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Research Article

Phytochemical analysis and anti-allergic activity of a combined herbal medicine based on bur-marigold, calendula and hawthorn

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Abstract

Using an experimentally selected extractant for balanced extraction of flavonoids, polysaccharides and polyphenols from bur-marigold herb, calendula flowers and hawthorn leaves and flowers, 3 suitable dry extracts and 1 combined extract in the selected ratio of raw materials were obtained. It was concluded that it was reasonable to standardize the combined herbal extract on the content of flavonoids in terms of luteolin-7-glucoside and content of polyphenols in terms of pyrogallol by UV method and the content of terpenoid compounds, in terms of oleanolic acid by densitometric method. The combined extract at a dose of 30 mg/kg in the model of anaphylactic shock has showed antiallergic activity at the level of tesalin and exceeds diazoline; the ability of the combined extract to reduce the permeability of skin capillaries at the level of the tesalin has established on the model of active cutaneous anaphylaxis and it significantly exceeded diazoline; the combined extract did not differ in membrane-stabilizing properties from the effect of desloratadine and was significantly superior to the reference drugs diazolin and tesalin in the test of indirect degranulation of mast cells. As a result, it was concluded that the mechanism of anti-allergic action of the combined extract is probably associated not only with antihistamine properties but also with the ability to stabilize cell membranes.

Keywords

Anti-allergic activity, combined herbal extract, flavonoids, immediate-type hypersensitivity, polyphenols, terpenoids

Introduction

Currently, there is an annual increase of allergopathologies worldwide that are mainly caused by adverse environmental conditions, changes in the body's immune reactivity, and poor nutrition. According to statistics, 20% of the population today suffers from various allergic diseases. The results of studies on the epidemiology of allergic diseases indicate their widespread and steady growth (Ernazarova and Adylova 2017; Ministry of Health Protection of Ukraine 2019). Drugs of the first generation that block histamine H_1 receptors, classic low selectivity blockers with a pronounced sedative effect (such as diphenhydramine, chloropyramine, and clemastine), and drugs of the second generation, relatively selective antagonists of peripheral histamine H_1 receptors (such as loratadine, astemizole, and cetirizine) are widely used for the treatment of allergic diseases. However, it should be noted that H_1-blockers have many undesirable effects, for example, the first-generation drugs have a sedative effect, and the second-generation drugs are not recommended for use in case of hepatic and/or renal diseases, which, of course, limits their use (Compendium 2020; Vityuk 2020).

Therefore, it is relevant to create anti-allergic medicinal products based on herbal drugs (HDs). A group of combined new galenic herbal medicines is particularly promising, they can be considered as an alternative and/or addition to the main treatment. Herbal products, due to the presence of various biologically active substances, can have a gentle effect on the body, have an effect on various aspects of the complex etiopathogenesis of allergies, and restore disturbed immune response functions. Special attention should be paid to the fact that the pharmaceutical market of Ukraine has no anti-allergic drugs based on herbal drugs, which, under conditions of their correct dosage and duration of use, are not inferior as for their effectiveness to synthetic molecules and significantly exceed them in terms of safety of use.

Given the above, extracts of *Bidens tripartite*, *Calendula officinalis*, plants of the genus *Grataegu*s can be considered as promising herbal drug preparations.

B. tripartita herb (bur-marigold) from these plants is the main one responsible for anti-allergic activity. The chemical composition of bur-marigold is represented by the following classes of biologically active substances (BAS): flavonoid compounds – flavonoids (luteolin, luteolin-7-glucoside) (up to 1.3%), flavanones (isocoreopsin, flavanomarein), associated chalcones (including butein); aurones (sulfuretin, sulfurein, etc.), polysaccharides carbohydrates (4.5–4.7%), and hydroxycinnamic acids (Kotova et al. 2021). Herbal preparations of the bur-marigold herb have antioxidant, anti-allergic, antimicrobial, antifungal, hepatoprotective, immunostimulating and hypotensive activity (Shikov et al. 2014; Al-Snafi 2015). The anti-allergic activity of this preparations is associated with the presence in the raw material of both polysaccharides (Karazhan et al. 2014) and flavonoids, primarily luteolin and its derivatives for which this action has been confirmed by many researchers (Seelinger et al. 2008; Liang et al. 2020).

Calendula officinalis flowers contain flavonoids, from 0.3 to 0.8% (isorhamnetin, quercetin, isoquercetin, narcissin, rutin (Olennikov et al. 2017). Quercetin displays high antioxidant and anti-inflammatory properties that have been proven by many in vivo and in vitro studies. Quercetin's anti-allergic mechanism of action through the inhibition of enzymes and inflammatory mediators has also been extensively studied (Mlcek et al. 2016). Also marigold flowers contain terpenoids (triterpene saponins from 2 to 10%, triterpene alcohols 1.2%, in particular, oleonolic acid glycosides-calendulosides A-H about 5%), faradiol derivatives which are known for anti-inflammatory and also anti-edematous action (Muley 2009; Schneider et al. 2015); water-soluble polysaccharides up to 15%, which are associated with immunostimulatory activity (Gruenwald et al. 2000).

Grataegus leaf and flowers have been used for a long time in folk medicine for the treatment of cardiovascular diseases, diseases of the central nervous system, immune system, etc. The chemical composition of hawthorn is represented by several classes of BAS: proanthocyanidins (catechin-like, up to 2.4%); flavonoids (up to 1.8%), in particular quercetin derivatives including O-glycosides (hyperoside (0.28%), rutin (0.17%), luteolin and apigenin glycosides which are assigned, among other things, anti-allergic action (Mlcek et al. 2016; Ngoc 2020)), 6-C- and 8-C-glycosides (vitexin (0.02%), vitexin-2”-*O*-alpha-L-rhamnoside (0.53%), vitexin-2”-*O*-alpha-L-rhamnoside-4”’-acetate)); triterpenes (up to 0.6%), including oleanolic acid, ursolic acid, crategolic acid, which have a beneficial effect on coronary circulation (Chevallier 2007; Williamson et al. 2009), hydroxycinnamic acids (chlorogenic and caffeic), etc. (Gruenwald et al. 2000).

