

**THE STUDY OF PHENOLIC COMPOSITION AND ACUTE TOXICITY,
ANTI-INFLAMMATORY AND ANALGESIC EFFECTS OF DRY EXTRACTS OF SOME
ELSHOLTZIA GENUS (LAMIACEAE) SPECIES**

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Abstract

The aim of this study was to investigate the *Elsholtzia Stauntonii* Benth. and *Elsholtzia ciliata* Thun. dry extracts acute toxicity, anti-inflammatory and analgesic activity. Phytochemical screening and identification of the main compounds of the dry extracts were performed. The main chemical compounds were determined with HPLC analyses and in total of 18 phenolic compounds (gallic acid, hydroxyphenyl acetic acid, chlorogenic acid, caffeic acid, syringic acid, benzoic acid, trans-ferulic acid, synapic acid, trans-cinnamic acid, chinic acid, rutin, quercetin-3-b glucoside, naringin, neohesperidin, quercetin, luteolin, naringenin and kaempferol) were identified and quantified.

Acute toxicity of dry extracts was studied by the Prozorovsky method. It is established that the studied dry extracts of both species belong to practically non-toxic substances (V class of toxicity according to Sidorov's classification). According to the obtained results, the dry extract of *Elsholtzia ciliata* in the model of carrageenan edema showed the most pronounced anti-inflammatory effect at a dose of 100 mg/kg, slightly inferior to the comparison drug diclofenac sodium. For *Elsholtzia Stauntonii* the extract is characterized only by a tendency to antiexudative activity.

Keywords: *Elsholtzia Stauntonii*; *Elsholtzia ciliata*; HPLC; flavonoids; acute toxicity; anti-inflammatory; analgesic.

Introduction

Ordinarily, treatment of algesia and inflammation depends on the use of nonsteroidal anti-inflammatory drugs (NSAIDs), adjuvants, and opioids. As noted by [1], most of the drugs are either expensive or inaccessible, and they often lead to adverse effects. Conventional drugs only provide a symptomatic relief, and they are often toxic to body tissues and organs, including the liver and kidney. In this regard, plant-based medicines can be used for treatment. This has necessitated the search for safer, affordable, and more effective alternatives to avert pain, inflammation and associated maladies. Natural products, especially those of plant origin, are more potent, easily accessible, affordable and are relatively less toxic compared to sythetic counterparts.

Genus *Elsholtzia* Willd. is related to Lamiaceae family and comprises of more than 30 species. These are mainly herbaceous plants and shrubs, common in East Asia, India, Africa, North America, and European countries. [2,3].

The mint family (Lamiaceae) is an important medicinal flowering plant family that contains about 236 genera and more than 6000 species [4].

As usual in plants, phenols belong to the largest group of secondary metabolites in Lamiaceae family, and they exhibit multidirectional biological activity [5]. Numerous studies have demonstrated various effects of polyphenols as antioxidants. Mainly they show cardioprotective, analgesic, anti-trombotic, anti-cancer, anti-diabetic, anti-aging, neuroprotective, anti-inflammatory, antiasthmatic, antihypertensive, hepatoprotective, cholesterol lowering, antioxidative, antifungal, antibacterial, and antiviral effects [6, 7, 8, 9, 10,11].

The extracts from *Elsholtzia Stauntonii* and *Elsholtzia ciliata* contain not only phenols, but also essential oils, steroids, and triterpenes [2]. The herbs are used as spices in cuisine and as remedies in folk medicine. In traditional medicine, *E. ciliata* has been used for the treatment of headache, fever, diarrhea, edema, blood clotting, gastralgia, dysphonia, nephritis, and throat infections [12,13]. According to scientific literature, *Elsholtzia* species are valuable bioactive sources of natural antioxidants, their extracts possess anti-

inflammatory, antiviral, antibacterial, antioxidant, anticancer, and vasorelaxant effects [14-17].

The aim of the present study was to investigate the *Elsholtzia Stauntonii* Benth. (Mint Bush) and *Elsholtzia ciliata* Thun. (Chinese Vietnamese Balm) dry extracts acute toxicity, anti-inflammatory and analgesic activity. Also, the study focused on the chemical predominant compounds of the obtained dry extracts.

