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Which *Humulus lupulus* drug samples meet the European Pharmacopoeia criteria: Cultivated, obtained from the herbalists or online shopping sites in Turkey?

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ABSTRACT: *Humulus lupulus* L. (Hops), which belongs to the Cannabaceae family, originates from Asia and grows naturally in geographies with temperate climates such as Europe and North America. *H. lupulus* might have been brought to Turkey from Germany to be used mostly in beer production. The plant is also used for medicinal purposes due to its sedative and phytoestrogenic effects all over the world. Pharmacopoeia analyzes are important to confirm whether the subject drog has the properties specified in the pharmacopoeia in order to check its suitability before it is used for health purposes. For this reason, we aimed to evaluate features of the variety of hops plants: 1-cultivated in Bilecik/Turkey; 2-sold by herbalists and sold on online shopping sites. Obtained samples of hops were analyzed according to the '*Lupuli flos*' monograph provided within the European Pharmacopoeia 8.0 (EP 8.0). Macroscopic, microscopic, thin-layer chromatography, matter extractable by ethanol, loss on drying analyzes and determination of total ash amount of the samples were conducted according to the EP 8.0. As a result, the hop samples, except those cultured in Turkey, were not suitable for the EP 8.0. Legal controls should be provided by the authorities on herbals sold by herbalists and on the internet. Samples of hops cultured for use in beer production in Turkey were suitable for pharmacopoeia. These samples should meet the public at secure sales points or in food supplements in Turkey.

KEYWORDS: *Humulus lupulus*; hops; European Pharmacopoeia; Cannabaceae; herbalist.

1. INTRODUCTION

Humulus lupulus L. (Hops) belongs to the Cannabaceae family and is a perennial vine that can reach 7-8 meters in length, which re-grows from its rhizomes every spring [1]. The leaves of *H. lupulus* are dark green, heart-shaped with 3-5 lobes, sharp-toothed with a smooth surface. The male and female flowers are on separate plants and can be easily distinguished by their different sizes. On the other hand, only female flowers are used due to their higher secondary metabolite rate. The female flowers are called strobile or cones. That are yellowish-green in color and consist of bracts, bracteoles and lupulin glands, which contain abundant resinous essential oil [2,3]. Hops, which grows naturally in Asia (China, Japan), Europe (Germany, Poland, England, Czech Republic, Slovenia), North America (mainly in Idaho, Oregon, and Washington), South Africa, Ukraine, Australia, and New Zealand, is cultivated in various provinces of Turkey such as Bilecik, Kayseri (Erciyes Region) and Kırklareli. It is grown in many regions with abundant sunlight, warm temperatures, high annual precipitation and fertile soil [1-8].

Hops were first used in brewing due to their antimicrobial effect in Europe. Later, it was used in beer production to add bitter taste originating from monoterpenes linalool and geraniol in its essential oil and to provide foam stability with xanthohumol (XN) in its composition [2,9,10]. About 97% of *H. lupulus* grown worldwide is used in beer production [11]. Hops strobiles have been used as food flavoring to prepare spices, sauces, tobacco and alcoholic beverages. Apart from the strobiles, such as leaves, stems and rhizomes were used as vegetables, in the production of paint, fabric and paper. Hops are traditionally used for healing earache, toothache, stomach problems, liver disorders, leprosy, and fever; especially the female inflorescences have been shown to have biological activity. Traditional Chinese Medicine and Ayurvedic Therapy included *H. lupulus* to treat problems such as indigestion and nervous tension [2,3,12]. In aromatherapy, *H. lupulus* applied for skincare, respiratory conditions, nervousness, nerve pain and stress related conditions. Topically, used for treating skin ulcers and injuries, relieving muscle spasms and nerve pain [3,4,13-17]. Excessive

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daytime sleepiness was observed in *H. lupulus* collectors and plant workers led to its use as a sedative, relaxant in beverages, moreover, hops-filled pillows have been used as a sleep aid [13].