Given the information about the pharmacological effects of these plants, it seems promising to create a combined herbal medicine on their basis having a multifunctional, anti-allergic in particular, action, which normalizes the functioning of the body's defense systems, increases non-specific resistance and normalizes immune status.

The purpose of this work was to research to develop a technology for obtaining a combined herbal medicine based on a mixture of HDs (bur-marigold herb, calendula flowers, hawthorn leaves and flowers), to study the phytochemical profile of its main BAS and methods of its standardization, and to study of its anti-allergic activity.

Materials and methods

Obtaining herbal extracts

The object of research was the samples of the bur-marigold herb, calendula flowers, hawthorn leaves, and flowers harvested on the territory of Kharkiv and Zhytomyr regions of Ukraine and analyzed for compliance with the requirements of national monographs of the State Pharmacopoeia of Ukraine (which is fully harmonized with the European Pharmacopoeia) “Bur-marigold herb” (State Pharmacopoeia of Ukraine 2021), “Calendula flower”, and “Hawthorn leaf and flower” (State Pharmacopoeia of Ukraine 2014). The samples were dried at 40 °C in an air-forced dryer (Binder drying cabinet ED 53T, Germany), grounded to powder in an excelsior mill (electric coffee grinder, art. 346AU, China), and sieved (3000 mesh).

The following mixtures of ethanol/water were used: ethyl alcohol 25% (1 extractant), ethyl alcohol 40% (2 extractants), and ethyl alcohol 60% (3 extractants). The crushed HDs were transferred to a percolator, filled with extractant to form a “mirror”, the content of the percolator was left to infuse for 12 h and then extracted for 48 h until the complete yield of percolate. The total extractants were used in an amount corresponding to the ratio of raw material: extractant 1:20 (DSR). The combined extracts were defended for 2 days at a temperature of 8 °C and then filtered from possible impurities. The criteria for evaluating the extraction degree of BAS in the obtained extracts were as follows: the content of the flavonoids, polysaccharides, and polyphenols.

The resulting aqueous-alcoholic extracts with DSR 1:20 were placed in a reactor, where the extractants were removed by evaporation at a temperature of 60–80 °C under reduced pressure to a final moisture content no more than 5% (dry extracts as components of a combined herbal extract). The technology for obtaining extracts was described in the previous work (Kotov et al. 2021a).

Phytochemical analysis

Total flavonoid contents

The quantitative determination of the flavonoid content was carried out by absorption spectrometry using HP-8453 UV-VIS Spectrophotometer (Hewlett Packard, USA) according to State Pharmacopoeia of Ukraine (2021). The total flavonoid content was estimated as luteolin-7-glucoside equivalents in g/100 g of dry weight. 5 mL of the extracts (DSR 1:20) (or 0.2 g of dry extracts) were placed in a 50-mL volumetric flask and adjusted to the mark with 60% (V/V) ethanol.

Assay of polysaccharides

20 mL of ethanol 96% were added to 10 mL of extracts (DSR 1:20). The resulting mixture was heated on a water bath at 35–40 °C for 5 minutes and then it was allowed to stand for 1 hour. Then, using previously adjusted to a constant weight and weighed glass filters (16), the precipitated polysaccharides were filtered under vacuum, washing the precipitate on the filter with a small amount of ethanol. Next, the filters were placed in an oven and dried at a temperature of 100 °C to constant weight.

Assay of polyphenols

The total amount of phenolic compounds was evaluated using the modified Folin-Ciocalteu reagent spectrophotometry method. Pyrogallol was used as a standard, for which the polyphenol content was calculated (State Pharmacopoeia of Ukraine 2015a). For analysis, 1 g of the obtained extracts (DSR 1:20) (or 0.1 g of dry extracts) each were taken, placed in a 25-mL volumetric flask, and the volume was adjusted to the mark with water.

Chromatographic fingerprint analyses by HPLC

The liquid chromatographic apparatus Waters Alliance with Waters 2690 separation module and with 996 PDA detector (Waters, USA) was used. Separation was achieved on a column Kinetex XB (C18), 4.6 × 250 mm (Phenomenex Inc., USA) with a pre-column (2 mm) containing the same adsorbent. The temperature of the column was kept constant at 30 °C and the mobile phase was delivered at a flow rate of 1.0 mL/min and the detection wavelength was set at 360 nm. UV spectra were carried out at wavelengths between 200 and 400 nm. The volume of the sample was 10 μL and each sample was analyzed in triplicate. The components were identified by comparison of their retention times and UV spectra to those of authentic standards under identical analysis conditions and the published UV spectra data (Olennikov et al. 2017; Alirezalu et al. 2018).

Sample solutions**.** 20 mL of methanol was added to 0.25 g of dry extracts, they were sonicated at 50 °C for 20 min. The extract was filtered through filter paper, and the residue left after extraction was washed with methanol. The combined filtrates were transferred to a 25-mL volumetric flask, and the volume was adjusted to the mark with methanol.

Reference solutions**.** The solutions of the pure compounds were prepared by dissolving 1 mg of reference standards in 10 mL methanol.

Mobile phase. The binary solvent system of the mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (acetonitrile/methanol (80:20, v/v)). A linear gradient program applied was as follows: 0–5 min, 10% B; 5–15 min, 10–18% B; 15–25 min, 18% B; 25–30 min, 18–25% B; 30–35 min, 25% B; 35–40 min, 25–35% B; 40–45 min, 35–60% B; 45–50 min 60–70% B; 50–53 min 70–10% B and 53–56 min with 10% B. A 5-min equilibrium time was allowed between the injections.

Assay of triterpens

Test solutions. An accurately weighed 0.3 g of dry extracts were hydrolyzed with 2 M methanolic hydrochloric acid (20 mL) under reflux on a water bath at 100 °C for 2 h. The extract was filtered through filter paper, and the residue left after extraction was washed with methanol. The combined filtrates were transferred to a 25-mL volumetric flask, and the volume was made up to the mark with methanol.

