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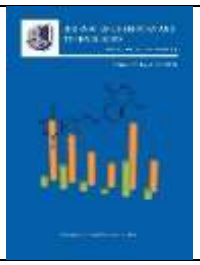

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ISOLATION AND CHARACTERIZATION OF PANCREATIC LIPASE INHIBITOR FROM RAPESEED SEEDS

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Abstract

Digestive enzymes and inhibitors of digestive enzymes are effective correctors of digestive processes in the body, the violation of which leads to various diseases (diabetes, hyperlipidemia, cardiovascular diseases, neoplasms and others). The present study identified the most promising plant objects characterized by the highest antilipolytic activity (ALA) in relation to pancreatic lipase. The experimental results indicate that the inhibitory activity (IA) of phenolic compounds of rapeseed is so much high and comparable to ALA "Orlistat", reaching 95.5 % of its activity. This determines the potential possibility of using the phenolic complex of rapeseed as an alternative to anti-lipolytic drugs of synthetic origin. The predominant component of the phenolic complex is low molecular weight phenolic compounds; polyphenolic compounds are almost equally represented by tannins – condensed and hydrolyzable. According to TLC data, the main components of low molecular weight phenols are glucopyranosylsinapate, sinapic acid and sinapin. Among the phenolic compounds of rapeseed seeds, sinapine and hydrolyzable tannins have the highest anti lipolytic activity against lipase. Significant ability to inhibit the action of pancreatic lipase is characterized by both low molecular weight and high molecular weight phenolic compounds of rapeseed.

Keywords: biotechnologie; pharmacology; pancreatic lipase inhibitor; rapeseed seeds; phenolic compounds; inhibitory activity; extraction and purification; composition and physico-chemical properties; pH-stability; temperature stable.

ВИДІЛЕННЯ ТА ХАРАКТЕРИСТИКА ІНГІБІТОРА ПІДШЛУНКОВОЇ ЛІПАЗИ З НАСІННЯ РІПАКУ

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Анотація

Травні ферменти та інгібітори травних ферментів є ефективними коректорами процесів травлення в організмі, порушення яких призводить до різних захворювань (цукровий діабет, гіперліпідемія, серцево-судинні захворювання, новоутворення та інші). У даному дослідженні визначено найбільш перспективні рослинні об'єкти, що характеризуються найвищою антиліполітичною активністю (АЛК) по відношенню до панкреатичної ліпази. Результати експериментів свідчать про те, що інгібуюча активність (ІА) фенольних сполук насіння ріпаку настільки висока і порівняння з АЛК «Орлістат», досягаючи 95.5 % її активності. Це визначає потенційну можливість використання фенольного комплексу ріпаку як альтернативи антиліполітичним препаратам синтетичного походження. Переважним компонентом фенольного комплексу є низькомолекулярні фенольні сполуки; поліфенольні сполуки майже порівну представлені дубильними речовинами – конденсованими і гідролізними. За даними ТШХ, основними компонентами низькомолекулярних фенолів є глюкопіранозилсинапат, синапінова кислота і синапін. Серед фенольних сполук насіння ріпаку синапін і гідролізовані таніни мають найвищу антиліполітичну активність проти ліпази. Значною здатністю пригнічувати дію панкреатичної ліпази характеризуються якнизькомолекулярні, так і високомолекулярні фенольні сполуки ріпаку.

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Ключові слова: біотехнологія; фармакологія; інгібітор панкреатичної ліпази; насіння ріпаку; фенольні сполуки; інгібіторна активність; екстракція та очищення; склад та фізико-хімічні властивості; pH-стабільність; температурна стабільність.

Introduction

Today, biologically active additives, which are effective tools for managing the composition of food rations and their correction, play a significant role in the arsenal of means for the prevention and treatment of morbidity. The development of technologies for the production of dietary supplements was the result of the realization of the impossibility of optimizing diets solely by increasing production volumes and expanding the range of traditional food products. Only concentration of the necessary nutrients in the form of dietary supplements is able to enrich the human diet or affect its health, taking into account the diversity of genotypes, differences in metabolism and physiology of each individual [1].

The market of dietary supplements in developed countries is growing rapidly, not being medicines, they have a diverse positive effect on the human body. If the first generation of dietary supplements were created exclusively as a means of correcting nutritional deficiencies, the second generation of dietary supplements were already able to solve more complex problems - optimizing the composition of the diet and the therapeutic effects of food. The strategy in the development of dietary supplements has led to the creation of modern third-generation dietary supplements, which have certain characteristics and are able to correct physiological processes in the body.