Methods

2.1. Plant Material and Extraction

The objects of this study were dry extracts of the aboveground parts of *Elsholtzia Stauntonii* and *Elsholtzia ciliata* cultivated in Grishko Botanical Garden, Kyiv, Ukraine. The plants were collected within August and October in the flowering stage in 2017-2018. The collected plant samples were cut into small fragments, air-dried in a well aerated room, with regular grabbing for 14 days, and ground into a coarsely powdered material using an electric mill. The powder was kept in well labelled manila bag and kept in a dry place awaiting extraction.

The main task of grinding raw materials can be considered the destruction of cell structure and increasing the extraction surface [18, 19].

Dry extracts of *Elsholtzia Stauntonii* and *Elsholtzia ciliata* were obtained using experimentally selected conditions.

The type of extractant affects not only the extraction of a certain group of substances, but also the total amount of extractives depends on the hydrophilicity of the extractant.

In plants most substances are hydrophilic, so polar extractants usually will extract more compounds. Alcohol extracts with low ethanol content contain many macromolecular compounds (water-soluble proteins, sugars, enzymes, pectins, mucus, starch), which must be removed. Considering the chemical composition of the herbs of both species, 20%- ethanol and 70%-ethanol were used as extractants.

Extraction methods also play an important role in the processing of bioactive constituents from plant materials.

Extracts were obtained by maceration, taking into account the absorption coefficient of the extractant

by raw materials, according to the pharmacopeial method. For *Elsholtzia Stauntonii* extract was used 70% -ethanol, in the ratio of raw material:extractant - 1:10, *Elsholtzia ciliata* extract was received by maceration with 20% -ethanol, in the ratio of raw material:extractant 1:25.

Dealcoholized dry herb extracts of both *Elsholtzia* species were obtained by evaporation of ethanol extracts. Evaporation was performed on a rotary evaporator under following conditions: temperature - 50 °C, rotation speed - 100 rpm, vacuum force - 900 mBar. These conditions allow to remove ethanol minimizing the impact of biologically active components on the composition.

Drying of the extracts was carried out in a sublimation apparatus type KS30. In the initial period of drying, the pressure in the sublimator was reduced from 1.10-1 to 1.10-5 mm Hg. Art. and the temperature up to + 35 - 40 ° C. The temperature of the product in the final period of drying did not exceed + 50 ° C. To obtain dry extracts, the total duration of drying averaged 28 - 32 hours. The obtained extracts are a powder varying from light brown to brown-green color with a specific odor.

2.2. High-Performance Liquid Chromatography (HPLC) Analysis

In our previous studies, we have found a high content of hydroxycinnamic acids and flavonoids in *E. Stauntonii* and *E. ciliata* herbs, therefore HPLC-PDA method was applied for the determination of dry extracts individual hydroxycinnamic acids and some other individual phenolic compounds detection.

In order to trace the chromatographic profile, the extract was solubilized. To determine the flavonoid composition a sample portion of 0.2-0.6 g was extracted into 5 ml of an 80% ethyl alcohol solution in an ultrasonic bath at 80 °C. for 5 hours in sealed glass vials with a Teflon lid. The obtained extract was centrifuged at 3000 rpm and filtered through disposable membrane filters with pores of 0.22 µm.

Liquid chromatography was performed on an Agilent Technologies 1200 liquid chromatograph. Acetonitrile (A) and a 0.1% formic acid solution in water (B) were used as the mobile phase. Elution was performed at a gradient mode:

0 min - A (5%): B (95%); 20 min - A (30%): B (70%); 30 min - A (60%): B (40%); 50 min - A (100%): B (0%); 60 min - A (100%): B (0%). Separation was performed on a Zorbax SB-C18 chromatographic column (3.5 µm, 150 x 4.6 mm) (Agilent Technologies, USA), column flow rate 0.25 ml/min, thermostat temperature 30 °C, volume injection 4 µl. Detection was performed using a diode-matrix detector with signal registration at 280 and 365 nm and fixation of absorption spectra in the range of 210–700 nm [20].

To determine the hydroxycinnamic acids, a sample portion of 0.1-1.0 g, was extracted into 5-10 ml of 60% methanol solution in an ultrasonic bath at 80 °C for 4 hours in sealed glass vials with a Teflon lid. The obtained extract was centrifuged at 3 thousand rpm and filtered through disposable membrane filters with pores of 0.22 µm.