Reference solutions. Methanolic solutions 0.06 mg/mL, 0.12 mg/mL, 0.18 mg/mL reference standard of the oleanolic acid.

TLC plate. TLC plates silica gel 60 F_{254} (Merck), 20 × 10 cm (Cat. No. 1.05729/0001, Batch No. HX98582029; Merck, Darmstadt, Germany).

Sample application. Linomat V Automatic Sample Spotter (Camag, Muttenz, Switzerland), speed application – 150 nL/s, volumes – 20 μL sample solutions (in triplicate on a TLC plate) and 10 μL – reference solutions.

Development. Glass twin trough chamber for 20 × 10 cm plates (Camag), in the mobile phase toluene-ethyl acetate-formic acid (7:3:0.2, v/v/v) for a distance of 8.0 cm at 25 °C. After development, the plates were dried in air at room temperature.

Detection method. The plates were dipped in about 10 mL freshly prepared anisaldehyde-sulfuric acid reagent for 1 min and heated at 100 °C for 7 min before scanning.

Densitometry. TLC Scanner 3 linked to WinCATS software (Camag), scanned densitometrically at 530 nm in absorbance mode using the tungsten lamp. The peak areas and absorption spectra were recorded. The calibration curve of oleanolic acid was obtained by plotting peak areas vs applied concentrations of oleanolic acid. The amount of oleanolic acid in test solutions was calculated using its calibration curve.

Reference standards and chemicals

The used reference compounds chlorogenic acid (100% purity), luteolin-7-glucoside (99% purity), luteolin (98% purity), quercetin (100% purity), caffeic acid (100% purity), hyperoside (100% purity), rutin (100% purity), vitexin (95% purity), oleanolic acid (purity 100%) were pharmacopoeial reference standards of the State Pharmacopoeia of Ukraine. Reference compounds (isorhamnetin-3-*O*-(2″,6″-di-rhamnosyl)-glucoside, isorhamnetin-3-O-rutinoside)) were obtained previously from *C. officinalis* (Derkach 1989).

The solvents (ethanol, methanol, toluene, butanol, acetonitrile, water, and ethyl acetate) and chemicals (anhydrous acetic acid, oxalic acid, sulfuric acid, anhydrous formic acid, boric acid, and anisaldehyde) used in the experiments were of analytical grade and were purchased from Sigma-Aldrich (Germany) and Merck (Darmstadt, Germany).

Statistical analysis

The data are expressed as mean of three replicates ± SEM. Statistical significance of the differences between the groups was analyzed using Student's t-test following the State Pharmacopoeia of Ukraine (2015b).

Study the anti-allergic properties in models of immediate-type hypersensitivity in vivo and in vitro

Experimental animals and their maintenance

The experiments were carried out on 48 guinea pigs of both sexes weighing 340–560 g and 24 white non-linear male rats weighing 200–250 g. Animals were raised in the NUPh vivarium nursery and kept under standard conditions: temperature 20–22 °C, humidity no more than 60–70%, air exchange volume (extract-inflow) 8/10, day/night light regime in standard aluminum cages no more than 5 animals each (Directive 2010/63/EU of European Parliament and Council on the protection of animals used for scientific purposes). The work with animals was carried out in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1986), «Procedure for conducting experiments, experiments on animals by scientific institutions» (Normative document of the Ministry of Education, Science, Youth and Sports of Ukraine (2012). The NUPh Bioethics Commission did not reveal any violations of moral and ethical standards during research work.

Reference drugs

As a reference drugs, we used: 1 – a histamine H_1 receptor blocker – Diazolin tablets (active ingredient – mebhydrolin) at a dose of 12 mg/kg (JSC FARMAK). 2 – Desloratadine, one of the 2[nd] generation antihistamines approved in Europe, which has a high safety and compliance profile (manufactured by PrAT "Technolog", Ukraine) at a dose of 0.5 mg/kg. 3 – Tesalin (manufactured by Max Zeller Zyone AG, Switzerland) at a dose of 1 mg/kg was chosen as a reference herbal preparation, which contains a native extract from the leaves of butterbur (*Petasites hybridus* L.). The doses of reference drugs were calculated by the method of Rybolovlev et al. (1981) based on their daily doses for an adult (Kovalenko and Victorova 2001).

Anaphylactic shock model in guinea pigs

Guinea pigs are the recommended type of animals for modeling systemic anaphylaxis, since in them, like in humans, activation of histamine H_1-receptors causes spasm of smooth muscles of the trachea and bronchi, increases vascular permeability (Atsapkina et al. 2014).

Guinea pigs were sensitized with 0.1 normal horse serum according to the scheme: the first subcutaneous and the second intramuscular injection every day, 0.2 ml per animal. After the incubation period on the 21[st] day from the moment of the first sensitizing injection, all experimental animals were injected intraperitoneally with a peritoneal dose of antigen, which was 0.6 ml of serum (Ado 1978).

All experimental animals were divided into the following groups: group 1 – intact control- animals that received injections of saline according to the sensitization scheme; group 2- control pathology – guinea pigs, which were sensitized with serum; group 3 – animals that received the studied extract at a dose of 30 mg/kg against the background of sensitization with serum (established as a conditional therapeutic dose for anti-inflammatory activity). groups 4–6 – animals that received diazolin at a dose of 12 mg/kg, desloratadine at a dose of 0.5 mg/kg and tesalin at a dose of 1 mg/kg against the background of sensitization with serum.

Substances were administered to experimental animals in a prophylactic mode – from the first day of sensitization intragastrically for 21 days, since the rapid development of anaphylactic shock practically does not allow revealing the therapeutic effect of the drug, which was introduced after injection of a permissive dose of antigen.

The severity of anaphylactic shock was determined according to Weige et al. (1960) on a 4-point scale: 0 points – shock has not developed, its signs are absent; 1 point – weak shock (some anxiety, rapid breathing, short-term scratching of the face, involuntary urination and defecation); 2 points – moderate shock (pronounced scratching, slight cramps, pronounced manifestation of bronchospasm); 3 points – severe shock (general convulsions, asphyxia, the animal loses its ability to hold on to its paws, falls on its side, involuntary urination and defecation, spastic cough, remains alive); 4 points – fatal shock.