Reports in international monographs indicate that *H. lupulus* flowers can be used to relieve the symptoms of mental stress and treatment of sleep disorders [18-20]. Physicians' Desk Reference (PDR) of Herbal Medicines, reported that the plant stimulates the secretion of gastric juices and has a strong spasmolytic effect on intestinal smooth muscles, moreover, antibacterial and antimycotic effects come from the bitter acids of the plant. Commission E has approved the use of the herb for the treatment of nervousness and insomnia, additionally, the plant is also included in homeopathic formulations used for the treatment of nervousness and insomnia [20].

Clinical studies on the effectiveness of *H. lupulus* in sleep disorders are present in the literature. A randomized, placebo-controlled multicenter study was conducted applying a standardized combination of hops extracts (83.8 mg) and valerian (374 mg) every night for 28 days with 184 patients having mild insomnia. The study concluded that the hops-valerian combination demonstrated a modest hypnotic effect, improving sleep without producing significant residual effects and rebound insomnia [21]. An herbal preparation containing lavender oil, lemon balm and oat extracts, as well as hops, exhibited a relaxing effect in healthy volunteers, documented by electroencephalographic analysis [22].

The phytoestrogenic property of *H. lupulus* was first described by Koch and Heim in 1953. Milligan et al., determined that 8-prenylnaringenin (8-PN) a derivative of desmethylxanthohumol, was the major estrogenic substance in Hops using Ishikawa cells [6,23]. In a randomized, double-blind, placebo-controlled study, Heyerick et al., administered *H. lupulus* extract at a dose equivalent to 100 µg of 8-PN for 6 weeks to postmenopausal women. In conclusion, the incidence of symptoms related to estrogen deficiency, such as hot flashes, sweating, insomnia, heart palpitations, and irritability, decreased in menopausal women [24-26]. Topical application of gel formulation containing *H. lupulus* extract, hyaluronic acid and vitamin E significantly reduced vaginal dryness in postmenopausal women [27]. Additionally, Keiler et al., observed that low-dose 8-PN increased libido in women [28].

The antioxidant effect of humulon and lupulon, which are bitter acids of hops, was studied by Tagira et al., and it was observed that they have radical scavenging effects similar to ascorbic acid and α-tocopherol [29]. Further, the antioxidant effect of XN and isoxanthohumol (IXN) was investigated by Yen et al., with the comparison of Trolox [30]. Consequently, XN was found to be more effective than Trolox in scavenging peroxyl and hydroxyl radicals, while IXN was more effective than XN in scavenging peroxyl radicals but showed the same effect as Trolox in scavenging hydroxyl radicals. XN and IXN have also been proven to inhibit nitric oxide production [31,32].

XN has been evaluated as broad-spectrum chemopreventive agent by (1) inhibiting metabolic activation of procarcinogens, (2) inducing carcinogen detoxifying enzymes, and (3) inhibiting early-stage tumor growth [23,33,34]. Moreover, it has been determined that XN can induce apoptosis causing cancer cells to die and inhibiting the migration of cancerous cells [4,35-40]. In a review article published by Harish et al., *in vivo* and *in vitro* results for the effect of XN on cancer types such as breast, cervical, colon, colorectal, esophageal, hematological, laryngeal, liver, ovarian, pancreatic, prostate and thyroid demonstrated that. They mentioned several human studies on the therapeutic effect and toxic dose of XN, emphasizing that XN is a good candidate for cancer drug development [5].

The anti-inflammatory and antiangiogenic properties of XN, IX and 8-PN, which are in the chemical composition of hops used in beer making, were evaluated by Negrão et al., in a rat skin wound healing test *in vivo*, with both histopathological and immunochemical tests. The anti-inflammatory and antiangiogenic effects of IX have been confirmed. However, 8-PN was observed to be a potent pro-inflammatory and angiogenic factor [41]. In mice whose brains were affected by lipopolysaccharide (LPS), encephalitis and depressive behaviors were inhibited by bitter acids, including α-acids, β-acids, and iso-acids [42].

