European Journal of Life Sciences

Volume: 3 • Issue: 1 • April 2024 • ISSN: 2822-5333

ANADOLU UNIVERSITY
Faculty of Pharmacy

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Volume: 3 • Issue: 1 • April 2024

ISSN: 2822-5333

European Journal of Life Sciences

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Publication Type
International peer-reviewed journal

Publication Frequency
Triannually

Language
English

Website
https://dergipark.org.tr/ejls

Publisher
Anadolu University Faculty of Pharmacy

Publisher Address
Anadolu University, Faculty of Pharmacy
Yunus Emre Campus, 26470 Tepebaşı/Eskişehir
Phone: +90 222 335 05 80
E-mail: ejls@anadolu.edu.tr
Web: https://eczacilik.anadolu.edu.tr

Publishing Services
Akdema Informatics, Publishing, and Consultancy Trade LLC
Address: Kızılay Mahallesi, Gazi Mustafa Kemal Bulvarı No: 23/8, 06420 Çankaya/Ankara
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European Journal of Life Sciences 2024; 3: 1-8

Research Article

The prevalence of helminth eggs in raw vegetables from street markets in Eskişehir, Türkiye

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https://doi.org/10.55971/EJLS.1398299

Received: 30.11.2023
Accepted: 05.03.2024
Available online: 30.04.2024

ABSTRACT

This research was performed on a total of 120 vegetables, with 12 samples taken from each available Eskişehir district markets. In this study, we aimed to determine the presence of contamination from helminth eggs in 10 types of vegetables: carrot, lettuce, parsley, arugula, cress, fresh onion, mint, cucumber, dill, and purslane. 200 g of material from each collected sample was prepared for the helminthological examination. The prepared samples were examined for helminth eggs with a light microscope that could be identified at 40x magnification. The data were analyzed using SPSS 16 software. Helminth eggs were detected in 20 (16.6%) vegetable samples. According to the research, the highest rate of helminth eggs was observed in spring onion (66.6%), while the lowest rate was found in mint (16.6%) and purslane (16.6%). *Ascaris lumbricoides* eggs were found in 11 (9.1%) of the samples, and strongyle type eggs in 9 (7.5%). In this study, we observed that infectious forms of parasites can contaminate many foods and potentially infect people after consumption if these foods are not washed well. The aim of this study was to assess the presence of helminth eggs in raw vegetables, which are commonly consumed produce sold in street markets in Eskişehir, Türkiye.

Keywords: Eskişehir, helminth contamination, street markets

1. INTRODUCTION

The World Health Organisation (WHO) reports that diseases caused by contaminated food are the most common health problems in the modern world [1]. Factors that can cause contamination in food include bacteria, viruses, fungi, and parasites [2]. Parasites are commonly found in freshwater sources contaminated by either human or animal faeces. Consumption of fruits and vegetables grown or washed with water containing infective parasites causes infections [3,4]. The origin of water contamination can be from animals or humans, which can lead to contamination of products during cultivation, collection, transportation, and storage. The rise in organic farming also increases the risk of parasitic contamination of vegetables and fruits [5-7]. Parasitic infections can adversely affect human health in all age groups [8]. Parasites are the main causes of childhood diarrhoea and growth delay, particularly in non-developed countries [9,10]. Appropriate disposal of domestic and animal husbandry waste is a basic strategy to prevent orally transmitted parasitic infections by preventing contamination of food or water. Complete or detailed washing of pre-cooked vegetables and fruits removes parasitic cysts, oocysts, and eggs. However, it can be difficult

to clean leafy vegetables and peeled fruits. Washing hands frequently, using clean utensils and tools, and taking precautions to prevent cross-contamination are important for clean food consumption [3]. Failure to establish an effective washing and hygiene standard in places with high consumption, such as hospitals, schools, restaurants, and hotels, increases the risk of parasite infection transmission to humans [6,11-14]. This study aimed to detection of helminth contamination in various vegetable samples.

2. MATERIALS AND METHODS

2.1. The study area

The study was carried out in the location of Eskişehir. Eskişehir is located in the Northwest of the Central Anatolia Region of Türkiye, at 39° 46′ 60″ North latitude and 30° 31′ 0″ East longitude. It has a hot and dry climate in the summer, while it is cold and snowy in the winter. The weather is hot and rainy in spring and autumn, and the precipitation is mainly in the form of rain. The average annual rainfall of Eskişehir is 336.7 kg/m^3, and the annual temperature is 11°C. Although industry is at the top of the list as a source of income in Eskişehir, agricultural production is significant in some parts. Mihalgazi and Sarıcakaya are two important districts of Eskişehir that have microclimate characteristics. Controlled greenhouse-grown seasonal vegetables are grown in these districts and nearby regions. Apart from these zones, in the province, especially grain and the production of plants such as sugar beet are important. Since most of the cultivated land is dry land and the amount of annual rainfall in the province is low, it is not possible to cultivate all of the owned land every year [15].

2.2. Sample collection

As research material, a total of 120 unwashed vegetable samples (12 samples of each type: carrot, lettuce, parsley, arugula, cress, spring onion, mint, cucumber, dill, and purslane) were collected from Eskişehir district markets (four district markets in total) between March and April in 2017. These samples were transferred to the laboratory in sealed plastic bags. Three of each vegetable were collected from each of the four district market vendors.

2.3. Determination of helminth eggs

All vegetable samples were prepared for helminthological examination with 200 g of each sample. The samples were washed in 1.5 litres of detergent solution (1% sodium dodecyl sulphate and 0.1% Tween 80) and left for 12 hours. Then, the washing solution was transferred to 50 ml polypropylene centrifuge tubes and centrifuged at 1500 g for 15 minutes. Sediments were combined into a single tube and washed twice with PBS. The Fülleborn saturated salt water flotation method was applied to the final sediment and examined for helminth eggs using a light microscope at 40x magnification, which was sufficient for identifying eggs [6,16].

2.4. The statistical analysis

Research data were analyzed with SPSS 16 software. Percentage, Chi-squared, and Fisher's exact tests were used to analyse the data, and the p-value less than 0.05 was considered as significant ($p \leq 0.05$).

3. RESULTS AND DISCUSSION

Helminth eggs were detected in 20 (16.6%) of all 120 samples. *Ascaris lumbricoides* eggs were found in 11 (9.1%) of samples, and strongyle type eggs were found in 9 (7.5%) samples (Table 1). We found that lettuce, arugula, spring onion, mint, and purslane were contaminated with at least one species of helminth egg. There were no helminth eggs found in any dill, cucumber, parsley, cucumber, or carrots. Spring onions had the highest percentage of helminth eggs (66.6%), whereas mint (16.6%) and purslane (16.6%) exhibited the lowest rates. Arugula has the highest percentage of *Ascaris lumbricoides* eggs (33.3%), while spring onions (8.3%) and purslane (8.3%) have the lowest rates. Strongyle type eggs were most prevalent in spring onions (58.3%) and the least in lettuce (8.3%) and purslane (8.3%). Lettuce, spring onion, and purslane (Table 1) contained more than one helminth egg type, including both *Ascaris lumbricoides* egg and strongyle type egg.

Table 1. Distribution of helminth eggs detected in the examined vegetable samples in street markets in Eskişehir

Sample (n)	*Ascaris lumbricoides* eggs		Strongyle type egg		Total	
	n	%	n	%	n	%
Lettuce (12)	3	25	1	8.3	4	33.3
Arugula (12)	4	33.3	0	0.0	4	33.3
Spring onion (12)	1	8.3	7	58.3	8	66.6
Mint (12)	2	16.6	0	0.0	2	16.6
Purslane(12)	1	8.3	1	8.3	2	16.6
Carrot (12)	0	0.0	0	0.0	0	0.0
Parsley (12)	0	0.0	0	0.0	0	0.0
Cress (12)	0	0.0	0	0.0	0	0.0
Cucumber (12)	0	0.0	0	0.0	0	0.0
Dill (12)	0	0.0	0	0.0	0	0.0
Total (120)	11	9.1	9	7.5	20	16.6

Table 2. Distribution of helminth eggs in vegetable samples according to street market

Sample (n)	Gökmeydan Street Market		Visnelik Street Market		Kumlubel Street Market		Seker Street Market	
	*A. l. n (%)	**S. T n (%)	*A. l. n (%)	** S. T n (%)	*A. l. n (%)	** S. T n (%)	*A. l. n (%)	** S. T n (%)
Lettuce(12)	1 (8.3)	0 (0.0)	2 (16.6)	0 (0.0)	0 (0.0)	1 (8.3)	0 (0.0)	0 (0.0)
Arugula (12)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	4 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)
S. Onion (12)	0 (0.0)	0 (0.0)	1 (8.3)	0 (0.0)	0 (0.0)	1 (8.3)	0 (0.0)	6 (50)
Mint (12)	2(16.6)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Purslane (12)	0 (0.0)	0 (0.0)	1 (8.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (8.3)
Carrot (12)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Parsley (12)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Cress (12)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Cucumber (12)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Dill (12)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Total (120)	3 (2.5)	0 (0.0)	4 (3.3)	0 (0.0)	4 (3.3)	2(1.6)	0 (0.0)	7 (5.8)

*_Ascaris lumbricoides_. ** Strongyle type.

Helminthological examination methods applied to vegetable samples identified Strongyle type eggs (Figure 1A) and *Ascaris lumbricoides* eggs (Figure 1B).

The number of helminth eggs in vegetable samples examined in the laboratory is shown in Table 1 and 2.

After examining the results of the samples from district markets, we determined that the highest number of infected samples came from the Seker market with seven (5.8%), and the lowest number from the Gökmeydan district market with 3 (2.5%) samples (Table 2).

The number of helminth eggs found in vegetables purchased from the Gökmeydan market was statistically significantly lower than the number of helminth eggs found in samples purchased from the Visnelik market ($p = 0.01$; Table 3). In addition, the same result was observed when comparing samples from the Gökmeydan ($p = 0.03$) and Visnelik ($p<0.01$) markets with the Kumlubel market (Table 3). The number of helminth eggs found in vegetables bought from the Seker market was statistically significantly higher than the number of helminth eggs found in vegetables collected from the Kumlubel market ($p<0.01$; Table 3). In this study, we examined the

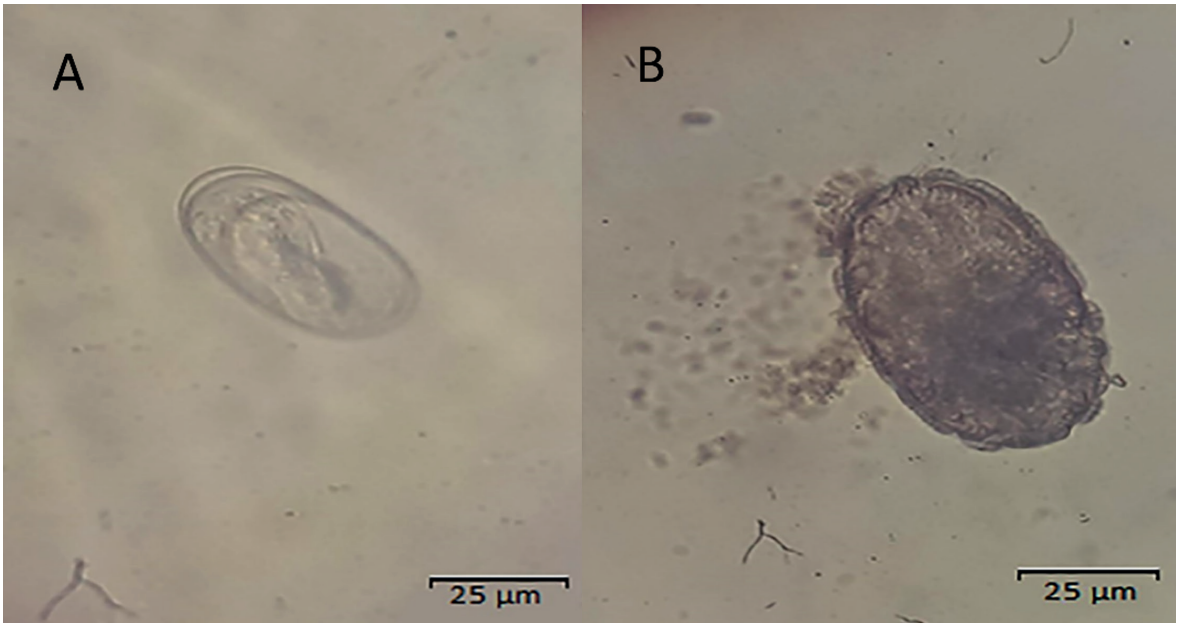

Figure 1. [A] Strongyle type egg and [B] *Ascaris lumbricoides* egg (40x magnification)

Table 3. Significance between the distribution of helminth eggs in food samples according to the street markets

	Visnelik Street Market (chi square /p*)	Kumlubel Street Market (chi square /p*)	Seker Street Market (chi square /p*)
Gökmeydan Street Market	8.000/ 0.01**	6.301/ 0.03**	2.857/ 0.20
Visnelik Street Market		10.355/ <0.01**	4.286/ 0.08
Kumlubel Street Market			7.532/ <0.01**

*Chi-squared test, Fisher's exact test. **p<0.05 was considered statistically significant.

contamination and frequency of helminth eggs in raw vegetable samples collected from district markets.

Ascaris lumbricoides [17,18] can cause infection in healthy people by consuming foods contaminated with the faeces of infected people. In the 120 samples examined in this study, the frequency of *A. lumbricoides* eggs was 9.1%, and that of Strongyle type eggs was 7.5%. *A. lumbricoides* eggs have been reported in numerous studies carried out in Türkiye. It has been reported that *Ascaris lumbricoides* eggs were found in 1% of 203 unwashed samples in a study conducted in Ankara [6], 2% in 199 unwashed samples in Bursa [19], 1.8% in 111 samples in Burdur [20], and 11% [21] in 100 food samples in Şanlıurfa.

The rate of egg detection varies by the number of samples, the country, or the region where the samples were taken. The frequency of *Ascaris lumbricoides* eggs detected in this study was higher than in many previous studies. *Ascaris lumbricoides* eggs were predominantly found in arugula (33.3%), the lowest in scallions (8.3%), and in purslane (8.3%); eggs were not detected in any carrots, parsley, cress, cucumber, or dill.