The anti-allergic activity of the combined extract was determined by its ability to reduce the severity of anaphylactic shock in comparison with the control pathology group and was expressed as a percentage by the formula:

$$AA = \frac{A_{ShK} - A_{ShD}}{A_{ShK}} \times 100$$

where: AA – anti-allergic activity,%;

A_{ShK} – the severity of anaphylactic shock in the group of animals with control pathology, points;

A_{ShD} – severity of anaphylactic shock in the group of experimental animals, points.

Guinea pig model of active cutaneous anaphylaxis

Sensitization of guinea pigs was carried out according to the following scheme: the animals of the experimental groups (except for the intact control group) were injected subcutaneously with 0.2 ml of 1% egg white solution three times after 2 days (Ado 1978). The investigated extract and the reference drug were administered intragastrically from the first day of sensitization during the 21st day.

Experimental animals were divided into the following groups: group 1 – intact control – animals that received injections of saline according to the sensitization scheme; group 2 – control pathology – guinea pigs of which were sensitized with egg white; group 3 – animals that received an extract at a dose of 30 mg/kg against the background of sensitization with egg white, groups 4–6 – animals that received diazolin at a dose of 12 mg/kg, desloratadine at a dose of 0.5 mg/kg and tesalin at a dose of 1 mg/kg against the background of sensitization with egg white. On the 21st day after the start of sensitization, all experimental animals were anesthetized with ether.

A resolving dose of 40 µl of an egg white solution prepared in physiological saline was injected intradermally into the clipped skin area on the right side. The control of the solvent was carried out by intradermal injection of 40 µl of saline on the left side area. Then the guinea pigs were intravenously injected into the femoral vein with 0.5 ml of a 1% solution of Evans blue. After 30 minutes, the animals were euthanized under ether anaesthesia, the skin was separated, and the area of colored spots on the inner surface of the skin at the injection site was determined (Habriev 2005). The severity of the allergic skin reaction was assessed by the area of stained spots at the injection site of the resolving dose.

In the blood of guinea pigs, we studied indicators that indicate the effect of the combined extract on the development of sensitization. For early detection of functional changes in the body during the period of sensitization, the leukocyte count was investigated according to the standard method (Menshikov 1987). Since the allergic reaction is accompanied by the formation of complexes "antigen-antibody", the immunological status of animals was assessed by the content of circulating immune complexes (a set of reagents manufactured by "Granum", Kharkiv, Ukraine) (Menshikov 1987).

Determination of the effect of the combined extract on mast cell degranulation in rats

Rats were sensitized (except for animals of the intact control group) with 0.2 ml of 1% egg white solution three times every other day by subcutaneous injections.

Experimental animals were distributed similarly to the model of active cutaneous anaphylaxis (4.4.). The test substances were administered to experimental animals in a prophylactic regime – from the first day of sensitization intragastrically for 21 days. On day 21, the rats were euthanized under ether anaesthesia and blood serum was obtained.

The degranulation reaction was performed on mast cells, which were obtained from the peritoneal exudate of intact rats. In previous experiments, the concentration of the allergen was selected, which causes no more than 10% nonspecific degranulation. The preparations were prepared on glass slides stained with a 0.3% alcohol solution of neutral red. To 0.03 ml of a mixture of mast cells were added 0.03 ml of serum from the experimental (sensitized) or control (intact) group of animals and 0.03 ml of an allergen solution. When setting up the reaction, the following controls were taken into account:

1. 0.03 ml of smooth muscle cell suspension, 0.03 ml of the studied sera and 0.03 ml of physiological solution;
2. 0.03 ml of smooth muscle cell suspension and 0.06 ml of saline.

Then the preparations were incubated for 15 minutes at 37 °C and examined under the light microscope "Biolam". In each chamber, 100 cells were counted. The results were assessed by the percentage of degranulated cells (Habriev 2005).

Results and discussion

The following results of the analysis were obtained for aqueous-alcoholic extracts (DSR 1:20) from all analyzed HDs with various extractants (Table 1). To select the optimal extractant that would provide a balanced extraction of the maximum amount of BAS, the dependence of the content of polyphenolic compounds, flavonoids, and polysaccharides in the obtained extracts (bur-marigold herb, calendula flowers, and hawthorn leaf and flowers) on the concentration of ethanol in the extractants was studied. These BAS are directly or indirectly responsible for the biological activity of the combined herbal medicine. As can be seen from the Table 1, the content of phenolic nature substances, namely polyphenols and flavonoids, increased predictably with an increase of ethanol concentration in the extraction mixture, on the other hand, with the transition from 40% to 60% ethanol, the amount of polysaccharides extracted decreased significantly. It was shown that 40% ethanol is optimal from the point of view of balanced extraction of BAS sum: flavonoids, polyphenols, and polysaccharides from all analyzed HDs.

The next step was to obtain dry extracts (see Materials and methods). Thus, 3 dry extracts (bur-marigold herb, calendula flowers, and hawthorn leaves and flowers) and 1 combined extract in the selected ratio of raw materials bur-marigold – calendula – hawthorn (6:3:1) were obtained. This ratio was chosen as a result of the previous phytochemical and phytopharmacological design given in the work (Kotov et al. 2021b). The obtained dry extracts were studied for their quantitative content of flavonoids

Table 1. The results of determination of the biologically active substances content in various aqueous-alcoholic extracts, %*.

HD	Extractant	Flavonoids	Polyphenols	Polysaccharides
bur-marigold herb	25% ethanol (1)	0.23 ± 0.03	0.52 ± 0.05	3.67 ± 0.19
	40% ethanol (2)	0.52 ± 0.04	0.85 ± 0.07	2.87 ± 0.14
	60% ethanol (3)	0.72 ± 0.07	0.99 ± 0.05	1.24 ± 0.12
calendula flowers	1	0.09 ± 0.01	0.59 ± 0.04	4.37 ± 0.22
	2	0.28 ± 0.04	0.68 ± 0.07	4.01 ± 0.21
	3	0.29 ± 0.03	0.69 ± 0.06	2.50 ± 0.12
hawthorn leaf and flowers	1	0.80 ± 0.04	1.32 ± 0.08	3.32 ± 0.10
	2	1.21 ± 0.05	1.80 ± 0.07	2.53 ± 0.10
	3	1.80 ± 0.07	2.05 ± 0.05	1.69 ± 0.12

*the results are given in % in terms of raw material.