Liquid chromatography was performed on an Agilent Technologies 1200 liquid chromatograph. Methanol (A) and 0.1% formic acid solution in water (B) were used as the mobile phase. Elution was performed in a gradient mode: 0 min - A (25%): B (75%); 25 min - A (75%): B (25%); 27 min - A (100%): B (0%); 35 min - A (100%): B (0%). Separation was performed on a Zorbax SB-Aq chromatographic column (4.6 mm ± 150 mm, 3.5 µm) (Agilent Technologies, USA), column flow rate 0.5 ml/min, thermostat temperature 30 °C, vol. injection capacity 4 µl. Detection was performed using a diode-matrix detector with signal registration at 250 and 275 nm and fixation of absorption spectra in the range of 210–700 nm [21].

Identification and quantitative analysis were performed using standard solutions.

The purity for all reference standards was over 98 %.

All solvents and reagents were of analytical grade: HPLC-grade acetonitrile and methanol Chromasolve®, formic acid was purchased from Sigma-Aldrich, USA. Ethanol was supplied by Bio-Pharma Ltd, Ukraine. Water was treated in a Milli-Q water purification system (Millipore system).

2.3 Determination of acute toxicity and pharmacological activity

Experimental studies were performed on white nonlinear female mice weighing 23-26 g (breeding vivarium of the "Institute of Pharmacology and

Toxicology of the National Academy of Medical Sciences of Ukraine”, Kiev), which were on the standard diet, with one route of administration - intragastric, which is intended for use in clinical practice [22].

Before the experiment, the animals were quarantined for 7 days. During the experiment, the animals were randomized into groups of four animals per cage. Each cage had a litter of wood shavings (changed daily). The animals had free access to a standard food ration and water. The animals were kept in a stable mode: 12 hours of light / 12 hours of darkness. All studies were performed in accordance with the requirements of the GLP, the Ministry of Health of Ukraine (MOH) and the rules of the European Convention for Protection of Animal Health and Experience "(Strasbourg, 1986) [23].

2.3.1 Acute toxicity study

The study of acute toxicity is a mandatory step in the study of new drugs, which allows to assess the health hazards of substances under conditions of short-term action and to determine the class of toxicity and the breadth of therapeutic action.

Acute toxicity of dry extracts of *E. Stauntonii* and *E. ciliata* was studied by intragastric administration. Distilled water was used as a solvent. The volume of the injected substance did not exceed 0.5 ml for mice when administered intragastrically. During the experiment, the condition of the animals and the timing of death were observed. The results were taken into account in an alternative form (the number of dead animals) 14 days after a single injection [22, 24].

Dry extracts were administered to animals in doses of 5000, 1000, and 500 mg/kg as an aqueous solution. The animals were observed for the first hour after administration, after 2, 4, 6, 8, 24 h, as well as once a day for 14 days of the experiment. Weight control was performed 3, 7 and 14 days after administration. On day 14 of the experiment, the animals were subjected to mortification, followed by visual examination of the main internal organs: brain, liver, kidneys, stomach, intestines, lungs, heart, pancreas. The mass of internal organs was estimated.

2.3.2 Pharmacological activity study

The test substances were administered once orally (p.o.) at a dose of 100 mg / kg as an aqueous solution. in the form of a water-ethanol emulsion using Twin-80 as an emulsifier. Ketorolac (5.5 mg/kg) and diclofenac (4 mg/kg) were administered once orally (orally) as a water-ethanol emulsion in doses of ED50 [25].

Analgesic activity dry extracts was evaluated by using acetic acid-induced writhing model. This model is thought to induce pain by indirectly acting on the abdominal release of prostaglandins and lipooxygenase products. These products are involved in the stimulation of nociceptive neurons sensitive to mild analgesics as indicated by Gyores and Knoll et al. [26]. The antinociceptive activity of the test substances was assessed by a decrease in the number of "writhing" caused by intraperitoneal injection of 0.6% acetic acid. The size of the experimental group was five animals. The control group received a solvent. The number of "writhing" of each animal was counted for 10 minutes. Inhibition of convulsions was compared with a standard drug (ketorolac).

Anti-inflammatory activity was investigated for carrageenan-induced inflammation in mice. It is known that "carrageenan" edema characterizes the cyclooxygenase component of inflammation. The obtained data do not exclude the influence of the studied extracts on the function of COX isoenzymes. The test substance was administered orally to mice 1h before subplantar administration of 0.05 ml of 1% carrageenan solution. The size of the experimental group was five animals. Three hours after administration of the studied dry extracts, mice were removed from the experiment. The hind legs with swollen and non-swollen feet were amputated at the level of the hip joints and their mass was compared. The control group received a solvent. The obtained data on antiexudative activity were compared with a standard drug (diclofenac).