While XN, IXN, 6-Prenylnaringenin (6-PN) and 8-PN in the composition of hops have antifungal and antibacterial activity, IXN also has antiviral activity. Among these chemical components, the most active is XN [6]. It has been observed that hops metabolites humulon, lupulon and XN have antibiofilm and strong antimicrobial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) chain. However, the strongest antimicrobial effect was seen with lupulon, followed by XN [2,5,43-45]. It has also been observed that hops extract and essential oil have antifungal effects [46,47].

It has been observed that iso-*a*-acids in hops improve hippocampus-related memory and cognitive function associated with the prefrontal cortex, and these functions are mediated by increased hippocampal dopamine levels and activation of dopamine D1 receptors [48].

In studies conducted to investigate the effects of hop secondary metabolites on obesity and type 2 diabetes, reductions in glucose, triglyceride and leptin levels and adipose tissue weight were observed. In 2017, Costa et al.'s study on a mouse model with XN and 8-PN showed that these two compounds were effective in patients with type 2 diabetes [49]. Thus, it was concluded that hops may play a role in natural treatment of obesity and type-2 diabetes [50-54]. There are studies on the analgesic, stomachic and antioxidant effects of *H. lupulus*, but studies on its sedative effect, phytoestrogenic effect and anticancer activity have come to the fore.

The female flowers of *H. lupulus* are rich in resin, essential oil and polyphenols. Hops resins contain bitter acids (*α* and *β* acids) in varying compositions and concentrations. Humulone (35-70% of total acids), cohumulone (20-65%) and adhumulone (10-15%) are named as *α*-acids; lupulone (30-55% of total acids), adlupulone and colupulone are named as *β*-acids [2]. *α*-acids are very important compounds for the quality of hops used in the brewing industry, they contribute to foam stability and have antibacterial activity [55]. *α* and *β* acids are also responsible for the plant's sedative and antidepressant-like effect. Essential oils and resins make the plant usable in the perfume and aroma industry [2,13,56].

H. lupulus flowers are registered as '*Lupuli flos*' in the European Pharmacopoeia (EP). In this study, it was aimed to determine whether the obtained hop samples have the characteristics specified in the EP 8.0 monograph and to evaluate their compliance with the pharmacopoeia. In this context, *H. lupulus* female flowers and dry pellets of two different varieties cultivated in Bilecik were provided by Agronomist Selahattin Yilmaz (Head of Medicinal Aromatic Plants Unit in Bursa Provincial Directorate of Food, Agriculture and Livestock). In addition, other samples were purchased, from herbalists and online stores. Pharmacopoeia analyzes were performed in all the samples and the results were compared.

2. RESULTS

2.1. Macroscopic analysis

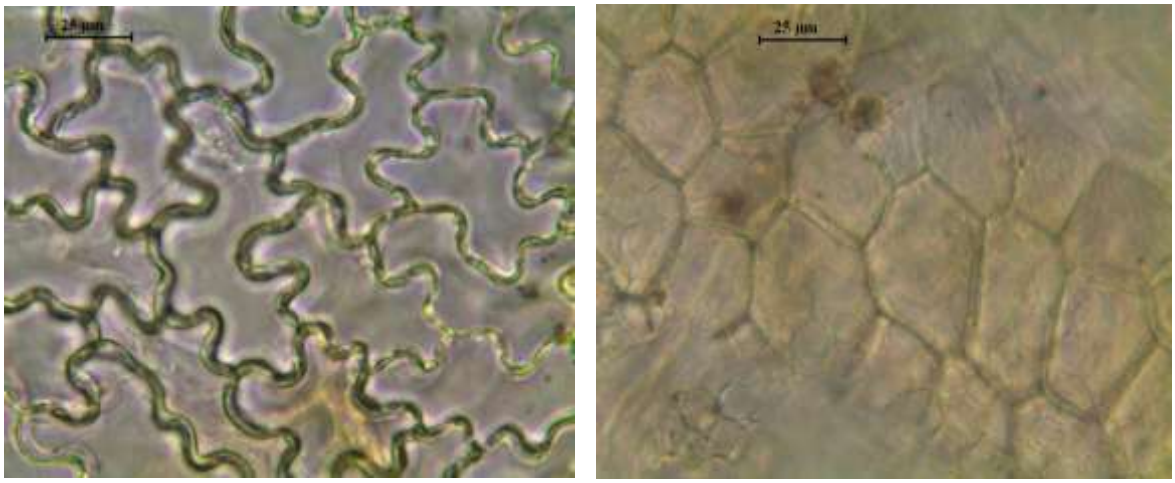
As a result of the analysis, the strobiles of samples 1 and 3 were greenish-yellow, oval, sessile, overlapping bracts as specified in the EP 8.0. The foreign matter was detected in samples 5, 6, 9, 10, 14 and 15. Samples 8, 12 and 13 contained brownish leaf fragments. In Example 15, there were only twigs and no female flower segments (Table 1).