Table 2. HPLC results for dry extracts of the bur-marigold herb, calendula flowers, hawthorn leaf and flowers and combined extract.

Reference	Retention time, min/ UV absorption maximum	Bur-marigold extract dry*	Calendula extract dry*	Hawthorn leaf and flower extract dry*	Combined extract*
Unknown acid (expressed as chlorogenic acid	7.9–8.2/210, 295, 325nm			2.7±0.10	1.3±0.07
Unknown acid (expressed as chlorogenic acid	12.7–13.0/212, 297, 325 nm			14.6±0.37	6.0±0.14
Chlorogenic acid	13.4–13.7/215, 295, 325nm		2.7±0.09	4.2±0.12	2.2±0.08
Caffeic acid	16.7–17.0/218, 298, 325mn			0.9±0.05	
Unknown acid (expressed as caffeic acid	19.0–19.1/215, 295, 325nm	2.4±0.07			1.0±0.07
Derivate of isorhamnetin (expressed as typhaneoside)	23.3–23.5/253, 265, 353 nm		1.3±0.10		
Vitexin 2”-***O***-rhamnoside (expressed as vitexin)	24.2–24.4/212, 267, 340 nm			12.9±0.16	1.8±0.11
Typhaneoside (isorhamnetin-3- *O*-(2″,6″-di-rhamnosyl)-glucoside)	25.0–25.3/253, 265, 355 nm		5.4±0.13		1.5±0.10
Vitexin	26.4–26.5/213, 268, 337 nm			0.5±0.08	
Rutin	28.2–28.5/210, 255, 354 nm			7.2±0.19	0.8±0.12
Calendoflavoside (isorhamnetin-3-*O*-(2″-rhamnosyl)-glucoside, expressed as narcissin)	29.5–30.0/253, 267, 355nm		3.8±0.12		1.1±0.08
Hyperoside	30.4–30.6/254, 352nm			4.6±0.13	2.5±0.09
Luteolin-7-glukoside	31.2–31.6/254, 265, 347nm	10.0±0.25		1.3±0.06	5.5±0.18
Narcissin (isorhamnetin-3-O-rutinoside)	34.5–34.8/253, 265, 355 nm		4.8±0.21		2.4±0.13
Derivate of Vitexin (expressed as vitexin)	36.6–36.9/212, 267, 337 nm			5.8±0.20	
Quercitin	45.2- 45.4/255, 365nm		0.4±0.06	0.8±0.07	
Luteolin	45.3–45.5/254, 265, 347nm	9.5±0.18			3.7±0.22
Total acids, mg/g,		2.4±0.09	2.8±0.11	22.4±0.55	10.5±0.28
Total flavonoids, mg/g		19.5±0.34	15.7±0.30	33.1±0.48	19.3±0.32

Note: the content was calculated as mg per g extract.

and polyphenols by the UV method, qualitative and quantitative content of phenolic compounds by the HPLC method, and the content of terpenoid compounds by the densitometry method to clarify the criteria for standardization of the combined herbal extract.

Chromatographic fingerprint analyses by HPLC

For a detailed study of the component composition of flavonoids and hydroxycinnamic acids an HPLC study of all dry extract samples (bur-marigold, calendula, hawthorn extracts, and the combined extract) was carried out. Fig. 1 shows a chromatographic profile for all extract samples obtained under the conditions of the “Chromatographic fingerprint analyses by HPLC” technique and Table 2 – the HPLC results obtained for all the samples studied. Analyzing the data of Table 2, it was first found that the bur-marigold extract, which is the main component of the combined extract, contained mainly flavonoids, namely luteolin and luteolin-7 glucoside (more than 90% of all detected peaks), which corresponded to a concentration of 1.95% in terms of dry extract. Iso-rhamnetin derivatives (almost 70%) were mainly identified in the calendula extract, which corresponded to a concentration of 1.5%. For hawthorn extract, it was found that it contained significant amounts of hydroxycinnamic acids (2.24% expressed as chlorogenic acid), as well as derivatives of vitexin (almost 42% of all peaks detected), which coincided with the literature data (Alirezalu et al. 2018). The total content of flavonoid compounds in the extract was 3.3%. For the combined extract, all the main components identified for the individual extracts were identified in its chromatogram. The content of hydroxycinnamic acids was 1%, and the content of flavonoid compounds -1.93%, which corresponded to the theoretical content (98%) taking into account the ratio of HDs in the combined extract.

Given that the HDs extracts used contain significant amounts of terpenoid compounds, which also contribute to the pharmacological activity of the developed combined herbal medicine, additional tests were performed to determine the content of terpenoids by the densitometric method. The method given by Anandjiwala et al. (2006) was used as a basis.

Fig. 2 shows the TLC chromatogram obtained under the conditions of the method of terpenoids content determination calculated as oleanolic acid. Fig. 3 shows the obtained densitogram for the calendula extract solution, oleanolic acid solution, and combined extract solution. When carrying out chromatography of tested extracts’ solutions obtained after acid hydrolysis, it was found that for extracts of bur-marigold and hawthorn, the conditions of determination did not allow quantitative determination due to insufficient concentration of the oleanolic acid in the samples, and increasing the concentration applied to the plate, did not lead to the desired result due to the resulting overload. Therefore, further quantification of the terpenoid content was performed for the samples of the calendula extract and combined extract, and their results are shown in Table 3. Thus, 0.44% of terpenoids calculated as oleanolic acid was found in the combined extract, which corresponded to 105% of the theoretical content, taking into account the ratio of calendula in the combined extract.

Table 3. The results of the terpenoids content determination expressed as oleanolic acid in calendula extract and combined extract.