Erez et al. (2022) conducted a study to determine helminth contamination of 508 vegetable samples, including lettuce, parsley, carrot, spring onion, spinach, cress, arugula, mint, dill, and purslane, in Afyonkarahisar. It was reported that they had detected Taeniid eggs in two (0.39%) vegetable samples, including 1 lettuce and 1 dill; *Toxocara* spp. in 2 (0.39%) samples, including 1 lettuce and 1 mint; *Toxascaris leonina* eggs in 1 (0.2%) arugula sample; hookworm and strongyle type eggs in 58 samples (11.42%), including 4 lettuce, 6 parsley, 3 carrots, 18 onions, 2 spinach, 9 cress, 4 arugula, 6 mint, and 6 purslane; *Dicrocoelium* spp. in 2 (3.63%) carrots,

1 (2.04%); *Moniezia* spp. in 2 (3.63%) carrot samples; and *Fasciola* spp. in 1 (2.08%) spring onion sample [22]. In this study, *Ascaris lumbricoides* eggs and Strongyle type eggs were found in the collected samples. We did not observe any other helminth eggs. The different irrigation methods in the regions where vegetables are grown may explain the absence of other helminth eggs. Avcioglu et al. (2011) detected helminth eggs in lettuce (5.6%) and parsley (4.3%), from 199 unwashed samples. No helminth eggs were found in 199 washed samples [19]. In our study, we found helminth eggs mostly in spring onions (66.6%) and the lowest in mint (16.6%) and purslane (16.6%).

In a study conducted in Burdur, *A. lumbricoides* eggs were mostly found in lettuce (9.09%) and parsley (7.69%) [20]. In contrast, we observed the highest rate of *Ascaris lumbricoides* eggs in arugula (33.3%), lettuce (25%), mint (16.6%), and less frequently in spring onions (8.3%) and purslane (8.3%).

Aydenizöz et al. (2017) observed a total of 9 samples positive for helminth eggs in 900 green leafy vegetable samples from Kırıkkale, also found that 0.44% were determined to be strongyle type eggs [23]. In our study, strongyle type eggs were found in 9 (7.5%) samples.

In many studies, parasitological analysis showed that foods contain both *Ascaris lumbricoides* eggs and Strongyle type helminth eggs. Studies conducted in other countries detected a higher frequency of helminth eggs compared to our study. According to the results of studies conducted around the world, it was easier for helminth eggs to infect multiple layers of vegetables, such as lettuce and spring onions. Green leafy samples taken from markets, grocery stores, and supermarkets in Brazil contained more than one helminth egg in lettuce, scallions, and arugula. Furthermore, Hookworm or *Strongyloides* spp. egg frequency was 12.9% [24]. Compared to our study, Strongyle type eggs were found at a rate of 7.5%.

In a study examining vegetables and fruits collected from four local markets in southern Ethiopia, the frequency of *Ascaris lumbricoides* was 20.8% [25]. Furthermore, in Nigeria, the frequency of *Ascaris lumbricoides* eggs was 41.1% and hookworm eggs (Strongyle type) was 8.9% in 124 vegetable samples [26]. Comparatively, the rate of *Ascaris lumbricoides* eggs (9.1%) was lower than that observed in these studies, while the frequency of Strongyle type eggs (7.5%) was similar to the mentioned studies. Another study in Nigeria of 300 samples found the frequency of *Ascaris lumbricoides* eggs to be 18.3% and hookworm eggs (Strongyle type) to be 1.67% [27]. We observed the frequency of *Ascaris lumbricoides* eggs to be lower but strongyle type eggs higher in comparison. In a study conducted in Nigeria where samples were collected from the market, helminth eggs were not found in carrots; however, *Ascaris lumbricoides* eggs were found in lettuce [28], whereas we found no *Ascaris lumbricoides* eggs in lettuce.

Helminth eggs have been reported in both lettuce and carrots [29]. Furthermore, Adamu et al. (2012) found hookworm (Strongyle type) eggs in 3% of 300 lettuces and 2.3% of 130 cucumbers [30]. Comparatively, we observed strongyle type eggs mostly in scallions (58.3%) and least in lettuce (8.3%) and purslane (8.3%), while we detected none in carrots, parsley, cress, cucumber, and dill.

The findings of the intestinal parasite examination conducted in Eskişehir (1993) reported that the rate of *Ascaris lumbricoides* was found to be 0.15% [31]. In contrast, a study conducted in 2008 did not find any *A. lumbricoides* eggs [32]. By eliminating the infrastructure problem and distributing drinking water in the city instead of tankers, the use of hygienic water bottles has reduced the incidence of this parasitic species. Moreover, actively preventing waste from mixing with the Porsuk River, which could be used for agricultural purposes, significantly contributed to the reduction.

Another study carried out to investigate the presence of intestinal parasites in primary school students in Eskişehir found *A. lumbricoides* in 2% of 124 samples [33]. Compared to previous studies, the latest findings show a higher percentage of observed *Ascaris lumbricoides* eggs. A probable explanation for this difference may be a variation in the ages of the collected samples. The high incidence of *A. lumbricoides* eggs in primary school students

suggests that children are not adequately conforming to hand hygiene along with general hygiene and nutrition rules. *Ascaris lumbricoides* eggs were found in 11 (9.1%) of the samples collected in this study. This rate is significantly higher than the rate observed in people in Eskişehir. One possible reason for this higher rate could be that people in Eskişehir consume vegetables after hygienic washing.

Water sources regularly used for drinking and farming in rural areas without sewage systems can become contaminated by human or animal waste. Furthermore, the use of animal fertilisers in growing vegetables can lead to the contamination of vegetables by zoonotic agents.

When comparing the helminth eggs detected by district markets, we determined the lowest egg frequency to be in the Gökmeydan district market (2.5%) and the highest egg frequency in the Seker district market (5.8%). We also observed that the number of helminth eggs found in vegetables bought from the Gökmeydan market was less than those from Visnelik, Kumlubel, and lastly, Seker markets, in that order. The differences in irrigation and fertilization practices of the soils from which the vegetables are obtained might explain the variation in the frequency of helminth eggs detected, despite the samples bought from the markets being the same in terms of variety and number. Some of the vegetables bought from different markets had no helminth eggs detected, while others had more than one helminth egg.

4. CONCLUSION

According to our findings, parasites can be found on many vegetables and herbs (particularly vegetables with multiple layers, such as lettuce and spring onions), which have the potential to cause parasitic infections in humans. This is likely the result of consuming vegetables without washing them adequately. The types and numbers of parasites observed in vegetables vary according to markets. The occurrence of parasitic infections varies depending on many factors, such as agricultural irrigation, access to healthy water resources, and socioeconomic structure. Considering this variability, it is necessary to develop correct and effective washing and disinfection methods for raw vegetables. In addition, there is a need to educate people with regards to thoroughly washing vegetables before consumption. Restaurants, hospitals, factories, schools, and anywhere such foods are offered for consumption in large quantities should establish effective washing processes. Authorities should manage and conduct thorough inspections of these establishments.

Ethical approval

Not applicable, because this article does not contain any studies with human or animal subjects.

Author contribution

Concept: KK, ME; Design: ME; Supervision: KK, ME; Materials: KK, ME; HG; Data Collection and/or Processing: KK, ME; HG; Analysis and/or Interpretation: HG; Literature Search: KK, ME; HG; Writing: KK, ME; Critical Reviews: ME.

Source of funding

This research received no grant from any funding agency/sector.

Conflict of interest

The authors declared that there is no conflict of interest.

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European Journal of Life Sciences 2024; 3: 9-15

Research Article

COX inhibitory profiles of a series of thiadiazole-benzothiazole hybrids

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https://doi.org/10.55971/EJLS.1443664

Received: 27.02.2024
Accepted: 15.03.2024
Available online: 30.04.2024

ABSTRACT

In an endeavour to identify small molecule COX-1 inhibitors, a colorimetric assay protocol was applied for the *in vitro* evaluation of COX-1 and 2 inhibitory potential of a series of thiadiazole-benzothiazole hybrids. The most potent and selective COX-1 inhibitor in this series was found as 2-[(5-amino-1,3,4-thiadiazol-2-yl)thio]-*N*-(6-chlorobenzothiazol-2-yl) acetamide (**7**) (51.36 ± 3.32% at 100 µM) compared to SC-560 (83.64 ± 3.76% at 1 µM). Compound **7** exerted weaker inhibitory effect on COX-2 (11.05 ± 1.69% at 100 µM). To explore its binding interactions at the active site of human COX-1 (PDB ID: 6Y3C), molecular docking studies were conducted. Compound **7** could establish hydrogen bonds with proper residues thanks to its amide C=O group. *In silico* studies were employed to shed light on their pharmacokinetic properties. Taken together, compound **7** can be considered as a potential lead compound for the generation of selective COX-1 inhibitors with enhanced efficacy.

Keywords: Benzothiazole, cyclooxygenase, molecular docking, thiadiazole

1. INTRODUCTION

Cyclooxgenases (COXs), which convert arachidonic acid (AA) into prostanoids in the AA cascade, are the key molecular targets for nonsteroidal anti-inflammatory drugs (NSAIDs) [1,2]. COX-1 and 2, two major isoforms of COX, are membrane-bound enzymes located in the endoplasmic reticulum and the nuclear envelope. COX-1 is the housekeeper isoform implicated in the maintenance of homeostasis, the protection of the gastric mucosa, the regulation of platelet aggregation, and renal perfusion [3]. On the other hand, COX-2 is the inducible isoform of COX that is associated with the disease-related inflammatory reactions [3,4].

Today, the paradigm of "good" housekeeping COX-1 and "bad" inducible COX-2 has lost its validity since it is known that COX-1 derived prostaglandins (PGs) also contribute to inflammation, COX-2 is also constitutively expressed in some tissues, and inhibiting COX-2 rather than COX-1 is not sufficient to prevent gastrointestinal (GI) toxicity [5-8].

Mounting evidence has demonstrated that COX-1 plays a key role in the pathogenesis of several diseases such as pain, neuroinflammation, cancer, and cardiovascular disorders. The inhibition of platelet COX-1 by low-dose aspirin is reported to be beneficial for preventing cardiovascular diseases. Furthermore, it is understood that the long-term use

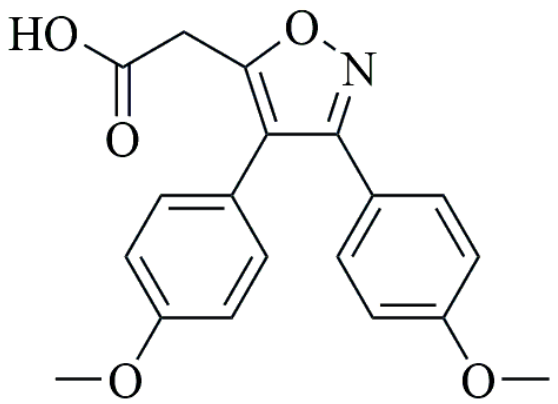


Figure 1. Mofezolac

of NSAIDs diminishes the incidence of some diseases including Alzheimer's Disease and some types of cancer. There is only one selective COX-1 inhibitor (mofezolac) (Figure 1) currently prescribed just in Japan for the treatment of pain and inflammation, and therefore the search for selective inhibitors of COX-1 has come into prominence [9-11].

The 1,3,4-thiadiazole has been emerged as a distinguished scaffold for the design of drug-like molecules due to its unique features (e.g. mesoionic nature and good liposolubility), which enable compounds with a thiadiazole motif to pass through cellular membranes and interact with biological targets properly [12-17]. In particular, 1,3,4-thiadiazoles have been reported to exhibit anti-inflammatory activity through several mechanisms including the inhibition of COXs [18-20].

The benzothiazole core serves as a privileged building block of small molecule ligands endowed with a broad range of biological activities such as anti-inflammatory activity targeting a plethora of crucial enzymes including COXs [21-28].

In the continuation of our recent studies on the discovery of selective COX-1 inhibitors [29,30], *in vitro* experiments were conducted to evaluate the COX inhibitory profiles of a series of thiadiazole-benzothiazole hybrids. Computational studies were also performed for the most active COX-1 inhibitor in this series.

2. MATERIALS AND METHODS

2.1. Chemistry

The reaction of 2-mercapto-5-methyl/amino-1,3,4-thiadiazole with appropriate 2-chloro-*N*-(benzothiazol-2-yl)acetamide in the presence of the base catalyst (potassium carbonate) afforded 2-[(5-methyl/amino-1,3,4-thiadiazol-2-yl)thio]-*N*-(benzothiazol-2-yl)acetamides (**1-10**). The synthetic procedure and the spectral data of these compounds were reported previously by Altintop *et al*. [31].

2.2. Biochemistry

2.2.1. Assessment of COX inhibition

The inhibitory effects of compounds **1-10** (at 100 µM) on COX-1 and 2 were examined using COX colorimetric inhibitor screening assay based on the instructions provided by the manufacturer (Cayman, Ann Arbor, MI, USA) as described earlier [32]. All experiments were performed in triplicate and the data were expressed as mean ± SD. The selective COX-1 inhibitor SC-560 (at 1 µM) and the selective COX-2 inhibitor rofecoxib (at 10 µM) were used in this study for comparison.

2.3. *In Silico* Studies

2.3.1. Molecular docking

Computational studies of compound **7** and mofezolac (selective COX-1 inhibitor) were performed on the X-ray crystallographic structure of the COX-1, which was retrieved from the Protein Data Bank (PDB) with the accession code 6Y3C [33,34]. The Protein Preparation Workflow of Schrödinger Release 2023-2 (Schrödinger, LLC, New York, NY, USA) was performed. The LigPrep module was used to prepare the ligands, which were drawn with 2D Sketcher. OPLS4 was selected as the force field. The Receptor Grid Generation was used to generate the grid box. Finally, the Ligand Docking was employed.

2.3.2. Prediction of pharmacokinetic profiles

The QikProp, the *in silico* absorption, distribution, metabolism, and excretion (ADME) module within

the Maestro suite (Schrödinger Release 2023-2, LLC, New York, USA), was used to estimate the pharmacokinetic profile of compound **7**.

3. RESULTS AND DISCUSSION

3.1. *In vitro* Studies

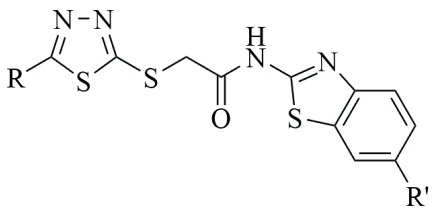
Among the tested compounds, compound **7** was found to be the most potent inhibitor of COX-1 (51.36 ± 3.32% at 100 µM) in comparison with SC-560 (83.64 ± 3.76% at 1 µM) (Table 1). The inhibitory effect of compound **7** on COX-2 (11.05 ± 1.69% at 100 µM) was found to be weaker than its inhibitory effect on COX-1.