Table 1. Macroscopic analyzes of the samples

Sample Number	General appearance
1	Female flowers: Greenish-yellow, 2-4 cm Strobiles: Oval, sessile, overlapping bracts External bracts: Symmetrical, flattened Characteristic, pleasant smell
2	Dry pellet: Green; characteristic, pleasant smell; 0.5- 2 cm
3	Female flowers: Greenish-yellow (more green), 2-3.5 cm Strobiles: Oval, sessile, overlapping bracts External bracts: Symmetrical, flattened Characteristic, pleasant smell
4	Dry pellet: Green (more green); characteristic, pleasant smell; 0.5- 2 cm
5	Powder; heterogeneous appearance; dark green; slight smell contains foreign materials such as soil.
6	Strobiles: Broken, greenish-yellow to brown, contains twigs, fragments of leaves and foreign plant parts
7	Strobiles are separated into bracts. contains twigs and leaves. Therefore, green color image is more.
8	Strobiles: Yellowish-brown contains long twigs.
9	Strobiles: Broken, yellowish-green contains long twigs, leaves and foreign materials such as insect.
10	Broken plant pieces, green contains twigs, fragments of leaves and foreign materials such as hair.
11	Strobiles separated into bracts and yellowish-green. contains leaves.
12	contains twigs and leaves, mostly green color
13	contains twigs and leaves. Some of the leaves were brown, but the general appearance color was pale green.
14	contains twigs, leaves and foreign materials such as animal feces.
15	contains twigs, leaves, foreign plant pieces and foreign materials such as plastic. It did not contain strobile and the general appearance was brown.

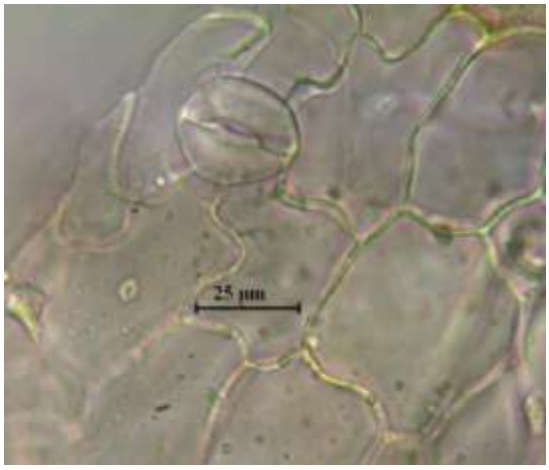
2.2. Microscopic analysis

Irregular-walled and wavy-walled epidermal cells were detected in all the samples (Figure 1). The samples had calcium oxalate crystals, glandular trichomes, and anomocytic stomata, except for sample number 15 (Figure 2). Leaf, root and stem elements (e.g. parenchyma cell, fungal and lignified tissue) were detected in all samples except the first 4 samples.