№	Terpenoids (expressed as oleanolc acid), %	
	Combined extract	*Calendula extract*
1	0.46	1.10
2	0.46	0.91
3	0.37	1.14
4	0.44	0.99
5	0.46	1.05
Mean	0.438	1.04
SEM	0.0390	0.0892

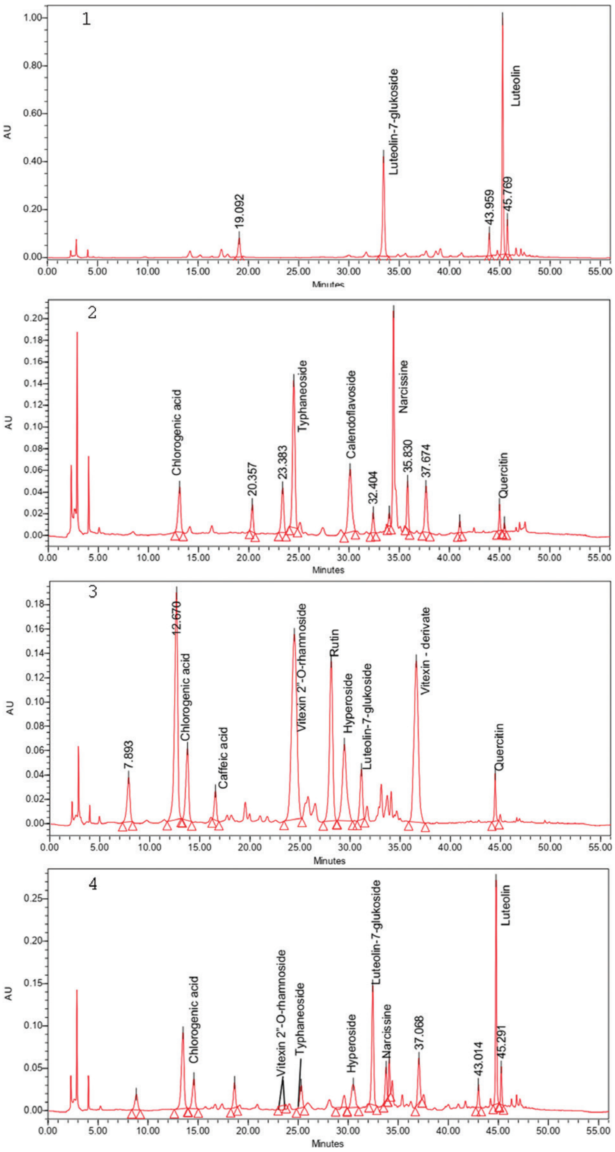

Figure 1. The chromatographic profile for the samples: **1** sample of the bur-marigold dry extract; **2** sample of the calendula dry extract **3** sample of the hawthorn dry extract; **4** combined extract.

As a result of the chromatographic study of the profile of flavonoid and hydroxycinnamic compounds, as well as the determination of the terpenoid compound content in the analyzed extracts, it was concluded that it is reasonable to standardize the combined herbal extract on the content of flavonoids in terms of luteolin-7-glucoside and content of polyphenols in terms of pyrogallol by the UV method and the content of terpenoid compounds, in terms of oleanolic acid by the densitometric method. Table 4 shows the results of determining the quality indicators selected for standardization in all HDs extracts tested.

Study of anti-allergic activity of the combined extract in the model of anaphylactic shock

The next step was to study the anti-allergic properties of the combined herbal extract in models of immediate-type hypersensitivity *in vivo* (model of anaphylactic shock and active each anaphylaxis) and *in vitro* (mast cell degranulation reaction). Anaphylactic shock – the general condition of the animal's body, caused by the introduction of a permissive dose of antigen, is manifested by the development of an immediate generalized hypersensitivity reaction arising from the accelerated massive release of allergy mediators (Zaikov and Bogomolov 2016).

Table 4. The results of determination of the BAS content for dry extracts of the bur-marigold herb, calendula flowers, hawthorn leaf and flowers and combined extract, %.

Extract	Flavonoids, %	Polyphenols, %	Terpenoids, %
bur-marigold herb	3.99 ± 0.03	5.51 ± 0.05	–
calendula flowers	2.12 ± 0.08	3.42 ± 0.09	1.04 ± 0.09
hawthorn leaf and flowers	5.43 ± 0.12	7.22 ± 0.11	–
combined extract	3.13 ± 0.09	5.03 ± 0.10	0.44 ± 0.04

In 8–10 minutes after the administration of a permissive dose of serum in animals of the control pathology group, manifestations of anaphylactic shock were observed, which was characterized by severe anxiety, piloerection, tremor, intense scratching of the face, and tousled coat. By the 20^{th} minute, the animals of this group developed severe anaphylactic shock, which manifested itself in convulsions, lateral position, Cheyne-Stokes breathing, asphyxia and death of all guinea pigs within 30 minutes. At the same time, the severity of anaphylactic shock in animals of the control pathology group was equal to 4.00 points (Table 5). Under the influence of all studied drugs, the survival rate of animals increased to 100%, but the severity of anaphylactic shock in the group of guinea pigs receiving the combined extract was at the level of the herbal preparation tesalin, anti-allergic activity was 38%. The studied combined extract by pharmacological action was 13% higher than the reference drug diazoline, which showed the least activity and only 12% inferior to the powerful antiallergic drug desloratadine (Table 5). Thus, the combined extract prevented the development of anaphylactic shock caused by sensitization, and was not inferior in anti-allergic activity to tesalin and exceeded the activity of diazolin.

Study of anti-allergic activity of the combined extract on the model of active cutaneous anaphylaxis

On the model of active cutaneous anaphylaxis on the twenty-first day of sensitization in animals of the control group pathology have observed a significant increase in the number of leukocytes in the blood (Table 6).

Table 5. Anti-allergic activity of the combined extract in the model of anaphylactic shock in guinea pigs, n = 6.