Compound **1** inhibited COX-1 and 2 with the percentages of 25.50 ± 3.54 and 22.47 ± 2.67%, respectively. The COX inhibitory profile of compound **1** can be described as non-selective. Compound **2** inhibited only COX-1 (7.58 ± 1.16%), while compounds **4** (10.85 ± 2.05%) and **9** (8.82 ± 1.42%) caused only COX-2 inhibition. On the other hand, compounds **3**, **5**, **6**, **8** and **10** did not exert any inhibitory action against both COXs. Based on the *in vitro* data (Table 1), it can be concluded that the substitutions on the benzothiazole scaffold as well as on the thiadiazole ring are important for the inhibition of both COXs. The incorporation of the methyl and the ethoxy substituents into the sixth position of the benzothiazole scaffold caused the loss of COX inhibitory potency.

3.2. *In silico* Studies

To provide an insight into the mode of action, compound **7** was docked into the active site of COX-1 (PDB ID: 6Y3C). According to the *in silico* data, the compound showed good affinity to the active site of COX-1 as depicted in Figures 2 and 3. The interactions of the amide C=O moiety of compound **7** with Arg120 and Glu524 led to the formation of

Table 1. COX inhibitory effects of compounds **1-10**, SC-560 and rofecoxib



Compound (100 µM)	R	R'	Inhibition (%)	
			COX-1	COX-2
1	CH_3	H	25.50 ± 3.54	22.47 ± 2.67
2	CH_3	Cl	7.58 ± 1.16	----
3	CH_3	CH_3	----	----
4	CH_3	OCH_3	----	10.85 ± 2.05
5	CH_3	OC_2H_5	----	----
6	NH_2	H	----	----
7	NH_2	Cl	51.36 ± 3.32	11.05 ± 1.69
8	NH_2	CH_3	----	----
9	NH_2	OCH_3	----	8.82 ± 1.42
10	NH_2	OC_2H_5	----	----
SC-560 (1 µM)	-	-	83.64 ± 3.76	nt
Rofecoxib (10 µM)	-	-	nt	96.48 ± 3.54

---- : no inhibition; nt: not tested.

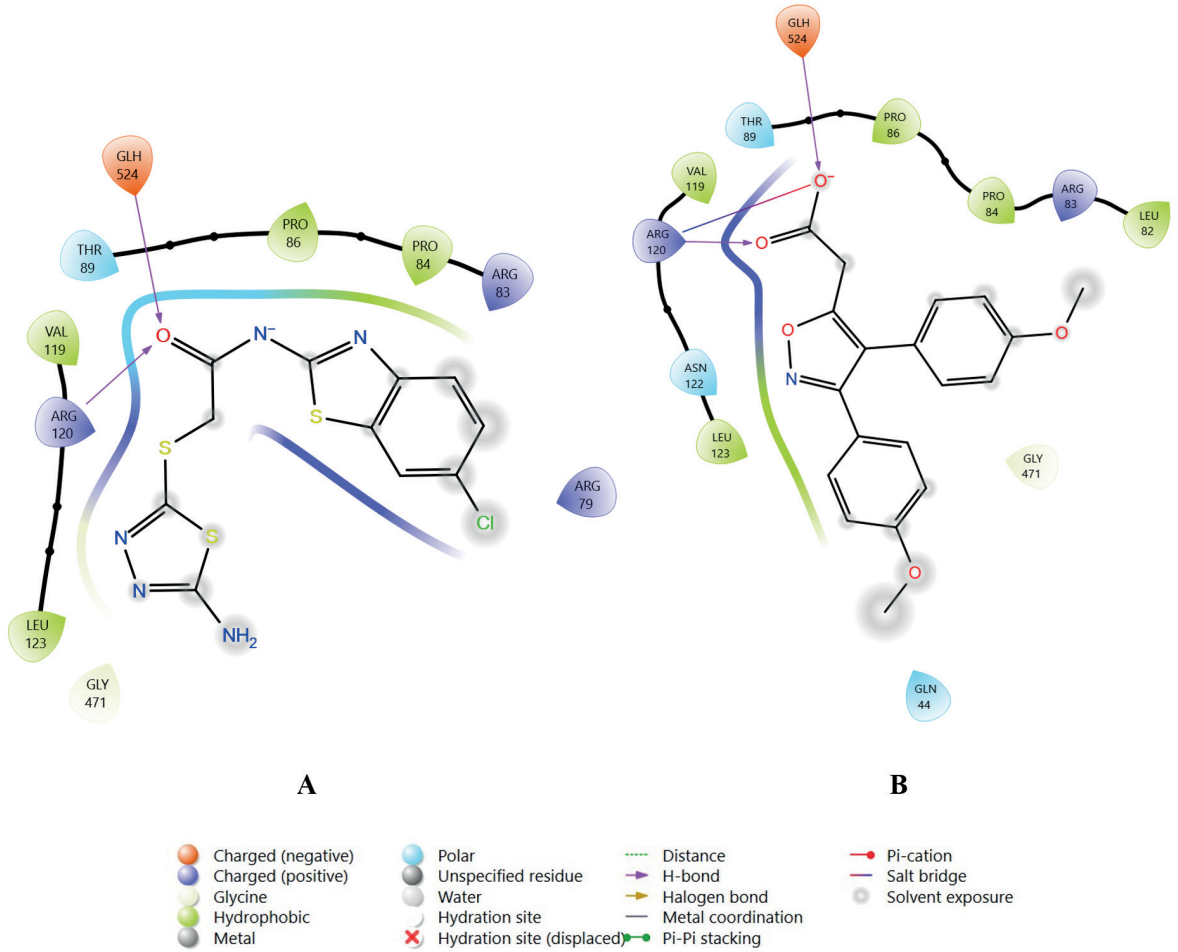


Figure 2. Docking interactions of compound **7 (A)** and mofezolac **(B)** in the active site of COX-1 (PDB ID: 6Y3C).

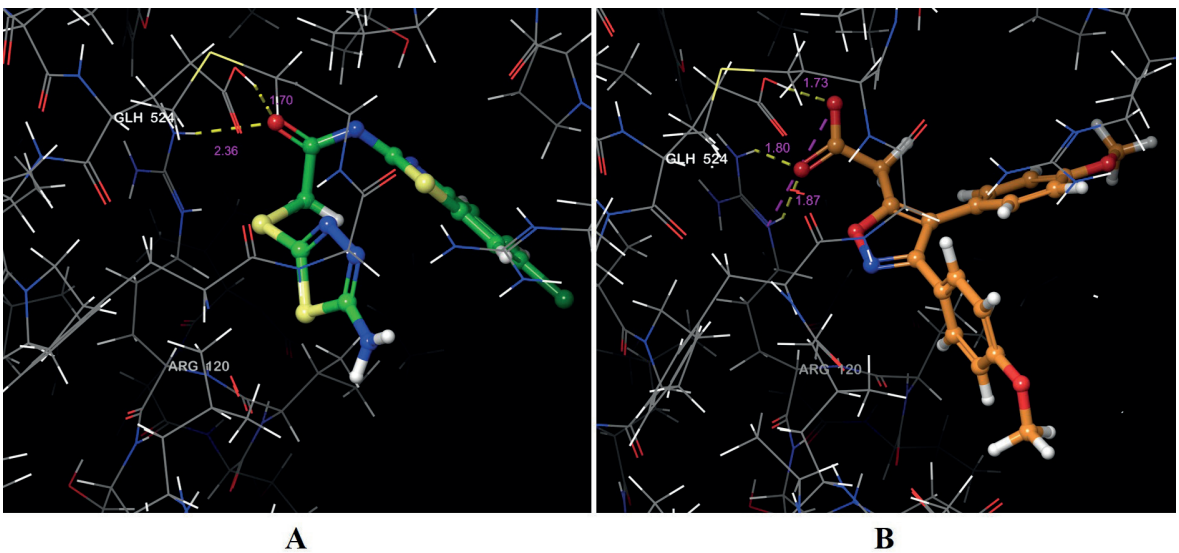


Figure 3. Docked poses of compound **7 (A)** and mofezolac **(B)** in the active site of COX-1 (PDB ID: 6Y3C) (Yellow and pink dashes represent the hydrogen bonds and the salt bridge, respectively).

Table 2. Docking scores (kcal/mol), glide gscores (kcal/mol) and glide emodel (kcal/mol) results of compound **7** and mofezolac for COX-1 enzyme (PDB ID: 6Y3C)

Compound	COX-1		
	Docking score	**Glide gscore**	**Glide emodel**
7	-3.696	-4.943	-53.325
Mofezolac	-5.047	-5.047	-50.495

hydrogen bonds. The selective COX-1 inhibitor mofezolac formed hydrogen bonds with Arg120 and Glu524. It can be concluded that these interactions (hydrogen bonds with proper residues) may be responsible for the observed COX-1 inhibitory activity of compound **7**. As illustrated in Figure 2, compound **7** does not form the salt bridge that mofezolac does with Arg120. This missed interaction may explain the lower COX-1 inhibitory activity of compound **7** compared to that of mofezolac [35]. The docking scores of compound **7** and mofezolac were found as -3.696 and -5.047 kcal/mol, respectively (Table 2).

ADME experiments require a large number of *in vivo* tests and, consequently, ethical procedures, and therefore these assays are expensive and time-consuming for a large number of chemical compounds. As a result, *in silico* procedures are frequently applied to evaluate the pharmacokinetic profiles of drug candidates [36]. In this context, compound **7** was subjected to a computational study for the prediction of its pharmacokinetic characteristics. As presented in Table 3, the SASA, CIQPlogS, QPlogBB, QPPMDCK values of compound **7** were predicted to fall within the range recommended by the QikProp. Moreover, this compound did not cause any violation of Lipinski's and Jorgensen's rules, making it a drug-like molecule with favorable oral bioavailability.

Table 3. Predicted pharmacokinetic features of compound **7**

Property or descriptor	Compound 7
SASA*	592.498
CIQPlogS*	-4.650
QPlogPo/w*	2.033
QPlogBB*	-1.271
QPPMDCK*	748.996
QPlogKhsa*	-0.236
Rule of Five*	0
Rule of Three*	0

* **SASA:** Total solvent accessible surface area in square angstroms using a probe with a 1.4 Å radius (Recommended range: 300.0 – 1000.0). **CIQPlogS:** Conformation-independent predicted aqueous solubility (Recommended range: –6.5 – 0.5). **QPlogPo/w:** Predicted octanol/water partition coefficient (Recommended range: -2.0 – 6.5). **QPlogBB:** Predicted brain/blood partition coefficient (Recommended range: –3.0 – 1.2). **QPPMDCK:** Predicted apparent MDCK cell permeability in nm/sec (< 25 poor, > 500 great). **QPlogKhsa:** Prediction of binding to human serum albumin (Recommended range: -1.5 – 1.5). **Rule of Five:** Number of violations of Lipinski's rule of five. The rules are molecular weight of the molecule < 500, QPlogPo/w < 5, hydrogen bond donor atoms ≤ 5, hydrogen bond acceptor atoms ≤ 10. Compounds that provide these rules are considered as drug-like molecules (maximum is 4). **Rule of Three:** Number of violations of Jorgensen's rule of three. The three rules are: predicted aqueous solubility (QPlogS) > -5.7, predicted apparent Caco-2 cell permeability (QPPCaco in nm/s) > 22 nm/s, # primary metabolites < 7 (maximum is 3). Compounds with fewer (and preferably no) violations of these rules are more likely to be orally available agents (Schrödinger Release 2023-2, LLC, New York, USA).

4. CONCLUSION

In conclusion, we described the *in vitro* inhibitory effects of a series of thiadiazole-benzothiazole hybrids on COX-1 and 2. Among the tested compounds, compound **7** was identified as the most potent and selective inhibitor of COX-1. The *in silico* studies suggest that the amide C=O moiety of compound **7** with Arg120 and Glu524 led to the formation of hydrogen bonds, which is considered to play a crucial role in COX-1 inhibitory activity. Based on the QikProp data, compound **7** was predicted to possess favorable drug-like characteristics and oral bioavailability. In the view of this research, a new class of selective COX-1 inhibitors could be designed *via* the molecular modification of compound **7** for the treatment of many illnesses in which selective inhibition of COX-1 is required.

Ethical approval

Not applicable, because this article does not contain any studies with human or animal subjects.

Author contribution

Concept: AÖ; Design: AÖ; Materials: AÖ, HET; Data Collection and/or Processing: AÖ, HET; Analysis and/or Interpretation: AÖ, HET; Literature Search: AÖ; Writing: AÖ; Critical Reviews: AÖ, HET.

Source of funding

This research received no grant from any funding agency/sector.

Conflict of interest

The authors declared that there is no conflict of interest.

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European Journal of Life Sciences 2024; 3: 16-20

Research Article

Evaluating attitudes of pharmacy students toward pharmaceutical side effect pictograms: Ankara sample

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https://doi.org/10.55971/EJLS.1445427

Received: 01.03.2024
Accepted: 29.03.2024
Available online: 30.04.2024

ABSTRACT

Pictograms are graphic symbols that facilitate international communications and express objects and the meaning of these objects. Using pictograms in health is essential to informing patients, especially in cases such as illiteracy, language problems, and multiple drugs. Pharmaceutical pictograms are thought to be helpful not only for pharmacists but also for patients.

In this regard, in this study, pharmacy faculty students' attitudes towards side effect pictograms are evaluated via the "Drug side effect pictogram attitude scale." As a result of exploratory factor analysis, a one-dimensional structure was obtained in parallel with the original scale with 0.906 Cronbach's alpha value. The mean values of the items in the scale were found above 3.70, which shows that students have positive attitudes toward side effect pictograms.

As a result, it is thought that pharmaceutical pictograms should be included more in pharmacy education to transform the positive attitudes of pharmacy faculty students towards pictograms into the behavior of using pictograms in patient counseling.

Keywords: Drug, Pharmaceutical pictogram, Pharmacist, Side effect

1. INTRODUCTION

Each patient's ability to understand health-related information varies. For this reason, pharmacist-patient communication is essential in ensuring that patients use medicine most effectively and safely. For the instructions in a prescription to be understood and implemented correctly, clear and understandable information must be provided to the patient. Sometimes, more than a verbal explanation is required in cases such as the patient being illiterate or having language problems. Montagne stated that drug information provided in written form improves the patient's knowledge, but more is needed to ensure patient compliance and enhance drug use knowledge [1]. Hence, additional information provided to patients can be beneficial. In this context, it is known that there is an increasing interest in pictograms, which consist of standardized graphics that make the messages more straightforward for the patient.

Pictograms are used in many fields, from transportation to marketing. Another area of use of pictograms is the healthcare sector. Pharmaceutical

pictograms were first discussed by the American Pharmacopoeia Commission in 1987, and 29 pictograms were presented in 1989 [2]. The American Pharmacopoeia Commission defines drug pictograms as "standardized graphic images that help communicate drug treatment instructions, precautions, and/or warnings to patients and consumers." This development was followed by 75 pictograms developed by Fédération Internationale Pharmaceutique (FIP; International Pharmaceutical Federation) in 1990 [2]. When these pictograms are examined, it is seen that the pictograms are generally prepared to provide information about the usage, form, and side effects of medications. Pictograms not only enable patients to understand drug information more accurately and easily but also provide great benefits to pharmacists when providing pharmaceutical care services [3]. There are also studies in the literature addressing the effects of the use of pictograms on the drug treatment and lifestyle of individuals with chronic diseases such as diabetes and tuberculosis [4]. Therefore, pictograms are thought to be very useful in pharmaceutical care services [5]. As Reijenen et al. stated, pharmaceutical pictograms are also useful for increasing patients' medication adherence [6].