Statistical processing of experimental data was performed by the method of variation statistics (t-test) OriginPro 8.0 (originLabCorporation, USA). Mathematical processing included calculations of arithmetic mean values (M) and their errors (m). Determination of the probability of differences in the values of the specific pharmacological indicator was performed using the parametric Student's t-test

and the method of analysis of variance (ANOVA). Differences were considered statistically significant at the level of $P < 0,05$ [27].

Results

3.1. HPLC Analysis

HPLC method was applied for separation and quantification of the major compounds in *E. Stauntonii* and *E.ciliata* dry extracts, which are ten phenolic acids (gallic acid, hydroxyphenyl acetic acid, chlorogenic acid, caffeic acid, syringic acid, benzoic acid, trans-ferulic acid, synapic acid, trans-cinnamic acid, quinic acid), eight flavonoid glycosides and flavonoid aglycones (rutin, quercetin-3-b-glucoside, naringin, neohesperidin, quercetin, luteolin, naringenin and kaempferol) (Fig. 1-2).

HPLC chromatograms of *E. Stauntonii* and *E.ciliata* dry extracts for hydroxycinnamic acids analysis are given in Figure 3-4 and showed peaks of the significant area.

The results of the hydroxycinnamic acids composition study in *E. Stauntonii* and *E.ciliata* dry extracts are specified in Table 1.

HPLC chromatograms of *E. Stauntonii* and *E.ciliata* dry extracts for flavonoid analysis are given in Figure 5-6.

The results of the flavonoids composition study in *E. Stauntonii* and *E.ciliata* dry extracts are specified in Table 2.

The obtained results showed similar qualitative composition of hydroxycinnamic acids content, in both dry extracts. At the same time flavonoids composition is significantly different in this two species dry extracts.

3.2 Acute toxicity and pharmacological activity

No animal deaths were observed in the acute toxicity study. The results of the study of acute toxicity of *E. Stauntonii* and *E.ciliata* dry extracts are given in Table 3.

The effects on the body of *E. Stauntonii* and *E.ciliata* dry extracts were similar. In the first 2h after administration of the studied extracts, the experimental animals were somewhat weak, motor activity was reduced. These symptoms resolved 2h after administration of the extracts. During further observations, the animals were neat, active, ate the

food given to them. In animals, positive dynamics of body weight was observed. According to the obtained data, the studied dry extracts can be classified as practically non-toxic.

The high content of phenolic carboxylic acids and flavonoids, which are part of the biologically active substances of extracts of *E. Stauntonii* and *E.ciliata* herbs, gave reason to believe that they have anti-inflammatory activity and slightly analgetic effect.

It has been shown that 20% of the extract is characterized by a significant anti-inflammatory effect, which is close to that of diclofenac sodium. For 70% of ethanol extract, the tendency of antiexudative activity is characteristic.

It has been shown that the *E.ciliata* extract is characterized by a significant anti-inflammatory effect, which is close to that of diclofenac sodium. For *E. Stauntonii* extract, there is tendency of antiexudative activity.

Based on the studied range of doses of acute toxicity for the study were selected doses of 100 mg / kg, amounting to 1/50 LD₅₀. Both extracts are inferior to the comparison drug ketorolac in terms of antinociceptive activity. However, *E.ciliata* dry extract has a significant antinociceptive effect (-45.45%). The *E. Stauntonii* extract shows a tendency to antinociceptive activity. Since the test of acetic acid "writhing" characterizes visceral pain in the pathogenesis of which inflammatory mediators play a significant role, we can assume the presence of anti-inflammatory component of the analgesic activity of dry extracts of both species.

Discussion

The results HPLC analysis of dry extracts of both *Elsholtzia* species confirm the presence of constituents which are known to exhibit medicinal as well as physiological activities.

On the basis of the *in vivo* study results, it is not possible to conclude that all the effects observed are true analgesic or anti-inflammatory effects. It seems safe, however to conclude that these parts do possess biological activities following oral administration. The above results need to be verified in other experimental models to be totally authentic.