Experimental conditions	Indicators		
	Animal survival, %	The severity of anaphylactic shock, points	Anti-allergic activity, %
Intact control	100	0	–
Control pathology (sensitization)	0	4.00 (4÷4)	–
Combined extract, 30 mg/kg + sensitization	100	2.50 (2÷3)*	38
Diazolin, 12 mg/kg + sensitization	100	3.50 (3÷4)*	25
Desloratadine,0.5 mg/kg + sensitization	100	2.00 (2÷2)*	50
Tesalin, 1 mg/kg + sensitization	100	2.50 (2÷3)*	38

Notes: n – the number of animals in the group; * – significant deviation in relation to the control pathology group, p < 0,05.

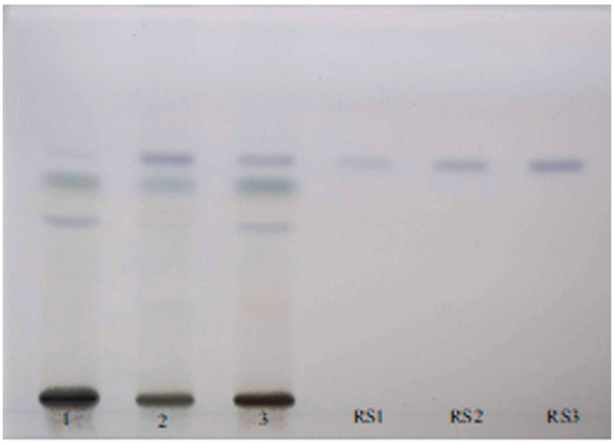

Figure 2. TLC chromatogram, obtained under the conditions of the terpenoids content determination in the analyzed extracts: **1** the solution of the hawthorn extract; **2** the solution of the calendula extract; **3** the solution of the combined extract, RS1–RS3 –solutions of the oleanolic acid (0.06 mg/ml, 0.12 mg/ml and 0.18 mg / ml as appropriate).

Since leukocytes are one of the first to appear at the site of inflammation and are the first line of cellular defense, already during the period of sensitization these cells accumulate in the blood (Dale and Foremen 1998). Leukocytes are involved in the phagocytosis of immune complexes, which are formed when the antigen interacts with the antibody. The resulting immune complexes contribute to the inactivation and elimination of antigens. A significant increase in circulating immune complexes in the blood during the period of sensitization (Table 6) in the control pathology group characterizes the physiological response of the body to excessive intake of antigen. The accumulation of circulating immune complexes during the sensitization period leads, after the introduction of a permissive dose of antigen, to the release of chemokines, and, as a result, to the development of symptoms of an allergic reaction (Menshikov et al. 1987).

In the mechanism of development of active cutaneous anaphylaxis, the antigen-antibody complex is involved, which leads to the release of one of the main mediators of allergy, histamine, from the cell depots. Histamine plays an important role in the processes of changes in vascular tissue permeability. The introduction of a dye solution (Evans blue), which quickly spreads throughout the body, and due to its release from the bloodstream at the site of tissue inflammation forms a blue spot, the area of which is an indicator of a local anaphylactic reaction (Habriev 2005).

In the group of control pathology, the size of the stained spot at the site of intradermal antigen injection was significantly increased compared with the indicator in the group of intact control. The data obtained showed the formation of an "antigen-antibody" complex upon administration of an antigen, which causes the release of histamine from the cell depot. Under the action of histamine, the permeability of the skin capillary walls sharply increases, which leads to the release of the dye into the tissue. Prophylactic administration of the combined

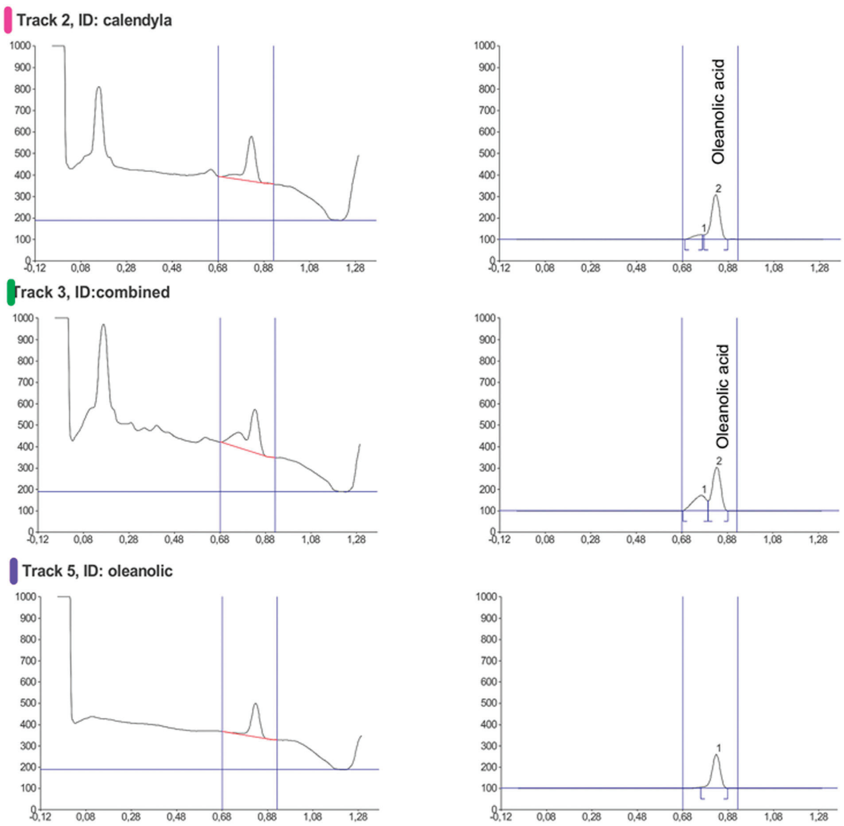

Figure 3. Densitograms obtained when determination the terpenoids content in terms of oleanolic acid: a solution of the calendula extract, a solution of the combined extract and a solution of the oleanolic acid.

Table 6. Results of studying the anti-allergic effect of the combined extract on a model of active cutaneous anaphylaxis in guinea pigs, n = 6.