The problem of digestive system dysfunction, which is regarded as epidemiological, is solved with the help of medicines, which are preparations of enzymes mainly of animal or microbial origin and synthetic inhibitors. The use of plant analogues, which are superior to them in many respects, is now considered as an alternative way to correct the functions of the digestive system. Thus, plant enzymes do not lead to inhibition of the production of the body's own digestive enzymes (do not cause the effect of "addiction"), are characterized by low allergenic potential and low toxicity. Therefore, it is timely to develop the scientific basis for the creation of dietary supplements containing enzymes and inhibitors of plant origin, and functional products based on them.

Given that the state of health of the population of Ukraine is directly dependent on the nutrition system, the development and use of domestic functional foods and dietary supplements is relevant and necessary.The prevalence of obesity is increasing at an alarming rate, but, unfortunately, only a few medications are currently on the market. Obesity is primarily regarded as a disorder of lipid metabolism and the enzymes involved in this process could be selectively targeted to develop antiobesity drugs. Recently, newer approaches for the treatment of obesity have involved inhibition of dietary triglyceride absorption via inhibition of pancreatic lipase (PL) as this is the major source of excess calories. Natural products provide a vast pool of PL inhibitors (PLI) that can possibly be developed into clinical products. Although widely regarded as a problem confined to the developed world, the obesity epidemic is, in reality, sweeping inevitably through the developing nations as well [2]. Obesity is becoming one of the greatest threats to global health in this millennium, with more than 1 billion overweight adults and of those, at least 300 million are clinically obese [3; 4]. The regulation of energy homeostasis for metabolic diseases is one of the most rapidly advancing topics in biomedical research today. Breakthroughs in understanding of the molecular mechanisms regulating body weight have also provided potential opportunities for therapeutic intervention and brought renewed hope and vitality for the development of antiobesity drugs [5; 6]. Despite the plethora of research data available on obesity, it still remains, largely, an unsolved medical problem [7–9]. The market for antiobesity drugs is potentially huge, as it accounts for 2–6 % of total health care costs in several developed countries. With its growing worldwide prevalence, the obesity market has been predicted to reach US$ 3.7 billion by 2008. The mushrooming market for these drugs and the vast sum of money at stake guarantee that research in this therapeutic area will not slow down within the foreseeable future [10; 11]. Several excellent reviews have appeared covering the detailed mechanisms associated with energy homeostasis, newly identified targets and current and investigational agents [12–22]. At present, the potential of natural products for the treatment of obesity is still largely unexplored and might be an excellent alternative strategy for the development of safe and effective antiobesity drugs [23]. Over the counter remedies for obesity, based on nutritional supplements, are extremely popular; however, although such treatments are widely used, none has yet been convincingly demonstrated to be safe and effective. Lipid

metabolism is elegantly balanced to maintain homeostasis [24; 25]. When the balance is lost, obesity or hyperlipidemia develops, leading to a variety of serious diseases, including atherosclerosis, hypertension, diabetes and functional depression of certain organs. Therefore, the control of lipid metabolism by drugs could be used to prevent or treat these diseases. A growing number of enzymes involved in lipid metabolic pathways are being identified and characterized; as such they represent a rich pool of potential therapeutic targets for obesity and other metabolic disorders [12; 25]. One of the most important strategies in the treatment of obesity includes development of inhibitors of nutrient digestion and absorption, in an attempt to reduce energy intake through gastrointestinal mechanisms, without altering any central mechanisms [22]. Since dietary lipids represent the major source of unwanted calories, specifically inhibiting triglyceride (TG) digestion forms a new approach for the reduction of fat absorption [26; 27].

