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Research Article

OBTAIN CUCURBITACIN FROM Ecballium elaterium and Cucurbita pepo and CONVERTING INTO A PHARMACEUTICAL FORM

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ABSTRACT

The squirting cucumber plant is widely used in the treatment of sinusitis, rheumatism, and hepatitis.It is reported that the liquid obtained from fruits is widely used in the treatment of sinusitis among people, especially in Turkey. The active components of Nigella sativa seeds are shown to be effective in inflammatory diseases caused by histamine. In this study, it was aimed to create a spray form by extracting Ecballium elaterium and Cucurbita pepo plants, which are easily found in almost every region of Turkey, mixing them with black cumin oil which is used widely and traditionally, and using inactive ingredients. Firstly, the amounts of cucurbitacin D, I, B, E from Ecballium elaterium were determined by HPLC and validation of cucurbitacin active substance analytical method was performed. Physicochemical properties of the nasal spray including pH, osmolality, specific gravity, and viscosity values were determined. Skin irritation, sensitization, and cytotoxicity tests were performed on the final product. The total amount of cucurbitacin D, I, B, E in squirting cucumber was found to be about 4% of the fruit juice. The proportion of these substances was also examined in squash juice and was found to be 0.033% for the respective amount. Skin irritation, sensitization, and cytotoxicity tests were performed in the obtained product and no adverse effects were observed. In addition, physicochemical parameters of the spray, such as pH, osmolality, specific gravity, and viscosity were measured. Results were found as 6.179, 630 mOsmol/L, 55.4 Mpa/s and 1,080 g/cm3 at ph 25 °C, respectively.

Keywords: squirting cucumber, squash, cucurbitacin, black cumin oil, spray

1. Introduction

While 292.000 tons of summer squash were grown in our country in 2004, a large part of the production is made in open areas under a small amount of cover. The summer squash is mainly consumed fresh, small amounts are used in the production of baby food as frozen and dried. Fruit characteristics vary according to varieties, while the fruit color is usually dark, medium, and light green, some varieties are in yellow-orange color (Vural et al., 2000).

Ecballium elaterium (L.) A. Rich. (Cucurbitaceae) is a pilous, perennial, herbaceous, and creeping Mediterranean plant with yellow flowers and grown pretty widely in Turkey. Ecballium is derived from a Greek word called Ekballein. Ekballein means to throw out. The drug prepared from its fruit juice is called "elaterium", which means to push in Greek. This name is thought to be derived due to the fact that the fruits squirt the seeds under the effect of internal pressure in maturity. The plant is given various names colloquially, such as squirting cucumber, exploding cucumber, etc. The plant is used in the treatment of sinusitis,

rheumatism, constipation, and hepatitis by people (Baytop, 1994, Çingi et al., 1983, Yeşilada et al., 1995). It is reported that the liquid obtained from fruits is widely used in the treatment of sinusitis among the people especially in Anatolia (Baytop, 1994, Çingi et al., 1983, Yeşilada et al., 1995, Sezik et al., 1982). It was found that the fruit juice showed an increasing anti-inflammatory effect depending on the dose and the substance that produced this effect was cucurbitacin B. (Sezik et al., 1982, Yesilada et al., 1988).

In Anatolia, the roots of this plant have been used for the treatment of hemorrhoids and as a pain killer, and its fruits for the treatment of diseases such as sinusitis, jaundice, nocturia, low back pain, and ear pain (Yeşilada et al., 1989, Steinegger et al.).

In the study carried out by Yeşilada et al., the dose-dependent increasing anti-inflammatory effect of E. elaterium fruit juice and cucurbitacin B on serotonin was found on serotonin, bradykinin, and acetic acid-induced edema in mice (14,37-38). Although the highest inhibition is observed at a dose of 400 mg / kg, this dose is very toxic to mice. While high anti-inflammatory activity was observed at 200 mg/kg dose, no toxic effect was detected (Yeşilada et al., 1988), ED50 values were found in mice to be 88.0 and 3.9 mg / kg for fruit juice and cucurbitacin B, respectively (Yeşilada et al., 1998).

