furneri et al. (2012) [13] reported that the major compounds were hydrocarbons monoterpenes like Limonene and Linalyl acetate and linalool [13, 17,18].

Due to the complex phytochemical composition, BEO has different biological activities such as reducing anxiety and stres, anti-iflamatory and anti-tumor effects in the literature.A major aspect of chemotherapic drugs are to reduce cell proliferation and to induce cell damage.. In the studies of Nair et al., (2017) [19] biocompounds from *Citrus bergamia* and *Citrus reticulata* were found to cause apoptosis in cancer cell lines A549 and DLA. In addition, Russo et al. (2014) [20] reported that BEO at 0.02% and 0.03% caused apoptotic and necrotic cell death in human SH-SY5Y neuroblastoma cells; conversly, no cytotoxic effects were observed at lower concentrations (0.005% - 0.01%). Besides, BEO induced modulation of autophagic markers in this cell line. When the cells were treated with the most abundant monoterpens d-limonene or linalyl acetate, d-limonene was found to act in the induction of autophagic markers. These studies indicate that mechanism of biologic activities of BEO changes acording to each individual constituent, concentration of the substance and type of cells used.

It has been known that BEO derived from *Citrus bergamia* Rissoet Poiteau consists of more than 345 compounds that can be divided into volatile fraction (93-96%) containing monoterpene and sesquiterpene and the non-volatile fraction (4-7%) including poly-methoxylated flavones, coumarins and psoralens, such as bergamottin and bergaptene [14,15] When volatile fraction was identified by GC/MS analysis in this present study, main constituents were found to be linalool (46.5%) limonene (22.3%) and linalyl acetate (15.4%). However, BEO from Italy and used by Russo et al. (2014) contained 22 compounds. Among them, limonene (39.7) and linalyl acetate (29.5%) were the major components [20]. Unlike our results, they found linalool as 6.7%. This is an important indication that geographic location, climate, altitude and ecological conditions result in a change in the level and variety of seconder metabolites in the same plant.

In this present study, essential oil from *C. bergamia* was identified and investigated for their cytotoxic activities against 4 different cancer cell lines and 1 normal cell line. Except Jurkat cells, BEO at 0.06% caused more than 70% cytotoxicity against A549,

HeLa and MCF-7 cells. In addition, BEO exhibited selective cytotoxicity against MCF-7 cancer cells and BEAS-2B normal cells in that MCF-7 cells had 0.013% LC_{50} value whereas BEAS-2B cells had 0.028% LC_{50} value. Future studies will examine the effects of each individual constituent of BEO or combinational effect of the constituents on different parameters of anticancer activities. For instance, Russo et al. (2013) indicated that combination of limonene and linalyl acetate caused significant cytotoxicity but no other combinations exerted significant effect on cytotoxicity of BEO against SH-SY5Y neuroblastoma cells. Therefore, isolation of pure compounds in a future study will show if each constituent alone or with different combinations may exhibit increased cytotoxic activity against cancer cells originated from different organs.

Conclusion

BEO of *C. bergamia* from Turkey exhibited antimicrobial activities against all microorganisms tested and it was more effective against *S. aerus* and *B. subtilis* than positive control erytromycin. In addition, BEO displayed significant cytotoxic activities against A549, HeLa, MCF-7 and Jurkat cancer cell lines. Especially selective cytotoxicity was detected between MCF-7 cells and normal cell line BEAS-2B. Additinal study is necessary to test BEO on a different number of pathogenic strains in order to identify a natural alternative for the bio-control of the pathogen. Future work will reveal the mechanism of cytotoxicity of BEO against cancer cells as well.

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