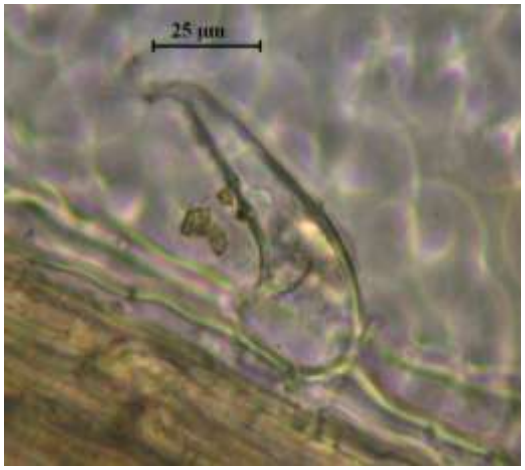


(1) (2)

Figure 1. (1) Wavy-walled epidermal cells, (2) Irregular-walled epidermal cells

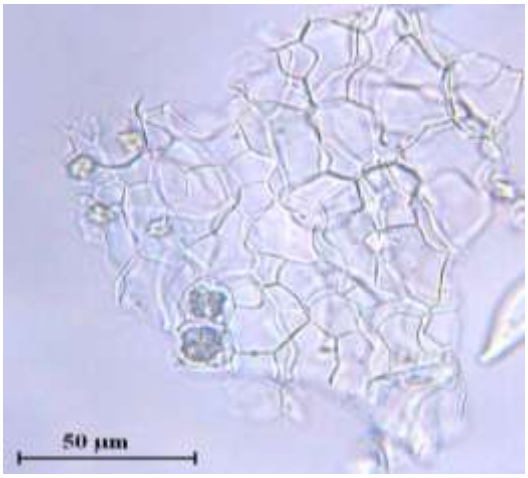


(1) Chloralhydrate (10×40)



(2) Chloralhydrate (10×40)

(3) Chloralhydrate (10×40)



(4) Chloralhydrate (10×40)

Figure 2. (1) Anomocytic stomata, (2) unicellular, curved and attached to an epidermis trichomes, (3) glandular trichomes, bicellular biseriate stalks and heads consisting of 8 small cells, (4) fragments of mesophyll containing small calcium oxalate cluster crystals

2.3. Thin-layer chromatography (TLC)

According to the results of TLC analysis, xanthohumol, humulon and lupulon were detected in samples 1, 3 and 4 (Figure 3). The plaque image obtained with 254 nm UV is shown in Figure 4 and the plaque image obtained with 365 nm UV is shown in Figure 5. The presence of these compounds was proven by all 3 detection methods. These compounds were not found in other samples. On the other hand, the tea samples of the *H. lupulus* plant cultivated in Bilecik, did not drift on the ITC plate.