Bitter fruits have been reported in several cultivars of the genus Cucurbita pepo. The principal bitter substance at the root of the harvested squash iscucurbitacin E and also contains trace amounts of cucurbitacin B and I. Seed-leafs contain cucurbitain B and contain trace amounts of cucurbitacin D and E (Rehm et al. 1957).

Among the cucurbitacin species, cucurbitacin R and dihydrocucurbitacin B have anti-inflammatory effects. They exhibit anti-inflammatory properties by inhibiting tumor necrosis factor(s) (TNF). (Escandell et al., 2008, Ríos et al., 1990). The glycoside form of cucurbitacin B and E is effective on lipid oxidation (Esterbauer et al., 1993, Tannin-Spitz et al., 2007). Cucurbitacin has cytotoxic, liver protective, and antidiabetic effects (Park et al., 2004).

Cucurbitacins are used especially as an anti-inflammatory and against sinusitis with the effects mentioned.//Black cumin contains 0.4 to 0.45% essential oil and more than 30% fixed oil. Thymoquinone constitutes 18-24% of essential oils (Al-Saleh et al., 2006). In addition, the essential oil contains thymol, carvacrol, nigellimin-N-oxide, nigellisin, nigellidine, and alpha-hederine (Randhawa and Alghamdi, 2011). Black cumin contains carbohydrates, fats, vitamins, minerals, proteins and eight of the nine essential amino acids.

The active components of Nigella sativa seeds are shown to be effective in inflammatory diseases caused by histamine. It is also known that these seeds have been used for many years as traditional folk medicine. In a clinical study, it has been suggested that the use of Nigella sativa oil as an adjuvant in the treatment of allergic diseases such as allergic rhinitis, bronchial asthma, and atopic asthma (Salem, 2005).

The most important cause of allergic asthma is reported to be allergic inflammation with respiratory hyperactivity of the respiratory tract, and Thymoquinone may reduce pulmonary inflammation induced by allergens in the respiratory tract (El Mezayen et al. 2006).

In this study, the amount of cucurbitacin active ingredient was determined by squeezing squash. The spray form was obtained by adding antiallergic black cumin oil (also due to its antiallergic properties) and inactive ingredient in the squash juice. Skin irritation, sensitization, and cytotoxicity tests were performed on rabbits and mice.

2. Materials and Methods

2.1. Squirting cucumber, Squash juice, and Oil samples

Squirting cucumber was obtained from pastures in Konya and squashes from a convenience store in Konya. Extruding was performed under laboratory conditions. Black cumin oil was accepted after quality control analysis and oil extraction was performed by ZADE VİTAL Pharmaceuticals Inc. with cold press method. The cold press is made without applying any chemicals and in such a way that the temperature does not exceed 40°C.

2.2. NaCl, Xylitol, Xantam gam, Eucalyptus oil

They have been purchased from various suppliers as Farma grade. NaCl and xylitol were used in the formulation to relieve the nasal mucosa; xantam gam, black cumin oil, and eucalyptus oil to form a homogenous formulation; and eucalyptus oil for its odor and refreshing properties.

2.3. Chemicals

Methanol and acetonitrile required for validation and analysis were obtained from Merck.

2.4. Standars

Cucurbitacin B, D, E, I standards were obtained from ChromaDex and used

2.5. Validation of the quantification of cucurbitacin active substances

In this study, HPLC method developed for the quantification of cucurbitacin belonging to Cucurbitacea family and validation studies were performed to prove its validity. In this scope:

- Selectivity
- Linearity
- Limit of Detection (LOD)
- Limit of Quantitation (LOQ)
- Accuracy
- Precision
- Limitations
- Robustness

parameters were realized.

2.5.1 Device conditions:

High Performance Liquid Chromatography (HPLC, Shimadzu DAD)

Column	:	4.6 mm * 0.25 cm; 5 µm
Column Temperature	:	60±1°C
Sensor	:	DAD
Wave length	:	232 mm
Flow Rate	:	0.5 mL / min
Mobile Phase	:	Acenitrile / ultra-pure water (50/50) (V / V)
Injection Volume:	:	10 µL
Analysis Time	:	55 min
Solvent	:	Methanol (HPLC grade)

2.5.2 Preparation of Mobile Phase

500 mL of acetonitrile was added in 1000 mL graduated cylinder and 500 mL of ultra-pure water was added to the volume. The prepared mobile phase was filtered with the help of vacuum and then taken into the mobile phase bottle and degassed.