This study aims to determine the attitudes of pharmacy faculty students regarding the pharmaceutical side effect pictograms and to evaluate the effects of gender and university on the students' attitudes.

2. MATERIALS AND METHODS

In line with the purpose of the study, a face-to-face survey was conducted with students of pharmacy faculties of three universities in Ankara. The survey took around 10 minutes to complete which included two sections. The first section comprised six questions for evaluating demographic characteristics. The second section includes the "Drug side effect pictogram attitude scale" developed and validated by Tarhan et al. [7]. There are ten items in this scale rated by a 5-point Likert scale. The Cronbach's alpha value of the original scale was 0.89.

The study population consists of 4th-grade students of the Faculty of Pharmacy of Hacettepe, Ankara, and Gazi Universities during the autumn term of the 2018–2019 academic year. The population consists of approximately 450 students. The sample size of this study was calculated with the acceptable error level method under the assumption that the sample statistics were normally distributed. Taking a confidence level of 0.05, z = 1.96, d (sensitivity) = 0.05, and p and q values as 0.50, the minimum sample size to reach was 207.

The obtained data was analyzed statistically with the help of the IBM Statistical Package for Social Sciences (IBM SPSS® Software) version 22. Descriptive statistics were first used, then explanatory factor analysis (EFA) was conducted. Finally, the factor scores obtained from EFA were subjected to independent sample *t*-test and ANOVA tests to examine whether gender and university had a statistically significant effect on them.

3. RESULTS AND DISCUSSION

To increase the reliability of the data obtained, the survey was applied to the maximum number of students that could be reached, and 253 students participated. 190 of the participants are female; 63 are male, and the distribution of the faculties they are enrolled in is given in Figure 1.

As can be seen in Figure 1, the university distribution of the students is very close to each other. In the survey, participants were asked whether they had heard of the term pictogram before, and it was

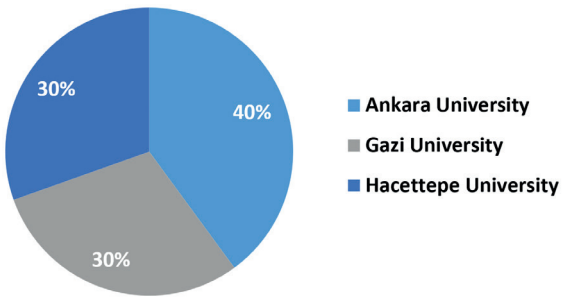

Figure 1. Distribution of universities

observed that only 33% of the participants answered yes. However, 91% of the participants think using pictograms will make it easier for pharmacists to provide pharmaceutical services. Additionally, 83% of the participants stated that it was necessary to acquire knowledge about pictograms during faculty education.

Following descriptive statistics, the data were subjected to exploratory factor analysis (EFA). First, the Kaiser-Meyer-Olkin (KMO) value was calculated to determine whether the sample size reached was sufficient for EFA. This value is generally desired to be above 0.5 [8]. As a result of the analysis, the KMO value was calculated as 0.877, which shows that the sample size is sufficient for EFA. As a result of EFA, a one-dimensional structure was obtained in parallel with the original scale, and it was determined that this structure explained 55.5% of the total variance. As a result of EFA, no item was removed from the scale, and the factor loadings of the statements are given in Table 1.

When the mean values of the responses to the items in the scale are examined, it is seen that the mean values are over 3.70 (with a maximum of 5.00). This shows that students have positive attitudes toward side effect pictograms. This result is similar with Tarhan et al. [7].

According to Table 1, all of the factor loadings are greater than 0.5. This result indicates that the "Drug side effect pictogram attitude scale" is also valid for the current sample. Additionally, Cronbach's alpha value was used to test the reliability of the scale, and this value was calculated as 0.906. Özdamar states that the scale reliability level is high when the Cronbach alpha value is between 0.80 and 1.00, and the obtained Cronbach alpha value shows that the scale is quite reliable for this sample [9].

Barros et al. put forth that pictograms are particularly helpful for older people and individuals with low health literacy [10]. Dowse and Ehlers found that pharmaceutical pictograms help to understand drug use information, especially for individuals with low literacy levels [2]. Kheir et al. revealed that pharmaceutical pictograms are useful for individuals who are illiterate and have language problems [11]. Dowse stated that pharmacy students found pharmaceutical pictograms useful [12]. The answers given by the respondents of the current study are that the pictograms will provide convenience to illiterate individuals, individuals with language problems, and elderly individuals.

Barros et al. stated that pictograms are effective in helping patients understand prescribed medications more accurately [10]. Additionally, Dowse emphasized that pharmacy students see pharmaceutical pictograms as an easy way to teach patients [11]. The findings obtained in this study also support this situation.

Yasmin et al. stated that the use of drug pictograms in patient counseling will reduce drug side effects [13]. Similarly, when the items in the scale used in this study and the answer averages are considered,

Table 1. Mean values and factor loadings of the items in the scale

Items	Mean values	Factor loadings
Side effects pictograms make individuals to understand drug-side effects easily.	4.020	0.845
Side effects pictograms make individuals to understand drug-side effects truly.	3.885	0.796
Side effects pictograms provide convenience for geriatrics.	3.758	0.795
Side effects pictograms provide convenience for nonliterate individuals.	3.770	0.769
Side effects pictograms increases patient compliance.	3.945	0.762
Side effects pictograms provide convenience for individuals with hearing loss.	4.111	0.760
Side effects pictograms provide convenience for individuals having language problems.	4.012	0.758
Side effects pictograms are effective.	4.059	0.724
Side effects pictograms should be included in medication package insert.	3.866	0.611
Side effects pictograms should be on drug boxes.	3.704	0.589

it is seen that the students think that the side effects of the drugs will be understood more efficiently and accurately by the patients while using pictograms.

Barros et al. pointed out that the use of pictograms will benefit healthcare professionals in providing better consultancy services to patients [10]. Montagne similarly emphasizes that pictograms are essential in helping patients to understand drug use more accurately [1]. In this regard, while the answers given by the participants to the question of whether drug side effect pictograms provide convenience to pharmacists were expected to create a statistically significant difference in the attitude factor, the result of the t-test was the opposite ($p>0.05$). It is thought that this result is due to the students' insufficient knowledge and training about the benefits of using pictograms.

Additionally, as a result of t-tests, there weren't any statistically significant differences between gender groups on factor loadings ($p>0.05$). Sharif et al. conducted a study about pharmaceutical pictograms with pharmacy and non-pharmacy students and similarly stated that gender did not affect the factors [3]. In this context, it seems that the result obtained is parallel to the existing literature.

Finally, an ANOVA test was applied to determine whether the universities where the students were studying had an effect on attitudes. A statistically significant difference was observed between universities at the 95% confidence interval. Tukey test was applied to determine the reason for this difference, and it was determined that the response averages of Hacettepe University students to the items in this factor were higher than the other two universities.

4. CONCLUSION

The results obtained in this study are of great importance in determining the opinions of pharmacy faculty students regarding illustrated drug use labels. Pharmaceutical pictograms are a fairly new concept for Türkiye. For this reason, in our country, where the rate of health literacy is low, it is thought that it would be beneficial for health authorities to work on pictograms in order to increase patients' compliance with treatment.

In this context, the following suggestions can be provided:

- Inclusion of topics related to pharmaceutical pictograms in pharmacy education curricula,
- Informing pharmacists about the developments regarding pictograms in Türkiye and around the world by including pharmaceutical pictograms in the in-service training of Pharmacist Regional Chambers,
- Recommend the use of pictograms by pharmacists, especially when counseling individuals who are elderly, illiterate, or have language difficulties,
- Carrying out local pharmaceutical pictogram development studies, especially for drug use and side effects.

Acknowledgements

This study was created by expanding the graduation project that the first author completed at Ankara University Faculty of Pharmacy under the supervision of Prof. Sevgi ŞAR.

Ethical approval

This study was conducted after Ankara University Health Sciences Ethics Committee approved the study ethically (Date:19/01/2018, Decision No: 02/27).

Author contribution

Concept: YP, MA, NT, SŞ; Design: YP, MA, NT, SŞ; Supervision: SŞ; Materials: YP, MA, NT; Data Collection and/or Processing: YP; Analysis and/or Interpretation: YP, MA, NT; Literature Search: YP, MA, NT; Writing: YP, MA; Critical Reviews: MA, SŞ.

Source of funding

This research received no grant from any funding agency/sector.

Conflict of interest

The authors declared that there is no conflict of interest.

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European Journal of Life Sciences 2024; 3: 21-30

Research Article

Comparison of apoptotic effects of lupeol on A549 and C6 cell lines

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https://doi.org/10.55971/EJLS.1466309

Received: 07.04.2024
Accepted: 22.04.2024
Available online: 30.04.2024

ABSTRACT

Lupeol compound is a cyclic triterpene alcohol that is widely found in plants. The compound lupeol has been reported to exhibit antitumor, anti-inflammatory, anxiolytic, neuroprotective and hepatoprotective effects. Recent research shows that lupeol could be a potential medicine for various diseases and also an adjuvant for intractable diseases. Cancer poses a health threat that is increasingly common around the world. Among new cancer cases, lung cancer is one of the most common and deadly cancers worldwide. In this study, the anticancer efficiency of lupeol on human lung adenocarcinoma A549 and glioma C6 cell lines were examined. Various concentrations of lupeol (500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.90 µM) effectually reduced cell viability in the A549 and C6 cell lines in a dose-dependent manner. These results showed that lupeol had selective anticancer activity against A549 and C6 cell lines.

Keywords: Adenocarcinoma, Cytotoxicity, Glioma, Lung cancer, Lupeol

1. INTRODUCTION

Cancer poses a health threat that is increasingly common around the World [1]. According to actual cancer epidemiological statistics collected from the official sources of the World Health Organization (WHO) and the American Cancer Society (ACS), cancers constitute the largest burden worldwide (244.6 million). It is prevalent in both men (137.4 million) and women (107.1 million), ensued by ischemic heart disease (203.7 million) and stroke (137.9 million) [2].

Lung cancer maintains the leading cause of cancer-related deaths around the world, driven by increased cigarette consumption [3]. Lung cancer is divided into two according to pathological types: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC), and NSCLC constitutes 85% of all lung cancer cases [4]. Current lung cancer therapeutic agents are expensive, ineffective, and have serious adverse effects and toxicity on non-cancerous tissue [5].

Gliomas are tumors originating compose of glial cells, which are the most extensive tumors of the central nervous system and constitute 81% of malignancies. They generally arise from glial or pioneer cells and evolve into astrocytoma, oligodendroglioma, ependymoma, or oligoastrocytoma, respectively. As regards to the WHO gradation, gliomas are segreated into four grades. Grade 1 and grade 2 gliomas describe low-grade ones, while grade 3 and grade 4 gliomas remark high-grade gliomas. In general, a relatively

high grade is associated with bad prognosis. While the average survival time for low-grade glioma is 11.6 years, the average survivance time for grade 3 glioma patients is approximately 3 years and for grade 4 glioma is 15 months. Glioblastoma is the most common type of grade 4 glioma [6].

In recent times, researchers have been increasingly interested in natural products, particularly compounds derived from plants, due to their potential as anti-tumor agents. The herb *Portulaca oleracea* L., used in traditional Chinese medicine to treat various types of inflammation and tumors in the digestive tract, contains lupeol terpenoid and has been proven effective like a therapeutic agent in the treatment of colitis associated cancer [7]. Lupeol compound is a cyclic triterpene alcohol (Figure 1) that is widely found in plants [8]. *S. surattense*, a Solanaceae plant, contains the phytochemical lupeol, and the phytochemicals in members of this family are known to generally have anticancer properties [9]. Lupeol is a pentacyclic triterpenoid found in *Ziziphi Spinosae Semen* oil, and it has been studied that the lupeol compound is effective in the plant's sleep-enhancing effect through neurotransmitter regulation and anti-oxidative stress mechanisms [10]. Lupeol is also found in pitaya (*Hylocereus undatus*) fruits and is the wax component of the fruit [11]. The compound lupeol has been reported to exhibit antitumor, anti-inflamatory, anxiolytic, neuroprotective and hepatoprotective effects [12]. The wound amelioration occasion of the ethanolic extract of the bark of *Thespesia populnea* L. was investigated and further phytochemical searches of the bioactive fractions caused the isolation of many compounds belonging to various chemical classes, with the compound lupeol acetate isolated and evaluated as potent wound healers through their antioxidant and anti-inflammatory activities as well as stimulating collagen synthesis [13]. Despite the limited water solubility and bioavailability of lupeol have restricted its therapeutic use, lupeol-chitosan nanoparticles encapsulated in cellulose acetate membranes were synthesized to triying their potential in treating diabetes and cancer. Not only did the *in vitro* assay using A431 human skin cancer cells reveal anticancer efficiency with an IC_{50} value as low as 54.56 mg/mL, the membrane also showed significant antidiabetic potential, effectively inhibiting carbonhyrate-digesting enzymes [14]. The synergistic effect and mechanism of a nanoliposome carrier combined with FDA-approved indocyanine green (ICG)-mediated optical therapy and the natural molecule lupeol in enhancing natural killer (NK) cell activity for colon cancer cell inactivation were investigated. This nanoliposome shows that it reduces colon cancer cell activity to 59.6% after 20 minutes of irradiation and to 43.4% after 20 minutes + 10 minutes of irradiation. When NK cells were added after 20 minutes + 10 minutes of irradiation, the activity decreased to 16.7%, providing a new approach in the treatment of colon cancer, proving its anti-cancer effect [15]. According to these data, we planned to examine the anticancer activity and apoptotic effect of lupeol on A549 and C6 cells.

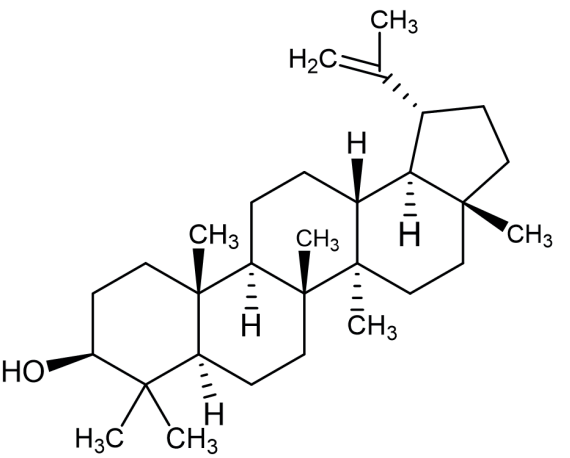

Figure 1. Chemical structure of lupeol.