Experimental conditions	Painted spot area, mm^2	Leukocyte count, 10^9/L	Circulating immune complexes, optical density units
Intact control	8.16±1.45	7.20±0.65	0.346±0.038
Control pathology (sensitization)	194.88±9.53*	13.81±1.06*	0.541±0.049*
Combined extract, 30 mg/kg + sensitization	79.07±6.34*/**/***	9.15±0.94**	0.191±0.020**/***
Diazolin, 12 mg/kg + sensitization	105.62±8.51*/**	10.62±0.82**	0.174±0.014**
Desloratadine,0.5 mg/kg + sensitization	35.22±4.39*/**/***	11.35±1,16*/**	0,256±0.038**/***
Tesalin, 1 mg/kg + sensitization	84.51±6.15*/**/***/d	11.05±0,93*/**	0,203±0.031**/***

Notes: * – significant deviation in relation to the intact control group, p < 0,05;
** – deviation is significant in relation to the control pathology group, p < 0,05;
*** – deviation is significant in relation to the group of the reference drug diazolin, p < 0,05;
d – significant deviation in relation to the group of the reference drug desloratidine, p < 0,05.

extract and the reference drugs during the sensitization period contributed to a significant decrease in the number of leukocytes in the blood and the level of circulating immune complexes (Table 6), which allows us to conclude about the ability of the studied substances to slow down the level of sensitization of animals and, as a consequence, to prevent the development of an allergic reaction

Under the influence of the combined extract, there was a significant decrease in the area of stained spots at the site of intradermal injection of egg white by 2.46 times compared with the control pathology (Table 6). By the ability to reduce the permeability of the walls of the capillaries of the skin, the combined herbal extract did not differ from tesalin and significantly exceeded diazolin by 1.33 times. Nevertheless, the combined plant extract, like tesalin, was 2.2 and 2.4 times inferior in antiallergic effect to deslorartadine, respectively, while diazolin was 3.0 times lower. Thus, the combined extract, when administered prophylactically during sensitization in a model of active cutaneous anaphylaxis, exhibits a pronounced anti-allergic effect at the level of tesalin which indicates its therapeutic efficacy in allergic reactions of an immediate type.

In "immediate" type allergic reactions, the target cells are connective tissue mast cells. Their degranulation under the action of the "antigen-antibody" complex leads to an intensive release of biologically active substances that cause allergy manifestations.

Determination of the effect of the combined extract on the degranulation of mast cells

In "immediate" type allergic reactions, the target cells are connective tissue mast cells. Their degranulation under the action of the "antigen-antibody" complex leads to an intensive release of biologically active substances that cause allergy manifestations (Klein and Sagi-Eisenberg 2019). Anti-allergic drugs – mast cell stabilizers, unlike receptor blockers, can prevent the development of allergies at the immunological stage, even before the release of mediators. In order to study the effect of the combined extract on the release of allergy mediators, an in vitro study was carried out on mast cells of rat peritoneal exudate. The results shown in Table 7 indicate that the combined extract significantly inhibited the degranulation of mast cells and was 2.2 and 1.49 times respectively superior to the reference drugs diazolin and tesalin in their ability to prevent the release of mediators of "immediate" allergic reactions. There were no significant differences between the number of degranulated cells in the combined extract group and the desloratadine group, which indicates their possible ability to suppress pro-inflammatory cytokines. Considering that the studied herbal remedy contains antioxidants of natural origin and comparing the results obtained from all experiments, it can be concluded that the mechanism of anti-allergic action of the combined herbal extract is probably associated not only with antihistaminic properties, but also with the ability to stabilize cell membranes, which is consistent with the data literature (Hiemori-Kondo 2020).

Table 7. Results of studying the anti-allergic effect of the combined extract on the model of mast cell degranulation, n = 6.

Experimental conditions	Number of degranulated cells, %
Intact control	6.19±1.22
Control pathology (sensitization)	25.43±3.91*
Combined extract, 30 mg/kg + sensitization	8,54±1,36**/***/t
Diazolin, 12 mg/kg + sensitization	18,75±3,05*
Desloratadine,0.5 mg/kg + sensitization	6.87±1.48**/***/t
Tesalin, 1 mg/kg + sensitization	13.72±1.64**/d

Notes: * – significant deviation in relation to the intact control group, p < 0.05;
** – deviation is significant in relation to the control pathology group, p < 0.05;
*** – deviation is significant in relation to the group of the reference drug diazolin, p < 0.05;
d – significant deviation in relation to the group of the reference drug desloratidine, p < 0.05;
t – significant deviation in relation to the group of the reference drug tesalin, p < 0.05.

Conclusions

Using an experimentally selected extractant for balanced extraction of flavonoids, polysaccharides and polyphenols from bur-marigold herb, calendula flowers and hawthorn leaves and flowers, 3 suitable dry extracts and 1 combined extract in the selected ratio of raw materials were obtained. The phytochemical profile of its main BAS and methods of its standardization have been studied and it was concluded that it is reasonable to standardize the combined herbal extract on the content of flavonoids in terms of luteolin-7-glucoside and content of polyphenols in terms of pyrogallol by the UV method and the content of terpenoid compounds, in terms of oleanolic acid by the densitometric method. The combined herbal extract on the model of anaphylactic shock has showed anti-allergic activity at the level of tesalin and exceeds diazoline. On the active model of cutaneous anaphylaxis the ability of the combined extract to reduce the permeability of skin capillaries at the level of the herbal antiallergic preparation tesalin has been established and it significantly exceeded diazoline. In the test of indirect degranulation of mast cells, the combined extract did not differ in membrane-stabilizing properties from the effect of desloratadine and was significantly superior the reference drugs diazolin and tesalin. The combined herbal extract is a promising subject for a new anti-allergic agent, which requires a deeper study of its mechanisms of action.

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Supplementary material 1

HPLC results for dry extracts of the bur-marigold herb, calendula flowers, hawthorn leafand flowers and combined extract

Authors: Semen Kotov

Data type: Table

Explanation note: HPLC results for dry extracts of the bur-marigold herb, calendula flowers, hawthorn leaf and flowers and combined extract. Note: the content was calculated as mg per g extract.

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