2.5.3 Preparation of standard solutions

1000 ppm cucurbitacin mix solution was prepared and then 200, 100, 50, 20, 10 and 5 ppm calibration solutions were prepared.

2.5.4 Preparation of Sample and Placebo Solutions

300 ml of squash juice was weighed into 500 mL beaker. It was then left o rest for 2 hours with equal amount of HPLC grade methanol. After phase separation was achieved, the organic phase fraction was removed and centrifuged at 4000 rpm for 30 min. The corresponding phase was treated in a rotary evaporator until the water was evaporated. The residue was then diluted with 1 ml methanol and put into the device.

2.6. Skin irritation, Sensitization, and Cytotoxicity tests

All the related tests were carried at the Scientific and Technological Research Council of Turkey (TÜBİTAK), Marmara Research Center, the Institute for Genetic Engineering and Biotechnology.

2.6.1 Skin irritation test

The irritation test of the spray sample was carried out using 3 female New Zealand rabbits with a weight of not less than 2 kg. The product was applied directly on the skin in accordance with its dense liquid form and structure. The application plan applied to experimental animals is shown in Figure 1.

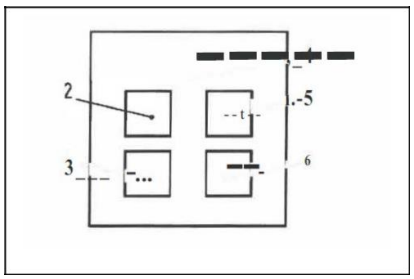

Figure 1: 2; test area, 3; negative control area, 5; test area, 6, positive control, 4; head of the animal.

• Positive Control

0.5 mL of 90% lactic acid, previously known to have a skin irritant effect, was used as the positive control.

• Negative control

As a negative control, 0.5 ml saline, previously known to have no skin irritant effect, was used.

After the experimental animals were shaved to provide a sufficient application area, the samples were applied as shown in Figure 1. After covering the samples with sterile gauze, the whole application area was wrapped with an elastic bandage.

Samples to be tested for 4 hours as stated in ISO 10993-1: 2010 were applied on the skin. At the end of this

period, bandages were opened, samples were taken, and application areas were marked. After that, the test sites were observed after 1, 24, 48 and 72 hours and the samples were evaluated considering the criteria specified in Chart 3.5 The results of the evaluation according to the scores obtained are given in Table 1.

2.6.2 Skin sensitization test

The product was directly tested as it was already in fluid liquid form. Sensitization test was carried out by using adult female guinea pigs (Cavia porcellus) between 300 and 500 g. As indicated in ISO 10993-10: 2010, the experiments were carried out by subcutaneously injecting 1 ml of test material. The topical application was applied to the left side of the animal on the 7th day of the test and to the right side on the 14th day on the area where no subcutaneous injection (intradermal induction phase) was performed. The application plan performed on experimental animals is shown in Figure 2.2.

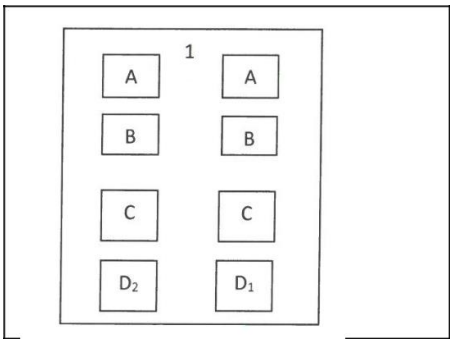

Figure 2: 1- Head of the experimental animal.

A-Test areas treated with Freund's Complete Adjuvant (FCA) and 50:50 mixing of physiological.
B- Test areas treated with test material only.
C- Test areas treated with 50:50 mixing of the sample applied in zone A and the test material applied in zone B.
D- Topical application of the test material was applied 0.3 ml to the intrascapsular area.

Applications to the A, B, C areas were made by a pair of 0.1 ml injections to the right and left areas of each animal. In the D region, the topical application was made on the left area on the 7th day and the right area on the 14th day.