2. MATERIALS AND METHODS

2.1. Materials

The A549 and C6 cell lines were bought from American Type Culture Collection. Lupeol (S957712) and Cisplatin were acquired from Sigma-Aldrich. Dulbecco's Modified Eagle Medium, penicillin streptomycin and fetal bovine serum were from Gibco, Phosphate-buffered saline tablets were from Sigma-Aldrich, MTT was from Alfa Aesar, and dimethylsulfoxide (DMSO) was from Sigma-Aldrich The Annexin -V FITC/Propidium iodide (PI) apoptosis detection kit was bought from BD Biosciences.

2.2. Methods

2.2.1. Cell Line Model

A549 and C6 cells were keeped in Dulbecco's Modified Eagle's Medium (DMEM) and was added with antibiotics (100 µg/ml streptomycin and 100 U/ml penicillin G) and 10% fetal bovine serum (FBS) in a incubator at 37°C (5% CO_2 and 95% air).

2.2.2 MTT Assay for Cytotoxicity

One of the most popular techniques for assessing cell viability, cytotoxicity, and proliferation is MTT [16]. Metabolically active cells reduce yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, or MTT) to purple formazan crystals, which forms the basis of this colorimetric assay. The A549 and C6 cells were cultivated for 24 hours at 37°C in a humidified environment with 5% CO_2 in the air at a density of $2x10^4$ cells per well on flat-bottomed 96-well plates with different concentrations (500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.90 µM) of lupeol and cisplatin.

Lupeol was applied at various concentrations (500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.90 µM) to a 96-well plate with $2x10^4$ L929, A549 and C6 seeded into each well. Following the incubation period, 20 µL of phosphate-buffered saline (PBS) was added to each well to dissolve the MTT powder (5 mg/mL). The cells were then incubated at 37°C for 2-4 hours. After the waiting period, 100 µl of DMSO was administered to each well after the medium was removed. Cells were measured with a Bio-Tek microplate reader at 540 nm. Cell viability was measured as a percentage relative to control cells for each concentration and this was repeated in three wells. The IC_{50} concentration that provided 50% inhibition of the cell population was found based on the computation findings.

2.2.3. Flow cytometric analysis: Annexin-V for early/late apoptosis

In a 6-well plate, $2x10^5$ cells/mL of A549 and C6 cells were seeded. It was incubated in a moisturized environment with 5% CO_2 in the air for 24 hours at 37°C. Next, cisplatin and lupeol at their IC_{50} doses were appended to the cells and incubated for a further 24 hours. At the end of this period, cells were collected from the plate and placed in flow tubes and then centrifuged at 1200 rpm for four minutes. Following centrifugation, the supernatant was removed with care, twice more centrifuged, and then cleaned with 1 milliliter of cold 1x PBS. After centrifugation, the supernatant was collected and 100 µl of the assay buffer (1x) was pipetted gently into each flow tube. Subsequently, 5 microliters of Annexin-V and 5 microliters of PI dyes were added, and the mixture was left in the dark for 15 minutes.

After this time, 400 µl of assay buffer was added, and the data was examined using FACS Diva Version 6.1.1 software and flow cytometry (CytoFLEX Beckman Coulter).

2.2.4. Statistical analysis

IC_{50} doses and percent inhibition were calculated compared to the control group. All data taken into account were indicated as the standard deviation (SD) of the mean from three repetition of the experiments. Microsoft Excel software and GraphPad Prism 8 were used to calculate the data.

3. RESULTS AND DISCUSSION

3.1. MTT assay for cytotoxicity

In our study, the cytotoxic impacts of lupeol on A549 and C6 cells were enquired using the *in vitro* MTT test On the other part, we used L929 cells to analyze the selectivity of lupeol. Cytotoxicity and IC_{50} values for lupeol and cisplatin in A549 and C6 cells were designated by MTT analysis. The dose and percent viability values used in the analysis are as shown in Figure 2, 3. This cell line was treated with a wide range of lupeol and cisplatin concentrations (500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.90 µM) for 24 hours. Lupeol suppressed A549 cell viability in a dose-dependent manner (Figure 2).

IC_{50} values of lupeol and cisplatin were determined in A549 cells as 483.24 µM and 49.78 µM for 24h respectively (Table 1). Although there are results supporting our findings, lupeol has been observed to exhibit different sensitivity with different cells. In a recent study, results supporting our findings were acquired. In the study directed by Aborehab et al. (2023), the IC_{50} value of lupeol in A549 cells was 344.8 µg/mL [17]. For example, in a study directed by Li et al. in 2013, the cytotoxicity of lupeol was examined in various cell lines such as A549, LAC, HepG2 and HeLa. Lupeol showed various cytotoxicity in these cell lines [18].

IC_{50} values of cisplatin and lupeol were specified in C6 cells as 287.23 µM and 29.84 µM for 24h respectively (Table 2). Also, IC_{50} value of lupeol was specified in L929 cells as 248.30 µM for 24h (Table 3). The IC_{50} value of lupeol was 248.30 µM for 24 h. in L929 cell line (Table 3 and Figure 4E). The dose-dependent percentage viability of lupeol in A549, C6 and L929 cell lines is shown in Figure 5.

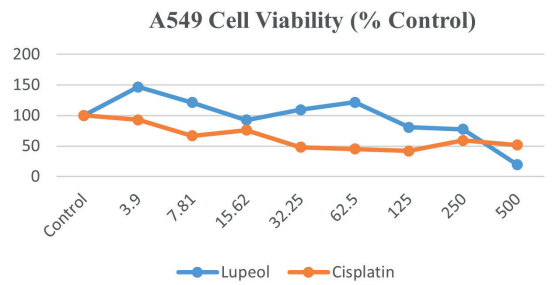

Figure 2. Dose-dependent percent viability graph of lupeol and cisplatin on A549 cells after 24 hours incubation.

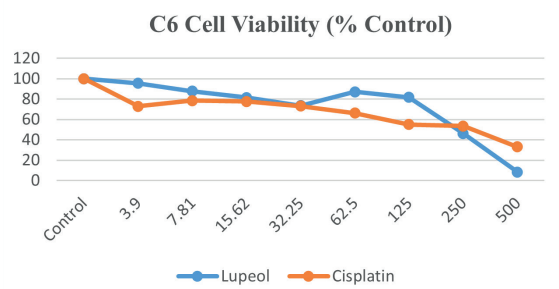

Figure 3. Dose-dependent percent viability graph of lupeol and cisplatin on C6 cells after 24 hours incubation.

Table 1. IC_{50} (µg/mL)[a] values of lupeol and cisplatin against A549 cell line

	IC_{50} (µM) A549	IC_{50}/2 (µM) A549
Lupeol	483.24 ± 148.93	241.62 ± 74.46
Cisplatin	49.78 ± 15.43	24.89 ± 7.71

[a]Cytotoxicity of lupeol and cisplatin. Incubation for 24 hours. The values represent mean ± standard deviation of triplicate determinations.

Table 2. IC_{50} (µg/mL)[a] values of lupeol and cisplatin against C6 cell line

	IC_{50} (µM) C6	IC_{50}/2 (µM) C6
Lupeol	287.23 ± 2.46	143.62 ± 1.23
Cisplatin	29.85 ± 4.05	14.92 ± 2.64

[a]Cytotoxicity of lupeol and cisplatin. Incubation for 24 hours. The values represent mean ± standard deviation of triplicate determinations.

Table 3. IC_{50} (µg/mL)[a] value of lupeol for L929 cell line

	IC_{50} (µM) L929
Lupeol	248.30 ± 2.64

[a]Cytotoxicity of lupeol. Incubation for 24 hours. The values represent mean ± standard deviation of triplicate determinations.

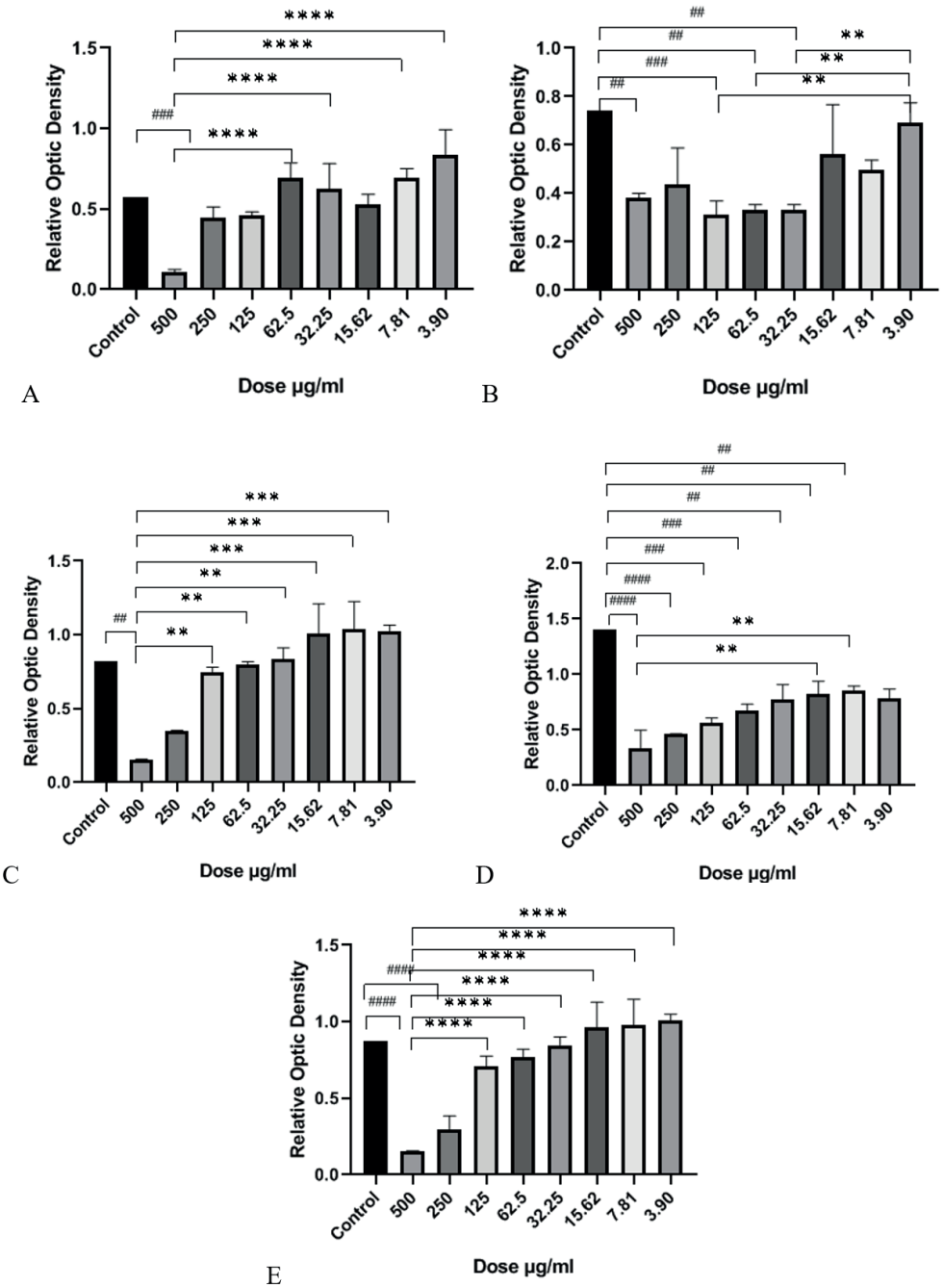

Figure 4. A) After MTT analysis relative optic density of lupeol on A549 cell line after 24 hours of incubation. ### p <0.005 significantly from control , ****p<0.0001 significantly different from 500 µg/mL dose **B)** Relative optic density of cisplatin on A549 cell line after 24 hours of incubation. ### p <0.005 significantly from control, ** p<0.005 significance values between groups **C)** After MTT analysis relative optic density of lupeol on C6 cell line after 24 hours of incubation. ## p <0.005 significantly from control, **, ***p<0.005 significantly different from 500 µg/mL dose **D)** Relative optic density of cisplatin on C6 cell line after 24 hours of incubation. ##, ### p <0.005, ####<0.0001 significantly from control, ** p<0.005 significantly different from 500 µg/mL dose. **E)** Relative optic density of lupeol on L929 cell line after 24 hours of incubation. The experiment was repeated three times and the data are presented as mean± S.D. #### p<0.0001 significantly different from control. ****p<0.0001 significantly different from 500 µg/mL dose.

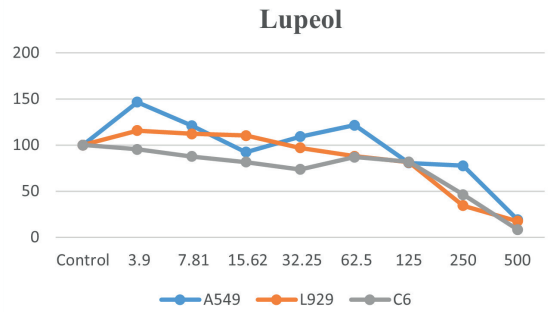

Figure 5. Dose-dependent percent viability graph of lupeol on A549, C6 and L929 cells. Lupeol was also used on L929 murine fibroblast cells and compared with A459 cells to establish a control group while determining the reliable IC_{50} dose. Data were obtained as a result of MTT analysis.

In 2019, Lemes et al. showed that the triterpene lupeol, one of the isoflavonoids they isolated from the *Deguelia costata* plant, was ineffective in the 48 hours cytotoxicity test on C6 glioma cells. However, in our study, activity was found after 24 hours of incubation. This suggests that the effect of lupeol varies over time [19]. The findings of our study indicate that lupeol has selective anticancer activity against A549 and C6 cell lines.

3.2. Flow cytometric analyses by annexinV-FITC

After 24 hours of incubation with IC_{50} and $IC_{50}/2$ doses of lupeol and cisplatin in A549 and C6 cells, readings were made using the kit procedure (BD, Pharmingen) on a flow cytometry device (Beckman Coulter) using Cytoflex Software.

According to the MTT result, we examined the mechanism of action of cell death caused by lupeol in A549 and C6 cells. IC_{50} and $IC_{50}/2$ values of lupeol were used to determine the apoptotic effects of lupeol (Figure 6 and Table 4). Annexin-V binding capacity was examined by flow cytometry by applying lupeol to A549 and C6 cell lines.