Pharmacodynamics studies should be undertaken to establish the mechanism of action of dry extracts of *E. Stauntonii* and *E. ciliata*.

Phytochemical investigation is also proposed in order to isolate the active fraction and eventually the pure compound.

Conclusions and Recommendations

The represented data shows the great potential represented by the compounds contained in two *Elsholtzia* species. Important pharmacological activities of dry extracts received from *E. Stauntonii* and *E. ciliata* herbal materials, that rich on phenolic and hydroxycinnamic acids compounds has been found. The main chemical compounds were determined with HPLC analyses and in total of 18 phenolic compounds (gallic acid, hydroxyphenyl acetic acid, chlorogenic acid, caffeic acid, syringic acid, benzoic acid, trans-ferulic acid, synapic acid, trans-cinnamic acid, chinic acid, rutin, quercetin-3-b glucoside, naringin, neohesperidin, quercetin, luteolin, naringenin and kaempferol) were identified and quantified.

Based on the results obtained in this study, it was concluded that the dry extracts of *E. Stauntonii* and *E. ciliata* herbs have anti-inflammatory and analgesic. Furthermore, this extracts have LD50 values of >5000 mg/kg bw and are thus nontoxic as per the guidelines of OECD [28].

Therefore, the the dry extracts of *E. Stauntonii* and *E. ciliata* herbs can be utilized as alternatives in the management of inflammation and pain, as claimed in traditional medicine.

Nevertheless, there is a need to bio-screen the studied dry extracts of *E. Stauntonii* and *E. ciliata* to identify and isolate the specific compounds with analgesic and anti-inflammatory activities. This way, new compounds might be discovered, which will be used for treatment of the two conditions: pain and inflammation. Additionally, future studies should focus on elucidation of the possible mechanism(s) for analgesic and anti-inflammatory actions. In addition to acute toxicity, there is a need to evaluate chronic toxicity to determine the safety of this extracts in animal models.

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Table 1. Quantitative estimation of hydroxycinnamic acids composition

#	Hydroxycinnamic acids	Extract	
		E. Stauntonii	E. ciliata 4
		Content, mkg/g	
1	gallic acid	-	231.41
2	hydroxyphenyl acetic acid	-	260.69
3	chlorogenic acid	604.16	452.18
4	caffeic acid	1857.40	7797.21
5	syringic acid	103.69	418.59
6	benzoic acid	459.88	2962.32
7	p-coumaric acid	-	-
8	trans-ferulic acid	144.45	492.34
9	synapic acid	1772.83	117.23
10	trans-cinnamic acid	1270.48	169.95
11	quinic acid	45.93	707.42

Table 2. Quantitative estimation of flavonoid composition

#	Hydroxycinnamic acids	Extract	
		E. Stauntonii	E. ciliata 4
		Content, mkg/g	
1	rutin	-	744.86
2	quercetin-3-b-glucoside	1651.93	-
3	naringin	1125.07	257.62
4	neohesperidin	29573.66	1402.25
5	quercetin	-	1601.38
6	luteolin	9309.76	-
7	naringenin	358.99	-
8	kaempferol	-	-

Table 3. Dynamics of changes in body weight of mice with a single intragastric injection of dry herbal extracts of *E. Stauntonii* and *E.ciliata*, n = 4

Dose, mg/kg	Statistical indicator	Dynamics of body weight change, g			
		Output data	Day 3	Day 7	Day 14
<i>E. Stauntonii</i> extr.					
5000	M	26,25	27	27,1	27,27
	±m	1,26	0,82	0,80	0,74
	%		2,85	3,24	3,90
1000	M	27,35	26,8	27,725	27,805
	±m	1,25	0,91	0,63	1,11
	%		-1,74	1,37	1,66
500	M	27,5	27,9	28,45	28,63
	±m	1,29	1,47	1,43	1,26
	%		1,64	3,45	4,09
<i>E.ciliata</i> extr.					
5000	M	27,25	26,7	26,65	27,45
	±m	1,25	0,96	0,66	1,38
	%	-	-1,83	-2,20	0,73
1000	M	26,62	26,3	26,67	27,51
	±m	0,95	0,52	0,47	0,36
	%		-1,03	0,19	3,32
500	M	27,62	27,6	27,8	28,45
	±m	0,47	0,79	0,53	0,13
	%		0,09	0,63	2,99
Control	M	26	26,7	27,5	28,125
	±m	1,15	0,5	0,57	1,03
	%		2,88	5,76	8,17