Negative Control
Negative control was performed comparatively in 2 different applications in 2 different areas
(Figure 3)

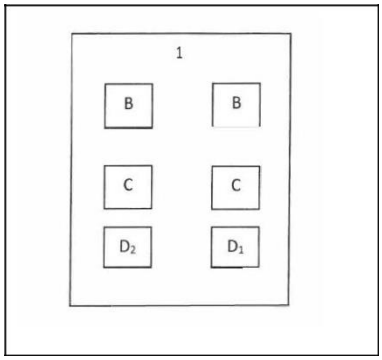

Figure 3:1-Head of the experimental animal.
saline solution.

B- Serum Physiological of 0.1 mL
C- (FCA) and saline solution were applied by mixing at 50:50 ratio.
D- 0.3 mL of saline was applied to the topical areas.

One day after shaving the experimental animals to provide sufficient application area, test materials were applied as shown in Figure 2, and on control animals, as shown in Figure 3. All applications were performed subcutaneously as 0.1 mL. The areas were not covered in any way after the application. In the topical application, test material was applied to experimental animals and 0.3 mL of saline was applied to the control animals. After application, the areas were covered with a sterile gauze bandage and all application areas were wrapped with an elastic bandage. Gauzes bandages were ensured to contact the area for 48 hours. At the end of the application, bandages were removed and reactions on the skin were noted. The second topical application was carried out 7 days later and the same experimental procedures were followed.

In the application, 10 animals were used for test material and 5 animals for control. Since there was one test material, a total of 15 animals were used in this test.

Table 1: Evaluation criteria and scoring

Reaction	Classification Scale
No visible changes	0
Specific or patchy redness	1
Moderate or confluent redness	2
Intense redness and blistering formation	3

Table 2: Average score values.

Samples	Results
Nasal Spray	0,5
Control application	0,4

2.6.3 Cytotoxicity test

Description of the cell lineage used and reason: L929 mouse cell line as one of the cell lines recommended by ISO 10993-5 and because of their suitability to represent the mammalian system.//Extraction conditions were carried out specifically in sterile, chemical-free indoor environments. Test samples were prepared and sterilized.

2.7. pH, osmolality, Specific gravity, Viscosity

European Pharmacopoeia was used.8.0 2.2.3 for pH, EP 8.0 2.2.35 for osmolality, EP 8.0 2.2.5 for specific gravity, and EP 8.0 2.2.10 for viscosity.

3. Results

3.1. Cucurbitacin active substance validation

The total amount of cucurbitacin D, I, B, E in squirting cucumber was found to be about 4% of the fruit juice. The proportion of these substances was also examined in squash juice and was found to be 0.033% for the respective amount.

3.1.1 Selectivity

The mobile phase, 100% sample solution, 100 ppm standard solution, and blank were prepared and 3 injections were given each.

3.1.2 Linearity

2 value should be 1.00 ≥ 2 ≥ 0.99. Three injections were given each at 6 different concentrations at 200, 100, 50, 20, 10, 5 ppm.

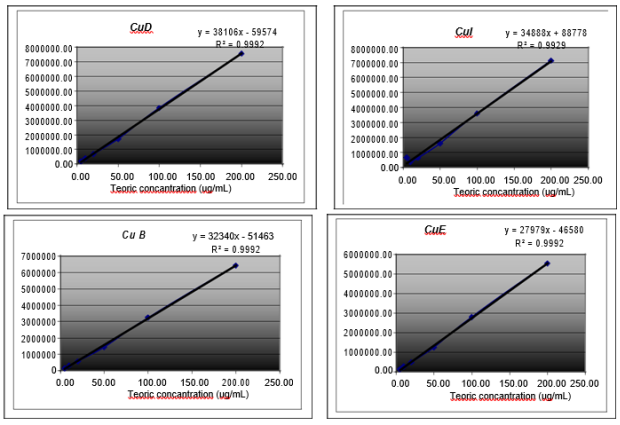

Figure 4: Cucurbitacin D, I, B, E linearity values

3.2. Irritation test results

Table 3: Evaluation criteria and scoring

No Rash	0
Low degree rash	1
Appreciable rash	2
Moderate degree rash	3
Serious degree rash and scar formation	4
Oedema	
No oedema	0
Low degree oedema	1
Appreciable oedema	2
Moderate degree oedema (abaout 1 mm)	3
Serious degree oedema (bigger than1 mm)	4
Total scor for irritation	8

Table 4: Evaluation of scoring results.