After incubation of A549 cells, lupeol and cisplatin at IC_{50} and $IC_{50}/2$ doses, respectively, early apoptotic cell percentages were determined as 13.21 %, 4.39 % and 7.21 %, 5.76 %. Lupeol is identified as an effective apoptosis-inducing agent in various cell lines. However, the mechanism of action varies depending on cell line types. Additionally, the doses of lupeol used to increase apoptosis percentages vary in different studies. For example, in the study of Jin et al., SMMC-7721 cells were evaluated in terms of untreated control cells. In mentioned study, the

Table 4. Percents of typical quadrant analysis of annexin V-FITC/propidium iodide flow cytometry of A549 cells treated with the lupeol and cisplatin

Groups	% Early	% Late	% Viable
Control (untreated)	3.37	3.91	90.66
Lupeol IC_{50} dose treated cells	13.21	21.70	59.46
Lupeol $IC_{50}/2$ dose treated cells	4.39	3.99	91.05
Cisplatin IC_{50} dose treated cells	7.21	4.42	87.96
Cisplatin $IC_{50}/2$ dose treated cells	5.76	3.37	90.68

Table 5. Percents of typical quadrant analysis of annexin V-FITC/propidium iodide flow cytometry of C6 cells treated with the lupeol and cisplatin

Groups	% Early	% Late	% Viable
Control (untreated)	4.54	1.24	94.21
Lupeol IC_{50} dose treated cells	7.30	1.31	91.38
Lupeol $IC_{50}/2$ dose treated cells	15.96	4.64	79.26
Cisplatin IC_{50} dose treated cells	9.95	1.63	88.41
Cisplatin $IC_{50}/2$ dose treated cells	4.95	0.98	94.06

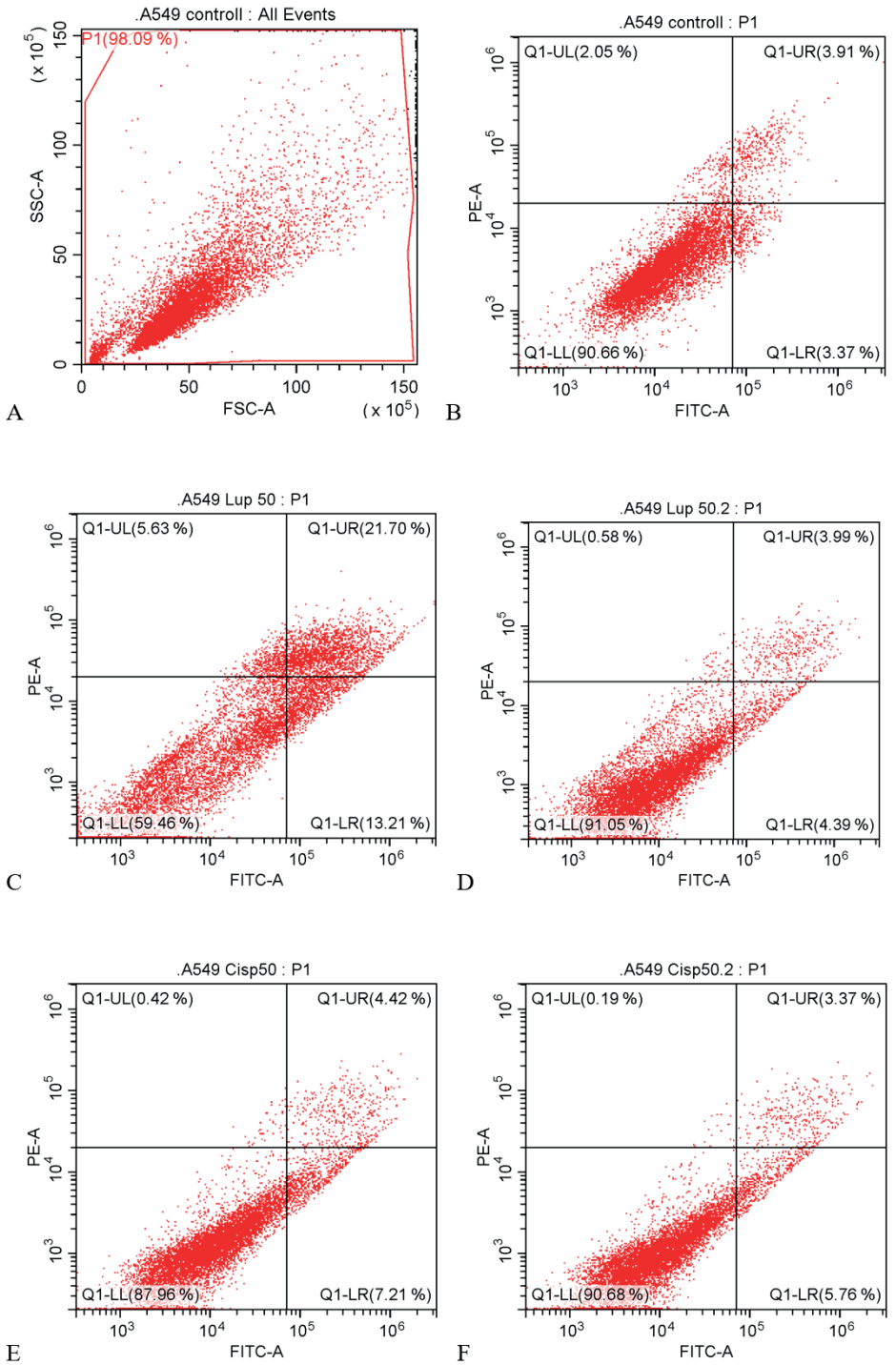

Figure 6. Flow cytometric analysis of A549 cells treated with IC_{50} and $IC_{50}/2$ concentrations of lupeol and cisplatin. A549 cells were cultured for 24 h in medium containing 48.24 and 241.62 µM lupeol and 49.78 and 24.89 µM cisplatin. At least 10,000 cells per sample were analyzed and quadrant analysis was performed. Q1-LR: Early apoptotic cell percentages, Q1-UR: late apoptotic cell percentages, Q1-UL: Necrotic cell percentages, Q1-LL: viable cell percentages. A) Main gate selected from the cell population, B) Control group, C) Apoptotic effect of IC_{50} dose of lupeol, D) Apoptotic effect of $IC_{50}/2$ dose of lupeol, E) Apoptotic effect of IC_{50} dose of cisplatin, F) Apoptotic effect of $IC_{50}/2$ dose of cisplatin.

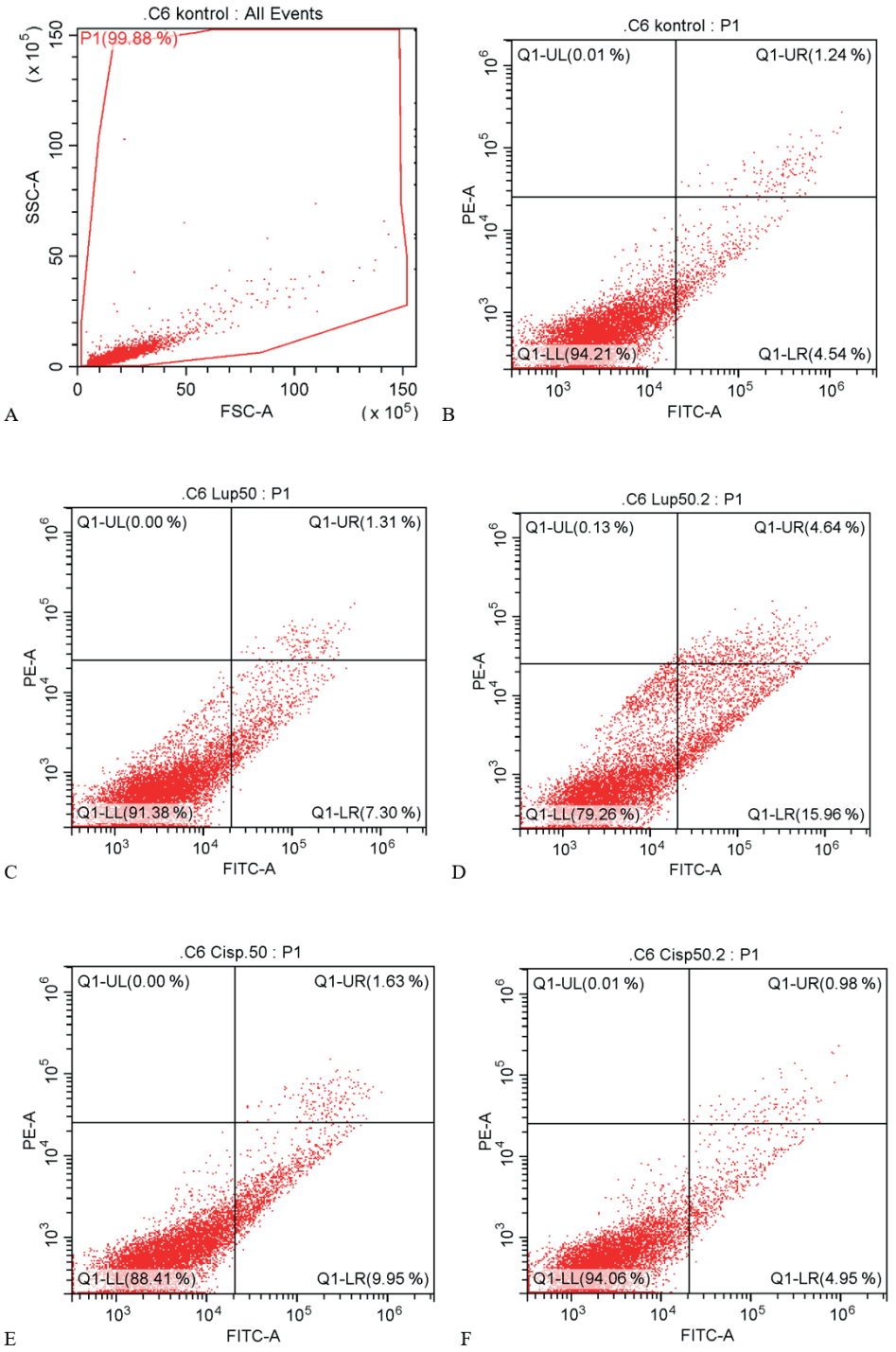

Figure 7. Flow cytometric analysis of C6 cells treated with IC_{50} and $IC_{50}/2$ concentrations of lupeol and cisplatin. C6 cells were cultured for 24 h in medium containing 287.23 and 143.62 and 241.62 µM lupeol and 29.85 and 14.92 µM cisplatin. At least 10,000 cells per sample were analyzed and quadrant analysis was performed. Q1-LR: Early apoptotic cell percentages, Q1-UR: late apoptotic cell percentages, Q1-UL: Necrotic cell percentages, Q1-LL: viable cell percentages. A) Main gate selected from the cell population, B) Control group, C) Apoptotic effect of IC_{50} dose of lupeol, D) Apoptotic effect of $IC_{50}/2$ dose of lupeol, E) Apoptotic effect of IC_{50} dose of cisplatin, F) Apoptotic effect of $IC_{50}/2$ dose of cisplatin.

researchers aimed to induce apoptosis in SMMC-7721 cells by combining lupeol treatment and radiation. As a result of lupeol treatment combined with radiation, an increase in apoptotic cells was observed (48.48% for 4 Gy plus lupeol, 10.65% for control) [20]. At the same time, Yuan et al. showed that lupeol can cause apoptosis in A431 and LNCaP cells via mitochondrial cell death [21].

After incubation of C6 cells, lupeol and cisplatin at IC_{50} and $IC_{50}/2$ doses, respectively, early apoptotic cell percentages were determined as 4.54 %, 15.96 % and 9.95 %, 4.95 % (Figure 7, Table 5). In line with all this information, it was observed that although lupeol triggered apoptosis dose-dependently in the A549 cell line, it triggered dose-dependent apoptosis in the C6 cell line.

4. CONCLUSION

According to our data, the lupeol compound is thought to have dose-dependent anticancer properties on A549 cell line. In addition to these results, it was determined that lupeol caused apoptotic cell death in A549 and C6 cells. The apoptotic mechanism of this cell death was determined by the interaction of the Annexin V protein that a calcium-binding protein, with the released phosphotidylserine, and the resulting necrotic and living cells can be distinguished by PI stain. When we evaluate our findings according to our literature review, lupeol shows a dose-dependent effect in the A549 cell line, but not in the C6 cell line. This suggests that this difference varies depending on the cell type. Although lupeol is thought to be a key agent with anticancer and antiproliferative properties in the light of this study, more studies are needed on this subject.

Acknowledgements

Figure 1 was created with ChemDraw19.0 software.

Ethical approval

Not applicable. Because in this article only cell lines were used that were obtained from ATCC.

Author contribution

Concept: İE, ŞKA, HET; Design: İE, ŞKA, HET; Supervision: İE, ŞKA, HET; Materials: İE, ŞKA, HET; Data Collection and/or Processing: İE, ŞKA, HET; Analysis and/or Interpretation: İE, ŞKA, HET; Literature Search: İE, ŞKA, HET; Writing: İE, ŞKA, HET; Critical Reviews: İE, ŞKA, HET.

Source of funding

This research received no grant from any funding agency/sector.

Conflict of interest

The authors declared that there is no conflict of interest.

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ANADOLU UNIVERSITY
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European Journal of Life Sciences 2024; 3: 31-43

Review Article

Review of an up-to-date, emphatic, and concise perspective on e-cigarettes

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https://doi.org/10.55971/EJLS.1368313

Received: 29.09.2023
Accepted: 14.11.2023
Available online: 30.04.2024

ABSTRACT

Electronic cigarettes entered the market in 2007 as an alternative to conventional tobacco products, and their use has increased significantly worldwide. Due to the emergence of various harmful effects and attracting attention, their sale was banned in our country and many other countries. However, according to the research, the use of e-cigarettes is increasing rapidly in the world and in Türkiye, and the rate of use at young ages is quite high especially. Its supply can be easily made through online sales on many websites. Despite this growing popularity, little is known about the potential effects of e-cigarettes on human health. This is particularly important in the presence of flavoring compounds, solvents, additives, and other ingredients that are added intentionally or unintentionally and whose long-term effects are uncertain. This is because every day a new product with new ingredients, flavors, and properties is introduced to the market. Previous studies have focused on measuring known toxicants, particularly those found in traditional cigarettes, while fewer have investigated unknown compounds and transformation products formed during the e-cigarette process in these diverse and ever-evolving products. It seems that the e-cigarette market is growing and developing rapidly in our country, as in the whole world, and product sales continue to increase despite all kinds of bans. However, when we look at the literature, the research conducted is insufficient, as mentioned above, and there are points that need to be further emphasized.