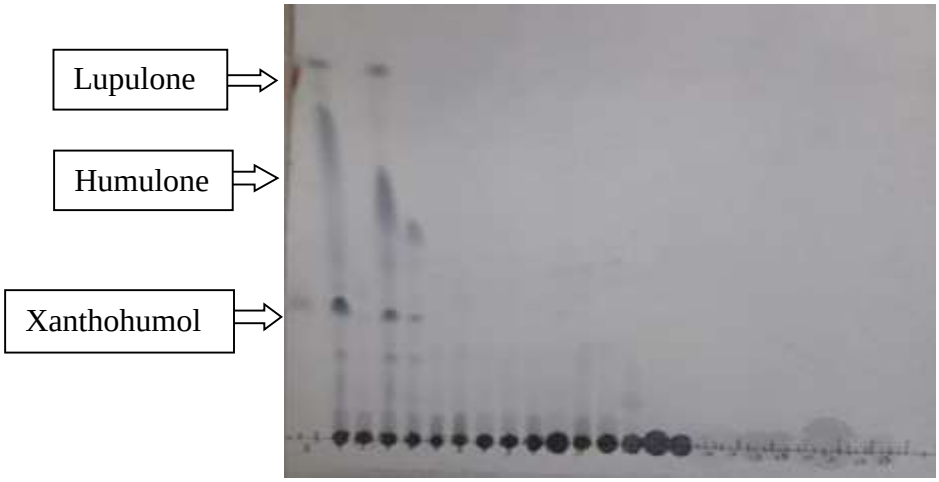


Figure 3. Plaque image obtained with dilute phosphomolybdotungstic reagent

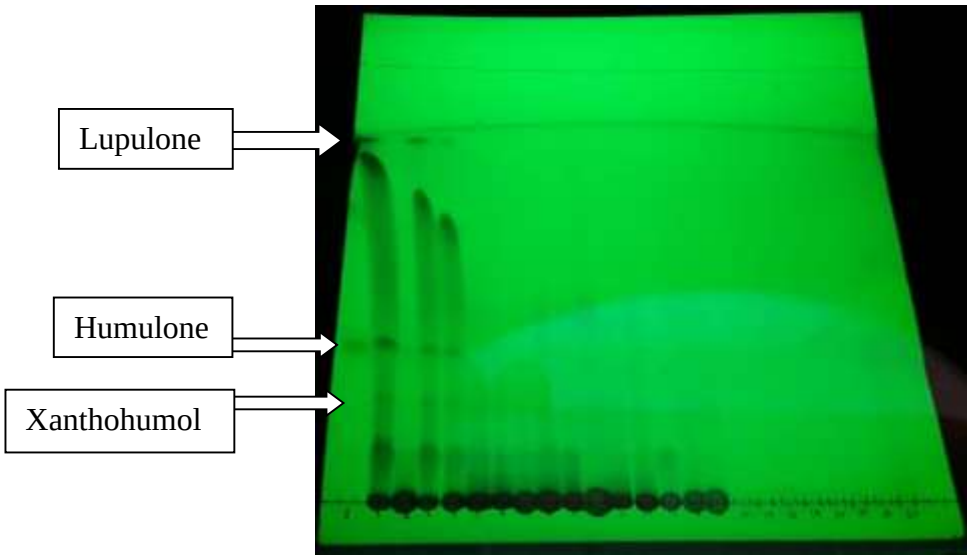


Figure 4. Plaque image obtained with 254 nm UV

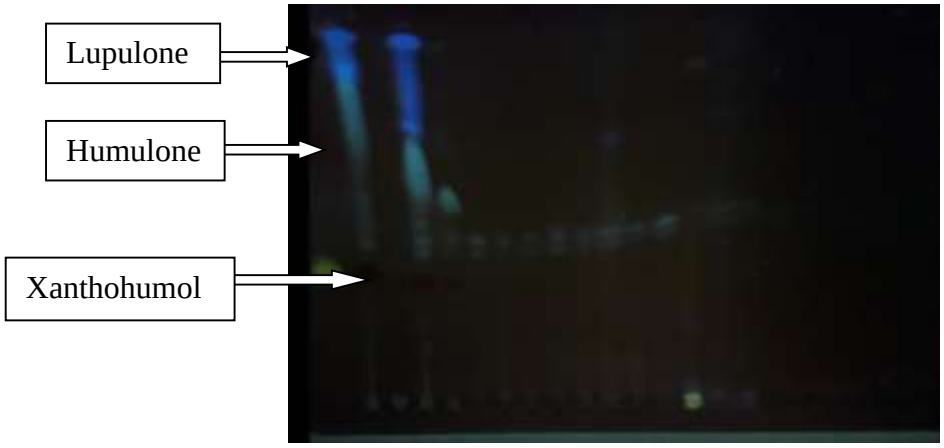


Figure 5. Plaque image obtained with 365 nm UV

Matter extractable by ethanol (70 per cent V/V)

According to the EP 8.0, the residue weighs a minimum of 0.250 g. Ethanol extractable amount of hop samples ranged from 0.1887 to 0.6810 (Table 2).

Table 2. Results of ethanol extractable substance, loss on drying and total ash amounts of hop samples

Sample Number	Matter extractable by ethanol	Loss on drying% (Average) ± SEM	Total ash% (Average) ± SEM
1	0,4117	8± 0,57	6 ± 3,07
2	0,4028	8± 0,46	9 ± 4,09
3	0,4217	8± 0,33	9 ± 4,08
4	0,4286	8 ± 0,35	9 ± 3,95
5	0,1887	3 ± 0,86	15 ± 7,39
6	0,2188	10± 0,81	13 ± 6,09
7	0,1879	10 ± 0,29	20 ± 9,83
8	0,6810	8 ± 0,16	19± 8,87
9	0,2056	9 ± 0,31	15 ± 9,01
10	0,1900	10 ± 0,36	17 ± 7,51
11	0,2373	5 ± 0,61	13 ± 4,85
12	0,2142	8 ± 0,81	17 ± 8,96
13	0,2194	8 ± 0,07	14 ± 6,04
14	0,2101	8 ± 0,53	13 ± 2,50
15	0,2111	11 ± 0,11	13 ± 5,97

2.4. Loss on drying

According to the EP 8.0, the amount of loss in drying of hop flowers should be at most 10%. This amount in hop samples varied between 3% ± 0.86 – 11% ± 0.11 (Table 2).