Average Point	Evulation of Category
0-0,14	Nominal
0,5-1,9	Barely
2-4,9	Moderate
4-8	Seriously

Values obtained as a result of the examination made with Binocular Lopus (3X) are given in Table 4. for "Nasal Spray". Primary irritation score and primary irritation index values obtained according to these observation values are given in Table 6. Table 5: Evaluation results for "nasal spray"

ID	Samples	Area	Observation (hours)							
			Rash				Oedema			
			1.	24.	48.	72.	1.	24.	48.	72.
1	Nasal Spray	Left front area	0	0	0	0	0	0	0	0
		Right front area	0	0	0	0	0	0	0	0
	Positive Control	Right back area	3	3	2	2	3	2	2	2
	Negative Control	Left back area	0	0	0	0	0	0	0	0
2	Nasal Spray	Left front area	0	0	0	0	0	0	0	0
		Right front area	0	0	0	0	0	0	0	0
	Positive Control	Right back area	3	3	3	2	3	3	3	2
	Negative Control	Left back area	0	0	0	0	0	0	0	0
3	Nasal Spray	Left front area	0	0	0	0	0	0	0	0
		Right front area	0	0	0	0	0	0	0	0
	Positive Control	Right back area	3	2	2	2	3	3	2	2
	Negative Control	Left back area	0	0	0	0	0	0	0	0

Table 6: Primary irritation scores and primary irritation index value for "Nasal Spray" and control samples.

Samples	Primary Irritation Scor			Primary Irritation Index (Pli)
	Rabbit 1	Rabbit 2	Rabbit 3	
Nasal Sprey	0	0	0	0
Positive Control	4,33	5,33	4,33	4,66
Negative Control	0	0	0	0

After four different observations were performed as expressed for the test material, the primary irritation index (Pli) was determined by averaging the values obtained. According to these results, it was found that the product which is tested according to the protocol and evaluation criteria specified in ISO 10993-10: 2010 document and which is defined as "Nasal Spray" does not have irritant properties.

3.3 Sensitization test results

The results obtained for the test and control samples were scored according to the evaluation and scoring criteria in Table 3. In the evaluation, there was no patchy redness on the skin of the animals in the group applied "Nasal Spray". However, a slight degree of hair loss was observed on the application area. No other significant changes were observed. As a result of the observations, the obtained sensitization score was 0.5. No significant weight loss in experimental animals and no significant negativity in general health conditions were detected.

3.4. Cytotoxicity test results

The tables indicating the result are given below.

Table 7: Cell response and other observations

Nasal Spray Solution Dilution ratios	Degree	Controls	Degree
1: 10.000	0	Control, DMEM-F12	0
1:5000	0	(+) Phenol (1:10000)	2
1:1000	0	(+)Phenol (1:5000-1:4)	4
1:500	0		
1:100	0		
1:10	4		
1:4	4		

Table 8: Quantitative evaluation

Degree	Reactivity	Condition
0	N/A	Intracytoplasmic granular is apparent, no cell lysis, no effect on cell growth.
1	Low	Presence of rounded, isolated lysed cells that exhibit poor adherence and / or lack of non-intracytoplasmic granules exhibiting morphological changes between is below 20%.

2	Weak	No presence of rounded extensive cell lysis with no intracytoplasmic granules, and growth inhibition is below 50%
3	Moderate	Less than 70% of the cells are rounded or lysed; and growth inhibition is not more than 50%
4	Serious	All or almost all of the cells are destroyed

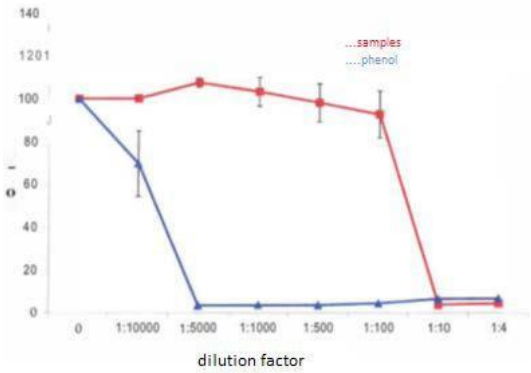

Figure 4: Evaluation criteria and scoring for the cytotoxicity test ("Nasal Spray" solution and (+) control (Phenol) viability graph (X-axis: viability %, Y-axis: Dilution factor)