Table 4. Anti-exudative activity of dry extracts of *E. Stauntonii* and *E.ciliata* in the model of carrageenan edema (n = 5) in mice

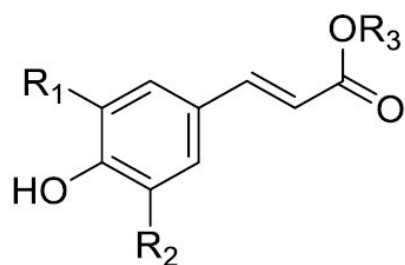
Substance	Dose, mg/kg	Control, weight (M ± m), mg	Experiment, mass (M ± m), mg	Change to control, %
Diclofenac sodium	4,0	54,70 ± 4,64	30,5 ± 5,62	-44,2***
<i>E. Stauntonii</i>	100,0	27,64 ± 4,92	20,94 ± 5,91	-24,24
<i>E.ciliata</i>	100,0	27,64 ± 4,92	15,5 ± 3,85	-43,9*

Note. *** P < 0.001 compared to control; * P < 0.05 compared to control.

Table 5. Analgesic activity of of dry extracts of *E. Stauntonii* and *E.ciliata* using acetic acid writhing method

Substance	Dose, mg/kg	Control, writhing number (M ± m)	Experiment, writhing number (M ± m)	Change to control, %
Ketorolac	5,0	15,5±1,88	2,2±0,44	-85,89***
<i>E. Stauntonii</i>	100,0	8,8±2,52	6,0±1,4	-31,8
<i>E.ciliata</i>	100,0	8,8±2,52	4,8±0,44	-45,5*

Note. *** P <0.001 compared to control; * P <0.05 compared to control.

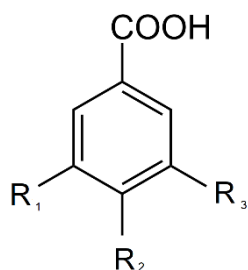
Figure 1.Chemical structures of ten phenolic acids