2.5. Total ash

According to the EP 8.0, the total ash content of hop flowers should be maximum of 12%. The analyzes showed that the total ash content of the hop samples was between 6% ± 3.07- 20% ± 9.83 (Table 2).

3. DISCUSSION

Lupuli flos is defined as the dried, usually whole, female inflorescence of *H. lupulus* L. in the EP 8.0. Hop is a plant that is frequently used because of its biological activities. It included in many pharmacopoeias, Codexis and monographs (EP, EMA, PDR). In this study, cultivated hops and 'hops' samples sold in herbalists and on the internet were examined in terms of pharmacopoeia suitability. Macroscopic, microscopic, TLC, ethanol extractable substance, loss on drying and total ash amount analysis were performed in the EP 8.0.

Macroscopic analyzes showed that hops flowers cultivated in Turkey were suitable for the EP 8.0, while the other samples were not suitable for the EP 8.0. Samples purchased from herbalists and internet contained foreign matters, as well as other parts of the plant. Their sizes could not be determined as most of the samples were fragmented or powdered.

According to the EP 8.0, the microscopic analysis of *Lupuli flos* shows irregular or wavy-walled epidermal cells; unicellular, conical, straight or curved covering trichomes with thin, smooth walls, fragmented or attached to an epidermis; anomocytic stomata; glandular trichomes, usually free, with bicellular biseriate stalks and heads consisting of 8 small cells, rarely attached to an epidermis; fragments of mesophyll containing small calcium oxalate cluster crystals. These images were detected in the microscopic analysis of hops cultured in Turkey, as shown in the book by İşcan et al [57]. In other samples, in addition to similar images, leaf, root and stem elements were also detected. However, since these plant parts are not specified in the hops monograph, samples other than those cultivated in Turkey are not comply with EP 8.0.

As a result of the analysis of the methanolic extract of *H. lupulus* flowers cultured in Turkey with TLC under the conditions given in the pharmacopoeia, it was determined that the extract contained XN, humulon and lupulon by comparing the extract with standard substances. These compounds were not found in commercially available samples. No drift was observed in the TLC analysis of the teas prepared in the form of infusion. To determine the content of teas, extracts can be prepared or analyzed using HPLC.

According to the EP 8.0, the amount of matter extractable by ethanol should be a minimum of 0.250 g. This study showed that the amount of matter extractable by ethanol of the samples cultured in Turkey and sample 8 purchased from the internet was greater than this value. Other samples showed results below pharmacopoeia and were not suitable.

As per EP 8.0, the amount of loss in drying of hop flowers should be at most 10%. All samples except sample number 15 showed results below 10% and were in compliance with the pharmacopoeia in this respect.

Determined by EP 8.0, the total ash content of hop flowers should be maximum of 12%. While the total ash content of the samples cultured in Turkey was less than 12%, it was higher in other samples. The high total ash amount proved that it was not suitable for pharmacopoeia and the samples contained pollution.

Considered as a whole, samples 1, 2, 3 and 4 cultured in Turkey were in compliance with the conditions specified in the EP 8.0. Unfortunately, samples of hops sold in herbalists and on the internet did not meet the pharmacopeial and scientific definition of a medicinal drug.

3. CONCLUSION

The presence of many foreign materials such as insects, hair, nylon and parts of other plants in commercially obtained samples poses a threat to public health. Therefore, inspections should be made and control should be provided. Hops samples grown in accordance with the pharmacopoeia in Turkey should be delivered to the public at secure sales points.