Viability was calculated according to control at dilution ratios of 1: 10,000, 1: 5,000, 1: 1,000,1: 500, 1: 100, 1:10, 1: 4 of the "Nasal Spray" solution sample, which was tested quantitatively according to the evaluation criteria given in Table 7.

Cytotoxicity of the "Nasal Spray" solution sample; Absorbance values of the samples were normalized using the absorbance values of the DMEM-F12 fresh medium incubated in parallel to the samples for 100% viability.

The data obtained for each sample was acquired by taking a sample from three bottles of solutions and preparing a dilution and working as triplicates in the experiments.

The "Nasal Spray" sample showed no cytotoxic effect at 1: 10000, 1: 5000, 1: 1000, 1: 500, 1: 100 dilutions according to the WST-1 cytotoxicity test, but it showed cytotoxic effect after 1: 10 dilution.

3.5 pH, Osmolality, Specific gravity, Viscosity

The extent of drug ionization is determined as it relates to pH. Therefore, the pH of the formulation is important. The nasal formulation should be adjusted to the appropriate pH to prevent irritation, to provide effective absorption, and to prevent the growth of pathogenic bacteria. The ideal formulation pH should be adjusted between 4.5 and 6.5. According to the monographs in the USP, butorphanol tartrate nasal spray monograph pH range should be between 4.0 and 6.0, while the other monographs indicate that the pH should be within the following limits: Desmopressin nasal spray ph 3.5-6.0 , fluticasone propionate nasal spray pH 5.0-7.0, flunisolid nasal solution pH 4.5-6.0, also topical nasal aerolusine Povidone-Iodine Topical aerolos should not be more than 6 pH. the pH of the xylometzoline hydrochloride nasal solution should be 5.0-7.5 (USP NF38). In the nasal spray with cucurbitacin, pH was analyzed as 6,179 at 25 °C.

The iso-osmolarity value suitable for the nasal mucosa is approximately 280mOsm / kg. The hypo-osmotic value is 50 mOsm / kg, which increases absorption but also the potential for damage to the epithelium. Hyper-osmotic is above 900 mOsm / kg and increases mucus secretion (Morimoto, 2015).

The values of some nasal sprays in the USP were also examined. For example; The value of osmolalite and osmomolarite in butorphanol tartrate nasal spray is the limit value is between 252– 292 mosmol / kg (USP NF38).//In this study, osmolality was found to be 630 mOsmol / L in the spray made with the active ingredient cucurbitacin.

According to the FDA guidelines, the viscosity should be controlled and be capable of maintaining its fluidity. The inactive ingredients can damage the nasal cavity by increasing viscosity. The viscosity value of the nasal spray was 55.4 MPa / s.

The density for flunisolide nasal spray was given as 17-33 µg per spray. The density in isoproterenol hydrochloride inhalation aerosol was found to be 1,456 g / ml (USP NF 38). In our study, the density was found to be 1,080 g / cm3.

4. Discussion and Conclusion

The squash, squirting cucumber, and black cumin are plants that can be grown easily in almost all parts of the world including our country. Black cumin is used in a wide range of applications including pharmaceuticals, cosmetics, and food for fatty acids and unique compounds in its seed and oil. Cucurbitacin compounds found in squash plants and squirting cucumbers also have an important place in the pharmaceutical industry. Taking into account the health effects of both plants, a

laboratory-scale nasal spray containing their active substances was produced. Skin irritation, sensitization, and cytotoxicity tests were performed for these sprays and as a result, no adverse effects were observed. In this way, it was aimed to produce a standardized product as a result, and the pilot study for the production of value-added traditional herbal medicinal products was completed using domestic raw materials. Stability studies of this product which was tested at laboratory scale and at pilot product tests.

Conflict of interest

The Author has no conflict of interest to declare.

Acknowledgment

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