Keywords: Analysis of e-cigarette, e-cigarette, e-cigarette compounds, e-cigarette laws

1. INTRODUCTION

The recognition of the detrimental effects of traditional combustion cigarettes has emerged as a significant societal concern in response to the increasing prevalence of non-combustible alternatives, such as electronic cigarettes (e-cigarettes) and heated tobacco products (HTPs). The e-cigarette was created by Herbert A. Gilbert in 1965. In his patent, Gilbert referred to it as a "smokeless non-tobacco cigarette" that serves as a substitute for traditional tobacco and paper combustion by generating heated, moist, and flavoured air [1]. The initial iteration of electronic cigarettes, known as first-generation e-cigarettes, exhibited a striking resemblance to conventional cigarettes. These devices were equipped with a rechargeable battery and an atomizer that housed

a nichrome wire and a filler substance designed to absorb an e-liquid solution. The composition of the e-liquid comprised propylene glycol and glycerol, which were supplemented with nicotine and various flavours. Subsequently, the advent of "second-generation e-cigarettes" occurred. The subsequent iteration of electronic cigarettes exhibited a larger form factor, resembling an instrument rather than adhering to the conventional shape of a tobacco cigarette. The components of the device included a battery and an atomizer, with the latter serving as a reservoir for e-liquid. This e-liquid was commercially available in bottles for the purpose of refilling the device. The smoke emissions of second-generation e-cigarettes were found to be higher in comparison to those of first-generation e-cigarettes. Subsequently, in more recent times, a new iteration of electronic cigarettes, known as third-generation e-cigarettes, has been introduced to the market. Typically, a third-generation electronic cigarette exhibits a substantial physical form and occasionally include batteries that may be adjusted to suit individual preferences. Certain components can be substituted, and the level of electrical power can be adjusted. These devices have the capability to generate a concentrated and voluminous emission of smoke particles, resulting in the formation of a "dense of smoke cloud." Nevertheless, the impact of electronic cigarettes on human health remains uncertain.

Electronic nicotine delivery systems (ENDS), colloquially referred to as electronic cigarettes or e-cigarettes, have gained widespread recognition as a comparatively less deleterious substitute for conventional cigarette smoking since their initial introduction to the market over sixteen years ago. E-cigarettes are composed of many components. These include a cartridge that contains an e-liquid, a heating element atomizer that is essential for heating the e-liquid, hence generating a vapour that can be breathed through a mouthpiece. Additionally, e-cigarettes are equipped with a rechargeable battery (Figure 1) [2]. Both electronic gadgets and various e-liquids can be easily obtained from retail stores or online vendors, and designed to provide nicotine to users. These products function by substituting tobacco with a solution containing nicotine benzoate salt, which is combined with a base of propylene glycol and glycerol. Additionally, e-cigarettes often incorporate various flavouring agents to enhance the user experience. The composition of the base consists of propylene glycol, glycerol, or a combination of both in different ratios, which are then diluted with distilled water [3].

E-liquid commonly consists of humectants and flavourings, which may or may not include nicotine. The mist generated by the sprayer bears resemblance to the act of smoking tobacco and is purportedly devoid of any adverse consequences [4]. Nevertheless, there have been reports indicating that the use of heat can result in the generation of novel breakdown chemicals that may pose potential hazards [5]. The concentration of nicotine, which is

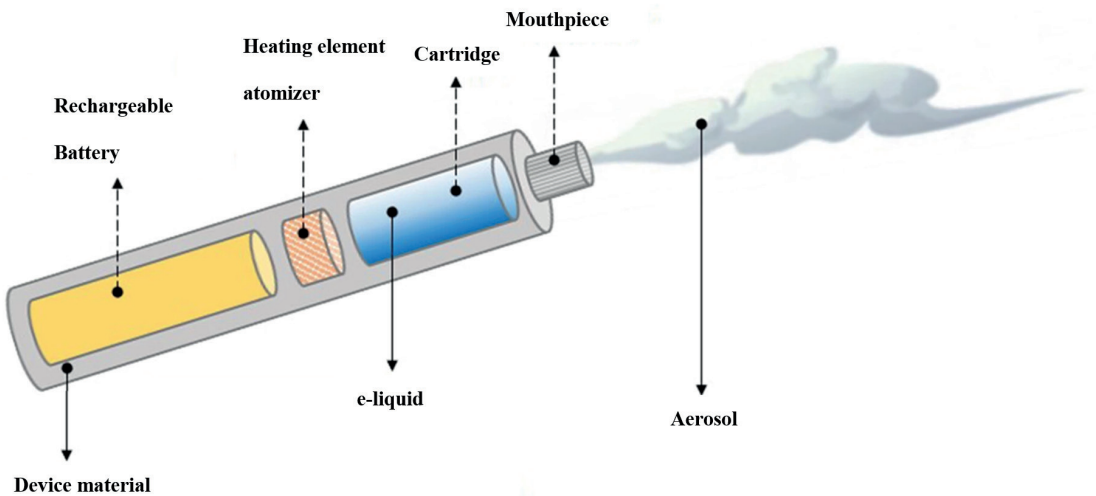

Figure 1. Structure of e-cigarette [2]

the primary addictive substance found in tobacco, exhibits variability between commercially accessible e-liquids, ranging from 0 mg/mL to 41.2 mg/mL [6]. Furthermore, it is worth noting that nicotine-free alternatives are also present in the market. Due to this rationale, electronic cigarettes are frequently regarded as a viable method for smoking cessation, as they have the potential to deter individuals from desiring conventional smoking. Nevertheless, this concept has yet to be substantiated by comprehensive evidence [7,8].

Due to the absence of combustion in e-cigarettes, which is responsible for numerous adverse health effects associated with tobacco usage, there is a prevailing belief that the act of using e-cigarettes, sometimes referred to as "vaping," is comparatively less hazardous than smoking conventional cigarettes [9]. Nevertheless, can they truly be considered devoid of risk? Is there enough toxicological data available for all the constituents utilized in e-liquids? Is the composition of the vapor inhaled during the heating process and its influence on health thoroughly understood? Can electronic cigarettes be utilized to decrease the consumption of tobacco? The questions mentioned do not have answers or waiting to be answered by scientists for the sake of public health, especially the health of adolescents and young people.

1.1. E-Cigarette Usage

The prevalence of combustible cigarette smoking in the United States has witnessed a notable decrease over the course of the last five decades. However, the electronic e-cigarette industry has experienced substantial growth since its launch in 2007 [10]. According to available data, the utilization of e-cigarettes among adults in the year 2018 was recorded at 3.2%, with a higher prevalence of 7.6% observed among young adults aged 18 to 24 years. Notably, the prevalence of e-cigarette use among high school students experienced a substantial rise from 1.5% in 2011 to a significant 27.5% by 2019 [10]. This increase in e-cigarette usage surpassed the prevalence of conventional cigarette use among the youth population. In 2019, over 30% of high school students in the United States disclosed their utilization of electronic cigarettes. Based on the findings of the "E-cigarette and Vape Market Size Report 2022-2030," it is evident that the global market for e-cigarettes and vapes reached a valuation of USD 18.13 billion in 2021. Furthermore, it is projected to exhibit a compound annual growth rate of 30.0% during the period from 2022 to 2030 [11]. The present paper is notable for its comprehensive analysis of the magnitude and potential of the e-cigarette industry. Additionally, the Center for Disease Control and Prevention (CDC) has provided data indicating that in the year 2020, approximately 20% of high school students and 5% of middle school students disclosed their engagement in e-cigarette usage [12]. In recent years, there has been a growing prevalence of electronic cigarette usage among young individuals. A research study including a sample size of 14,352 university students hailing from Belarus, Lithuania, Poland, Russia, and Slovakia revealed that a proportion of 1.1% of the respondents acknowledged using electronic cigarettes [13]. According to a study conducted in Canada, the prevalence of e-cigarette usage was found to be 6.5% [14]. Likewise, there have been reports indicating that in China, while the prevalence of traditional cigarette smoking has declined among adolescents, there has been a notable rise in the prevalence of current e-cigarette use. Specifically, among junior high school students, the rate of e-cigarette use increased from 1.2% in 2014 to 2.7% in 2019. Furthermore, among senior high school students, the rate of e-cigarette use experienced a 3.0% increase by 2019 [15]. As a result, a recent study estimated the number of vapers in the world to be 68 million by 2020 [16].

1.2. E-cigarette Usage in Türkiye

The introduction of e-cigarettes to the Turkish market commenced in 2007, with their distribution being facilitated through various channels such as media advertisements and direct marketing strategies. Nevertheless, it is worth noting that research conducted on e-cigarettes in Türkiye predominantly focuses on the adult population [17-19]. According to recent studies, the prevalence of e-cigarette usage among Turkish students stands at 2.9%, while among individuals aged 18 years and above, the rate is reported to be 19% [18]. Within the existing body of

literature, a notable absence of studies investigating the prevalence and utilization of electronic cigarettes among adolescents in Türkiye was observed.

The adolescent population is a significant demographic that the tobacco industry actively seeks to engage in Türkiye. Based on the findings of the 2017 Global Youth Tobacco Survey, it was observed that the overall prevalence of smoking among individuals aged 13-15 years is 7.7%. Further analysis revealed that the prevalence of smoking is 9.9% among males and 5.3% among females within this age group [19]. The researchers conducted on high school students in Türkiye was documented varying rates of smoking prevalence, ranging from 13.4% to 35.7% [20]. The tobacco business endeavors to sustain reliance on the novel goods it releases into the market.

Consequently, most individuals commenced utilizing electronic cigarettes to cease their smoking habit. While individuals first choose electronic cigarettes to cease smoking, the efficacy of electronic cigarettes in facilitating smoking cessation remains uncertain. The proliferation of electronic cigarette commercials, along with the online selling and marketing of these products, has led to a significant expansion in their usage. When an e-liquid purchase is typed in Turkish into the Google search engine, many products appear on the screen and can be easily purchased via e-shopping. Figure 2 shows an example purchase search. Hence, it is imperative to implement public health safeguards and procedures. The growing prevalence of e-cigarette usage, sometimes referred to as vaping, particularly among the younger population and individuals who have never smoked, is also generating apprehension around potential exposure to hazardous compounds.

1.3. E-Cigarette Products

E-cigarette devices work by heating a liquid mixture (e-liquid) with a metallic coil to produce a fine aerosol that the user inhales. The e-cigarette device is a device that can be used for a long time. The liquids filled in it are materials that are constantly consumed. E-liquid typically contains propylene glycol, glycerol, nicotine, flavourings, and other chemicals. Cinnamaldehyde, benzaldehyde, ethyl vanillin, ethyl maltol, and vanillin are specific chemicals that have been linked to cytotoxic effects on respiratory cells [21]. Diacetyl and acetylpropionyl, which can be found in e-cigarettes, have been linked to lung diseases. Ethyl maltol, the most commonly used flavouring agent, can be found in toxic concentrations [22]. Many flavouring chemicals are present in e-liquids, and some chemicals produce aldehydes by thermal decomposition [23].

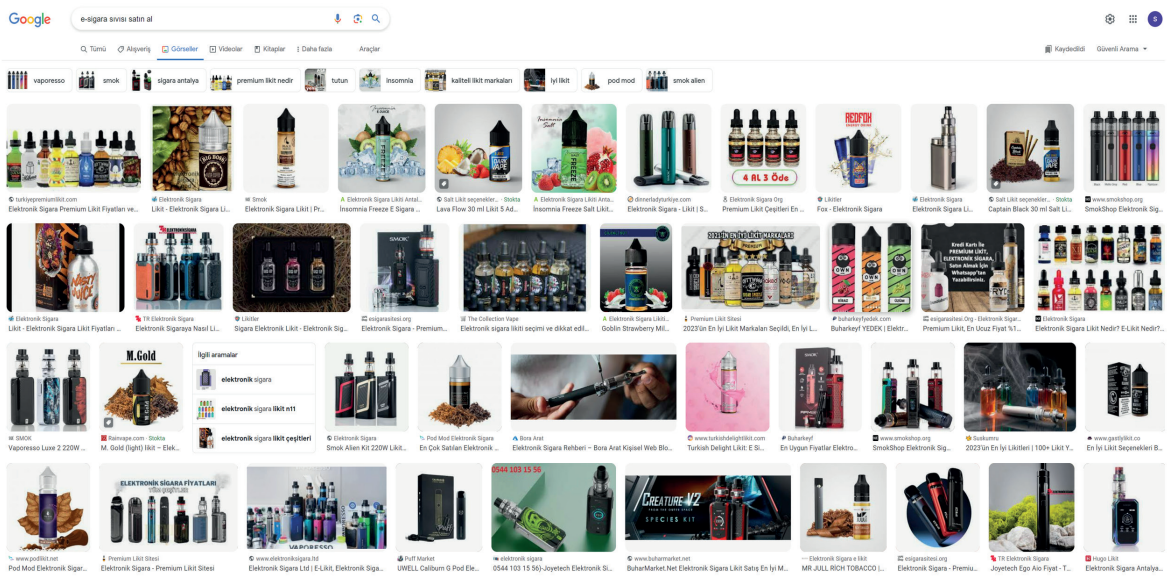

Figure 2. An example purchase search in Türkiye (Date of access: 21 September 2023).

The potential health risk is difficult because the concentration of flavouring chemicals is unknown. Manufacturers are not required to report chemicals or concentrations, and FDA regulations do not recommend guidelines for e-cigarette ingredients. However, information on chemical additives is typically not disclosed by manufacturers. Therefore, aerosols inhaled during e-cigarette use are complex mixtures of solvents and additives, as well as compounds formed during the e-cigarette process. Numerous brands of e-cigarette devices and a wide variety of e-liquid formulations, including more than 8,000 e-liquid flavors, are commercially available, and new e-cigarette devices and liquids are proliferating rapidly [24,25]. Although e-cigarettes have been proposed as a reduced-harm alternative to traditional tobacco-based products due to the reduced presence of well-studied toxicants produced during tobacco combustion, studies have begun to suggest that e-cigarette use has risks associated with vaping, such as lung injury, respiratory dysfunction, inhalation of carcinogenic carbonyls, and changes in gene expression [5]. Furthermore, since e-cigarette emissions are not completely inhaled, there is a potential for witness or secondary exposure to non-users from exhaled aerosols entering the environment [26]. Recent research has also provided insights into how e-cigarette components and emissions affect indoor air quality and exposure pathways [27,28].