Caffeic acid R1=OH, R2=H, R3=H

trans-Ferulic acid R1=OCH3, R2=R3=H

Chlorogenic acid R1=H, R2=OH, R3=3-quinoyl

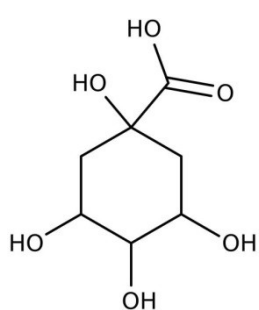
Synapic acid R1=R2=OCH3, R3=H



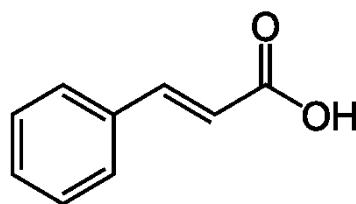
Benzoic acid R1=R2=R3=H

Gallic acid R1=R2=R3=OH

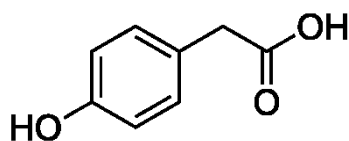
Syringic acid R1=R3=OCH3, R2=OH



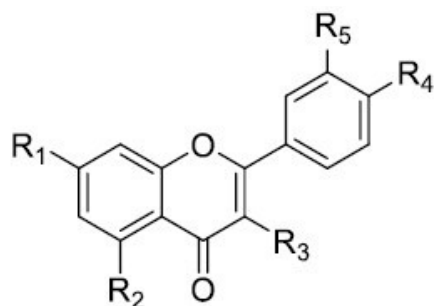
quinic acid



trans-cinnamic acid



hydroxyphenyl acetic acid

Figure 2. Chemical structures of the eight flavonoids

Rutin R1=R2=R4=R5=OH, R3=O-Glu-O-Rha

Quercetin R1=R2=R3=R4=R5=OH

Quercetin-3-b-glucoside R1=R2=R4=R5=OH, R3= O-Glu

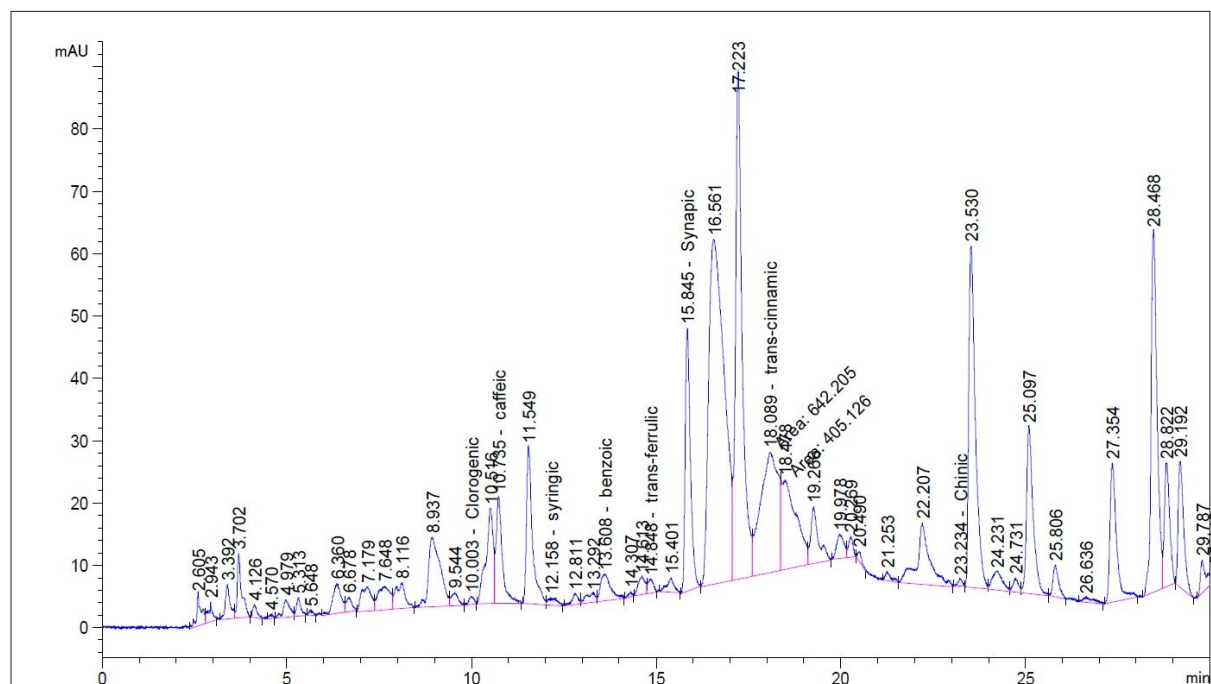
Kaempferol R1=R2=R4=OH, R3=R5=H

Luteolin R1=R2=R4=R5=OH, R3=H

Naringenin R1=R2=R4=R5=OH, R3=H

Naringin R2=R4=OH, R3=R5=H, R1= Glu-Rha

Neohesperidin R2=R5=OH, R4=OMe, R1= Glu-Rha

Figure 3. HPLC chromatogram of dry extract of *E. Stauntonii* herb at 275 nm**Figure 4.** HPLC chromatogram of dry extract of *E. ciliata* herb at 275 nm