5. MATERIALS AND METHODS

Total of 15 different samples were evaluated in this study. Four samples were obtained from Bilecik (Pazaryeri) where hops were cultivated. *H. lupulus* female flowers and dry pellets of two different varieties cultivated in Bilecik were provided by Agronomist Selahattin Yilmaz (Head of Medicinal Aromatic Plants Unit

in Bursa Provincial Directorate of Food, Agriculture and Livestock). Samples 1 and 3 were female, while samples 2 and 4 were dry pellets from those flowers. The other samples were purchased as packaged products from the internet or herbalists. Only one product containing *H. lupulus* was available in the form of individual tea bags. The characteristics of the hop samples and the places they were purchased are shown in Table 3.

Table 3. Characteristics of hop samples and place of purchase

Sample Number	Characteristic	Place of purchase
1	Brewersgold variety- female flower	Bilecik (Pazaryeri)
2	Brewersgold variety- dry pellet	Bilecik (Pazaryeri)
3	Aroma variety- female flower	Bilecik (Pazaryeri)
4	Aroma variety- dry pellet	Bilecik (Pazaryeri)
5	Tea bags	Internet
6	Packaged plant	Internet
7	Packaged plant.	Internet
8	Packaged plant	Internet
9	Packaged plant	Internet
10	Packaged plant	Internet
11	Packaged plant	Ankara- herbalist
12	Packaged plant	Ankara- herbalist
13	Packaged plant	Ankara- herbalist
14	Packaged plant	Ankara- herbalist
15	Packaged plant	Ankara- herbalist

For the analyzes, "*Lupuli flos*" monograph in the EP 8.0 was followed.

5.1. Macroscopic analysis

The samples were examined in terms of their morphological characteristics such as width, length, shape, color, etc.

5.2. Microscopic analysis

Powdered *H. lupulus* samples were examined under microscope at 10×40 magnification using chloralhydrate solution.

5.3. Thin-layer chromatography (TLC)

Chromatographical analysis conditions were specified according to the EP 8.0.

Test solution: 1 g of powdered samples was mixed with 3 mL of distilled water and 7 mL of methanol. After shaking for 15 minutes, it was filtered. In addition, 0.5 and 1 g infusion of the samples 1, 2, 3 and 4 cultivated

in Turkey was prepared and used as a test solution. These doses were determined according to the European Medicines Agency (EMA) data.

Reference solution: 2 mg of curcumin, 2 mg of dimethylaminobenzaldehyde, and 1 mg of Sudan orange dissolved in 20 mL of methanol.

Mobile phase: 2 ml of anhydrous acetic acid, 38 ml of ethyl acetate and 60 ml of cyclohexane were mixed.

Test and reference solutions were applied as 20 µL bands on to a silica gel F_{254} plate which was dragged as 15 cm in the mobile phase and air dried. Bands were detected as follows;

Detection 1: Examined in ultraviolet light (UV) at 254 nm and compared with references.

Detection 2: Examined in UV at 365 nm and compared with references.

Detection 3: Dilute phosphomolybdotungstic sprayed and exposed to ammonia vapor. It was then viewed in daylight and compared with references.

All the below studies were repeated at least 3 times.

5.4. Matter extractable by ethanol (70 per cent V/V)

Powdered samples (10 g) were weighed, further, 300 mL of 70% ethanol was added and heated on a water-bath under a reflux condenser for 10 min. Then samples were allowed to cool and filtered. According to EP 8.0, the first 10 mL of filtrate was discarded, remaining 30.0 mL of filtrate was evaporated to dryness on a water bath. Then it was dried in an oven at 105 °C for 2 hours, cooled in a desiccator and finally weighed.

5.5. Loss on drying

Powdered samples were weighed (1 g) exactly and were kept in a 105 °C oven for 2 hours. Cooled in a desiccator and weighed exactly. The difference between the 1st weighing and 2nd weighing was calculated.

5.6. Total ash

Powdered samples were exactly weighed (1 g), then, they were kept in the ash furnace at 600°C for 4 hours. Cooled in a desiccator and weighed exactly again. The difference between the 1st and 2nd weighings were calculated.

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