The current state of e-cigarette marketing, focusing specifically on promotional methods and themes employed on social media platforms. The study confirmed the utilization of promotional tactics that are consistently employed by the e-cigarette industry, such as price promotions, the incorporation of youth-appealing themes, the inclusion of various flavors, and the utilization of celebrity/influencer marketing [29,30]. Additionally, the other study identified emerging promotional strategies, including the establishment or promotion of pro-vape communities through the use of vape community- and identity-related hashtags, as well as the incentivization of friend tagging [31]. It has been proposed that there is a necessity for implementing limitations on the marketing of e-cigarettes on social media platforms [32]. This measure aims to mitigate the influence of social media in promoting the commencement of e-cigarette use among young individuals and reinforcing their behavioral patterns. The implementation of restrictions should encompass the prohibition of promotional content related to e-cigarettes on social media platforms, specifically targeting individuals who are underage [30]. Additionally, it is imperative to enforce the inclusion of warning labels on pro-e-cigarette information disseminated through social media channels. Furthermore, it is crucial to develop and disseminate counter-messaging initiatives on social media platforms with the aim of deterring the use of e-cigarettes among young individuals and adults in this age group [31].

1.4. Laws of E-Cigarettes

The establishment of a list containing 93 “harmful and potentially harmful ingredients” (HPHCs) in cigarette smoke, cigarette filler, and smokeless tobacco products was undertaken by the US Food and Drug Administration (FDA) in 2012 and [33]. The draught guidance of Section 904(a)(3) of the Federal Food, Drug, and Cosmetic Act (FD&C Act) provided specific information regarding a representative subset of 20 Harmful and Potentially Harmful Constituents (HPHCs) that tobacco product makers are required to disclose [34]. Today, it continues to work to provide an adequate and sufficiently broad perspective on e-cigarette (Figure 3). It is important to note that this reporting requirement applies solely to combustible tobacco products. Furthermore, the Premarket Tobacco Products Applications for Electronic Nicotine Delivery (PMTA) guideline under Section 910 of the FD&C Act draught guidance specifies a total of 29 Harmful and Potentially Harmful Constituents (HPHCs) [35]. The concept of “ends” refers to the ultimate goals or objectives that individuals or organizations strive The Tobacco Products Directive (TPD) 2014/14/EU5 was implemented on May 4, 2016, with the aim of establishing regulations for nicotine-containing electronic cigarettes and refill boxes (Article 20). These regulations were put in place to safeguard human health and ensure compliance with the World Health Organization’s Framework Convention on Tobacco Control, as mandated by the European Union. The enforcement

Figure 3. FDA's webpage on e-cigarette (Date of access: 21 September 2023).

of the majority of rules outlined in Article 20 is carried out by the Medicines and Healthcare Products Regulatory Agency (MHRA) in the United Kingdom [36]. The transposition of the European Union Tobacco Products Directive (EU TPD) into the national legislation of other member states within the EU has been carried out, accompanied by the establishment of competent authorities responsible for its enforcement and supervision [37].

In 2020, FDA implemented sales limitations within the United States; however, these policies were limited in scope, targeting specific flavours and items [38]. Due to the presence of heterogeneity, complexity, and limited transparency in relation to e-cigarette products, there exists a lack of comprehensive understanding regarding the exposures resulting from e-cigarette usage. Consequently, predicting the health consequences associated with such usage becomes a challenging task, as exemplified by the EVALI outbreak in 2019, which was characterized by lung injuries linked to the use of e-cigarette or vaping products [39]. Given the increasing number of individuals utilizing e-cigarettes, the mounting proof linking e-cigarette usage to elevated smoking rates, and the absence of prior governmental oversight, there exists a substantial imperative to establish regulations pertaining to present e-cigarette utilization [40]. Certainly, it is imperative to address the existing knowledge gaps pertaining to chemistry, toxicological, as well as clinical and behavioral patterns in order to enhance our understanding of e-cigarette consumer safety and associated dangers [41]. Nevertheless, our current understanding of the comprehensive chemical composition of the vapor generated during the act of vaping, as well as the intricate mechanisms responsible to produce these substances, remains incomplete. In addition, the extensive assortment of e-cigarette products and diverse range of flavor offered on the market, along with frequent product modifications resulting from the growing popularity of e-cigarettes, pose considerable obstacles in conducting research on e-cigarettes and forecasting potential risks for users [16].

Based on recent developments, Mayor London Breed has officially enacted a municipal regulation that effectively prohibits the commercial transaction of electronic cigarettes within the jurisdiction of

San Francisco, located in the state of California, United States of America [42]. According to the World Health Organization, the utilization of nicotine-containing goods, including Electronic Nicotine Delivery Systems (ENDS), is deemed dangerous for young individuals and pregnant women [43]. The demise of an individual who had engaged in the usage of electronic cigarettes and had been admitted to a medical facility due to a grave respiratory ailment was recently documented by the Illinois Department of Public Health (IDPH) [44]. Ongoing investigations are being conducted by the Center for illness Control and Prevention (CDC) and FDA into the prevalence of severe lung illness among individuals who use electronic cigarettes [45]. According to the CDC, there have been a total of 380 documented occurrences of lung disease linked to the utilization of e-cigarette products throughout 36 states and one United States territory [46]. Additionally, six fatalities have been reported in the states of California, Illinois, Indiana, Kansas, Minnesota, and Oregon [46]. The Pharmaceuticals and Medical Devices Act prohibits the selling of e-liquids containing nicotine in Japan [47]. In 2008, the Ministry of Health in Türkiye made a rule to classify e-cigarette cartridges as pharmaceuticals and e-cigarette machines as medical equipment, mostly due to their elevated nicotine levels. Consequently, these entities are not eligible for inclusion within the framework of free trade. Both e-cigarettes and heated tobacco are tobacco products that have been found to have detrimental effects on human health. The year 2013 witnessed the implementation of a comprehensive set of regulations in Türkiye, which included the prohibition of e-cigarette advertising, smoking, and sales within enclosed spaces. The publication of Law No. 2149 on 'Electronic Cigarettes and Similar Devices and Certain Tobacco Products and Imitation Tobacco Products' occurred on February 25, 2020.

1.5. Analysis of E-Cigarette

It is evident that electronic cigarettes possess the potential to cause harm to the human body, even in the absence of nicotine. To date, a variety of well-documented hazardous substances, including as metals, carbonyls, free radicals, and phthalates, have been found and quantified in e-liquids and aerosols [36,48]. Several carbonyl compounds, including formaldehyde, acetaldehyde, and acrolein, have been identified in the emissions of electronic cigarettes. E-cigarettes have been documented to contain hazardous substances, including nitrosamines and diethylene glycol, which have the potential to cause adverse effects on human health. Aerosols emitted from electronic cigarettes have been found to include carcinogens and hazardous substances, including carbonyl compounds. Additionally, there have been reports indicating the presence and impact of carbonyl compounds and free radical generation in e-cigarette smoke. The length, volume, and generation of radicals during respiration are somewhat influenced by aerosol creation at elevated temperatures. However, it has been noted that excessively high levels of free radicals are detected at temperatures ≥100 °C, even when aerosol production is limited [49]. The manufacturing of aerosols and chemicals is contingent upon the availability of electrical power. When considering the power applied to the coil, the quantity of aerosols generated per puff varies between 0.27 and 1.1 mg/watt [50]. In relation to the initial and subsequent iterations of electronic cigarettes, it has been shown that these devices inadvertently generate carbonyl chemicals within the emitted vapor [51]. One potential factor contributing to the generation of carbonyl compounds is the process of liquid oxidation within electronic cigarettes. One potential factor contributing to the creation of carbonyl compounds is the oxidation process that occurs when certain liquids, such as GLY and PO, meet the heated nichrome wire in the atomizer of e-cigarettes. This contact leads to the conversion of these liquids into various carbonyl compounds, including formaldehyde, acetaldehyde, acrolein, glyoxal, and methylglyoxal. Moreover, the emission of e-cigarette aerosol is augmented by elevating the applied voltage from 3.2 to 4.4 V. Carbonyl compounds are synthesized when voltages beyond the threshold of 4 V, and their concentration exhibits a significant augmentation at 4.8 V. The observed values of carbonyl compounds exhibit variations among the five items as well as within the three assays conducted for each product. Dicarbonyl compounds, including glyoxal and methylglyoxal,

as well as oxide compounds like as propylene oxide and glycidol, are suggested for the purpose of thermally decomposing e-liquid. Nevertheless, there is a scarcity of analytical findings pertaining to these chemicals [52].

The primary focus of previous research on e-cigarette exposure has been to measure the quantities of specific elements that are commonly found in high concentrations in e-liquids, notably those present in combustible cigarettes [53]. The analyzes of these compounds in aerosols and the method used are summarized in Table 1. These compounds include tobacco alkaloids, polycyclic aromatic hydrocarbons, and formaldehyde [54,55]. Nevertheless, an increasing body of research has also examined chemicals that are special to e-cigarettes. These include substances that are produced through the breakdown of solvents found in e-liquid [56,57], impurities [2] and flavorings originating from the packing of e-liquid [48], and components of e-cigarette devices [58].

The assessment of chemical exposures from electronic cigarettes necessitates the identification of both recognized toxicants and unknown compounds present in e-cigarette aerosols. These unknown chemicals encompass unreported additives, transformation products, and pollutants that are produced during the vaping process. To facilitate proper regulatory measures and mitigate inadvertent dangers to those using e-cigarettes, it is imperative to possess a thorough comprehension of chemical exposures. Prior research has primarily concentrated on the quantification of established harmful substances, specifically those present in conventional cigarettes. However, there is a scarcity of studies examining unidentified compounds and the byproducts generated during the act of vaping, particularly in relation to the wide range of constantly evolving vaping devices. Previous investigations utilizing Gas Chromatography (GC) in conjunction with quadrupole or time-of-flight mass spectrometry have mostly concentrated on the characterization and detection of semi-volatile and volatile constituents within e-cigarette samples [59,60]. Also, the new studies have drawn attention to a very important point. They conducted quantitative analysis of various types of cannabinoids [61] in e-cigarettes and proved that there are products on the market that contain such compounds [62-64]. The application of Liquid Chromatography combined with Electrospray Ionization (ESI) High-Resolution Mass Spectrometry (LC-HRMS) has been employed for the qualitative examination of industrial chemicals, next generation insecticides, food additives, and pharmaceuticals. Furthermore, the utilization of LC-HRMS for off-target analysis offers notable advantages in terms of mass accuracy, sensitivity, and specificity owing to its exceptional resolving power [56,57]. Consequently, this technology is well-suited for sophisticated chemical fingerprinting applications. The integration of this comprehensive methodology has been previously employed for the detection and characterization of organic chemicals

Table 1. Puffing of EC generates smoke [53]

Volatile organic compounds	Carbonyls	Tobacco-specific nitrosamines	Polycyclic aromatic hydrocarbons/ phthalates	Heavy metals	Nicotine	Flavoring compound
GC-FID	HS-GC-MS	GC-MS	GC-MS	ICP-OES	GC-MS	GC-FID
GC-PID	SPME-GC-MS	LC-MS/MS	SIM-GCMS	ICP-MS	GC-NPD	TD-GC-MS
GC-MS	HPLC-UV	UPLC-MS	LC-MS		GC-NSD	
GC-IT-MS	HPLC-DAD		LC-MS/MS		GC-FID	
HS-GC-MS					GC-TSD	
TD-GC-MS					HS-SPME/GC-MS	
SPME-GC-MS					VUV-AMS	
SIFT-MS					TD-CGC-NPD	
					HPLC-UV	

in bodily fluids such as urine and blood, as well as in various environmental matrices including wastewater, food, and plant samples. However, its application to the study of e-cigarette samples has not yet been explored [65-67].

In conclusion, there are methods available in the literature that can analyze quantitatively various compounds of proven risk in cigarette analysis with high accuracy and precision. However, the main and most important question is what exactly is in these products that are legally restricted but frequently encountered on social media, the internet and sometimes on the street? What do individuals inhale in these products? What is the result of combustion in these products? The most important and dangerous question is what is added to the products that come up with a new flavor every day during the production process? Which banned substances do e-cigarettes make more accessible to users? These are the main questions that we scientists often need to answer. E-cigarettes appear every day under a different cover and present dangers that can cause irreversible damage to public health, especially the health of young people. Therefore, we urge analysts to look at the analysis of these substances with more intensity and effort.

2. CONCLUSION

The utilization of e-cigarettes has emerged as a subject of public health discourse both within the United States and on a global scale. Although e-cigarettes have the potential to aid in smoking cessation, their utilization is associated with heightened health hazards and an increased likelihood of addiction, particularly among adolescents. Numerous legislative measures at the federal, state, and local levels have exerted an influence on the realm of tobacco retailing, with a specific focus on the domain of electronic cigarettes. Nevertheless, the existence of legal loopholes and the increased availability of these products due to globalization contribute to their accessibility. Undoubtedly, a salient consideration pertains to the inherent lack of control associated with social media advertisements. It is advantageous to highlight public health concerns pertaining to various aspects, including the accessibility of tobacco products to young individuals, the exposure of consumers to a diverse array of tobacco products and marketing strategies within retail environments where they may seek smoking cessation aids, the exposure of consumers to unregulated or inadequate products, and the potential undermining of federal, state, and local regulations due to consumer access to prohibited products through online platforms and mail services.

Currently, the number of recognized compounds included in e-liquids and aerosols exceeds 400, with concerns regarding the existence of numerous yet unidentified constituents. The way these substances are incorporated into the production process, combined with other constituents, or introduced through alternative means remains uncertain. Although several substances included in this study are deemed to be safe for consumption, the potential effects of inhaling these chemicals remain uncertain. It is evident that certain factors have the potential to induce a range of health complications. The designation of inhalation of these compounds as an alternative route of exposure is justified and necessitates additional evaluation of their toxicity.

The endorsement of electronic cigarettes as a viable substitute for traditional cigarettes lacks formal validation. The presence of compounds inside aerosols can give rise to the potential hazard of second-hand exposure. The potential prolongation of industry regulation could result in the inadvertent exposure of a significant number of individuals to unfamiliar substances, so subjecting them to health risks that are yet undetermined. Although many compounds found in e-liquids are deemed acceptable for oral use, there exists a dearth of scientific investigation about their potential health implications when inhaled. Exposure to these substances by inhalation is expected to be correlated with various outcomes. The designation of chemicals as unique exposures and the need for urgent investigation is justified by this route of exposure. It is advisable to conduct additional comprehensive analyses to identify and quantify the ingredients. This is crucial to prevent the widespread adoption of these products, which

has led to a significant increase in nicotine addiction among the youth and has been associated with the recent surge in respiratory illnesses. It is imperative to exercise prudence in this matter.

Author contribution

Concept: SÖ, EGÖ; Design: SÖ, NÖC; Supervision: SL, NÖC; Data Collection and/or Processing: SÖ, SL; EGÖ; Literature Search: SÖ, SL; Writing: SÖ; EGÖ; Critical Reviews: SL, NÖC.

Source of funding

Egemen Güvenç ÖĞÜT and Saniye ÖZCAN would like to thank the TÜBİTAK 2209 program for supporting them for their practical work on this subject.

Conflict of interest

The authors declared that there is no conflict of interest.

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