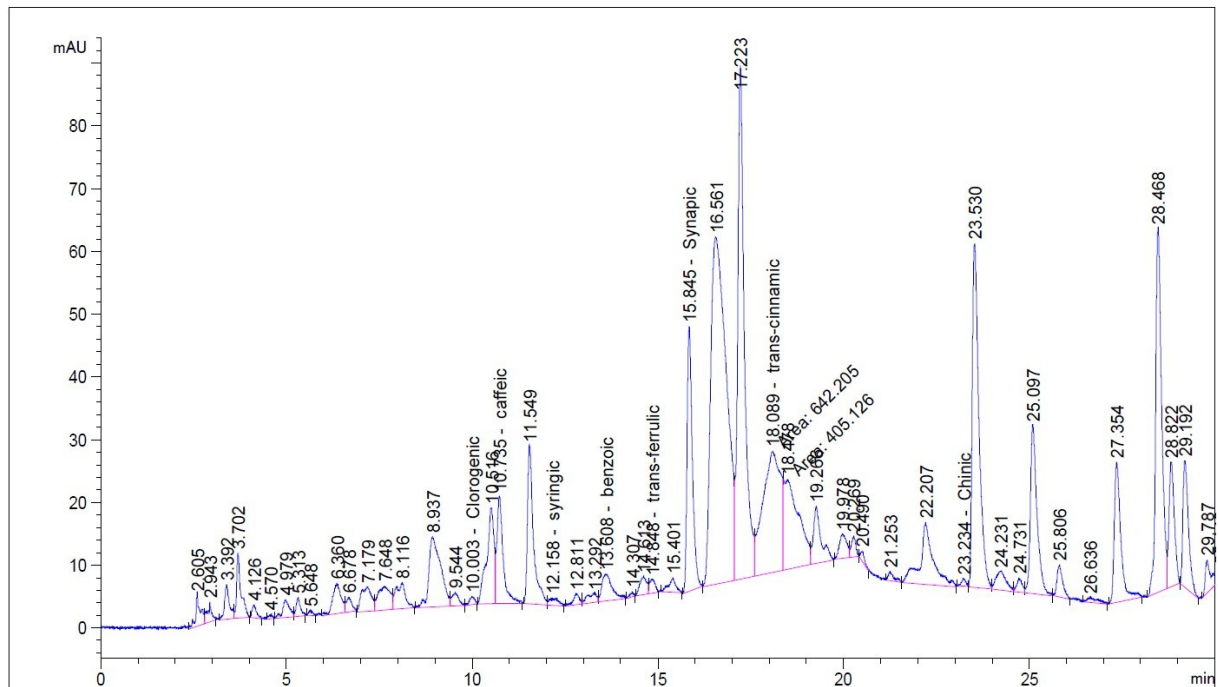


Figure 5. HPLC chromatogram of dry extract of *E. Stauntonii* herb at 280 nm

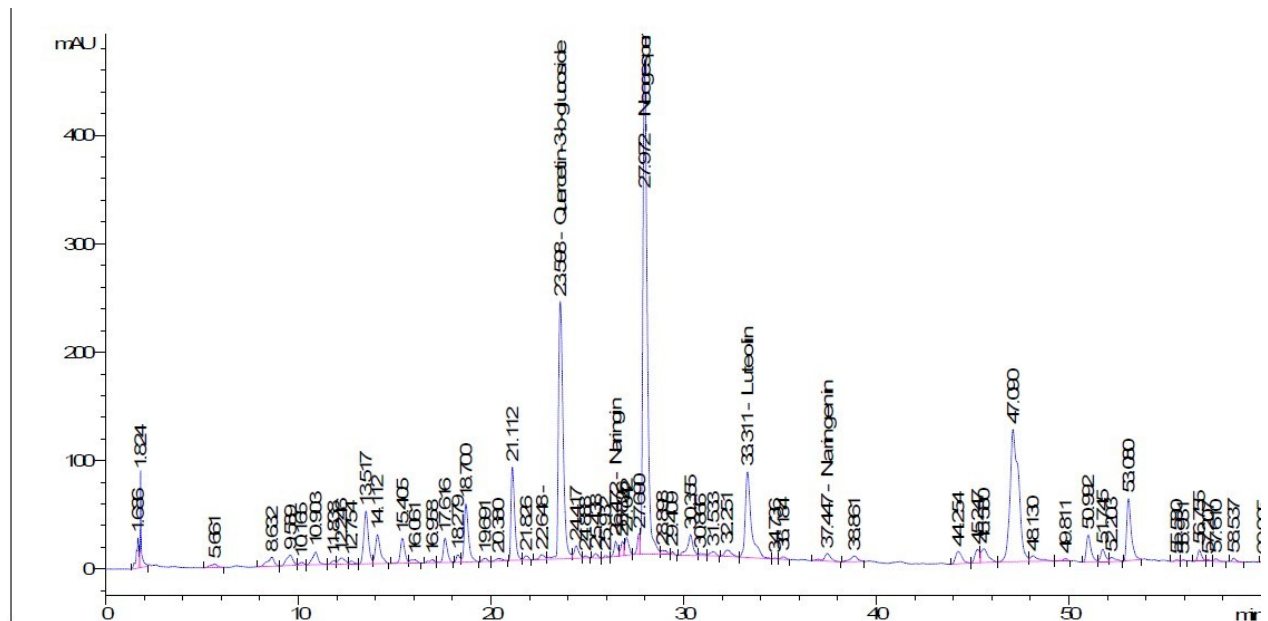


Figure 6. HPLC chromatogram of dry extract of *E. ciliata* herb at 280 nm