The fat included in our diet is mainly composed of mixed triglycerides. These mixed triglycerides are required to be cleaved into free fatty acids by lipase enzyme before absorption by intestinal enterocytes. The human lipase superfamily includes gastric, pancreatic, hepatic, endothelial and lipoprotein lipases. The digestion of fat starts in stomach where gastric lipase causes partial hydrolysis of triglycerides. The gastric lipase is secreted by the mucosal cells of stomach. Lingual lipase is present in trace amount in humans and has minimal effect on the metabolism of fat. After that, pancreatic lipase secreted from pancreatic acinar cells is responsible for complete hydrolysis of dietary fat in small intestine. The post prandial triglycerides are metabolized by other lipases. First, lipoprotein lipase which is produced by adipocytes, cardiac cells, muscle tissue and macrophages, converts chylomicron into chylomicron remnants and very low-density lipoprotein (VLDL) into IDL in liver. It requires apolipoprotein CII (Apo CII) as cofactor for activity. Second, hepatic lipase converts triglyceride of intermediate-density lipoprotein (IDL) into cholesterol rich low-density lipoprotein (LDL). It also converts triglyceride rich high-density lipoprotein (HDL2) particles to HDL3 that is poor in triglyceride content. It is produced by hepatocytes in liver. It does not require any cofactor for its activity. Further, endothelial lipase is produced by the endothelium, liver, lung, kidney, and placenta. Endothelial lipase shares their similarity with lipoprotein lipase (45 %), hepatic lipase (40 %) and pancreatic lipase (27 %), but it differs functionally from these lipases. It has a phospholipase A2 activity instead of triacylglycerol activity [26]. It is very much cleared that the pancreatic lipase enzyme plays major part in the metabolism of the dietary fat and prevents its absorption through the small intestine. The excess fat stored in body as white adipose tissue in body and cause obesity. The inhibitors of this enzyme directly influence root cause of obesity. Hence, it is a wellrecognized target for the management of obesity and related morbidities. The inhibition of this enzyme attracts the researchers globally because the only food and drug administration (FDA) approved antiobesity drug (orlistat) is a pancreatic lipase inhibitor [28]. In some of studies, the human gastric lipase activities of synthetic compounds were also given which are also included here because inhibitors of human gastric lipase (HGL) also affect the metabolism of fat and of course obesity eventually. The reviews on pancreatic lipase inhibitors are either related to natural pancreatic lipase inhibitors or related to approaches used in discovery of pancreatic lipase inhibitors [29–37]. They all categorized the constituents of natural pancreatic lipase inhibitors into polyphenols, saponins, alkaloids, triterpenes and flavonoids etc. A countless number of pancreatic lipase inhibitors have been screened from natural sources; plant extract as well as microbial products. The only reason behind this rat race is that orlistat is a pancreatic and gastric lipase inhibitor. Orlistat is developed from a microbial (*Streptomyces toxytricini*) product lipstatin (1) which is an irreversible inhibitor of pancreatic lipase enzyme [38; 39].

The anti-obesity drug Orlistat is a powerful lipase inhibitor, however, has some safety issues. This drug has some serious side effects such as oily spotting, fecal urgency, steatorrhea, insomnia, flatulence and fecal incontinence [40]. It is also associated with vitamin deficiency and liver diseases [41]. These unpleasant gastrointestinal side effects affect its patient compliance [42]. There have been reports that naturally-occurring compounds can inhibit pancreatic lipase [43; 44]. Nowadays, researchers have paid attention to natural inhibitors of lipase activity due to their potential inhibitory effect with low toxicity. Many studies have focused on polyphenols from herbal and fruit sources [45; 46]. Some have been isolated from leaves of Mental viridis and Eucalyptus globulus [47], from leaves of Morus alba [48], and from other plant sources [49; 50]. The studies on pancreatic lipase inhibition could

be used to produce data for the discovery of novel inhibitory compounds of either pharmacological or biotechnological interests [33]. The search for more efficacious lipase inhibitors from natural sources is still necessary. Although there are many studies that have clearly reported the potential pancreatic lipase inhibition from natural resources, very few extracts or compounds have reached clinical studies because of a lack of the understanding of their mode of action. The study of the mode of inhibition will provide data for further investigation in clinical trials [50–80].

It is well-known that polyphenols from plants have an affinity for proteins, primarily through hydrophobic, as well as hydrogen, bondings. Thus, hot water extracts of various plant materials could exhibit inhibitory activity for enzymes, because of aggregation of enzyme proteins [81; 82]. Many polyphenolics such as flavones, flavonols, tannins and chalcones are active against PL. Luteolin, a commonly occurring flavonoid, has been shown to be a weak inhibitor of PL [83]. 3-Methyletherganglin and Mangiferin flavonoids obtained from rhizomes of Alpinia officinarum and from fruits of Mangifera indica, respectively, showed moderate inhibition of PL [84; 85]. Hesperidin isolated from the peels of Citrus unshiu inhibited PL with IC50 value of 32 mg/ml. Other flavonoids from the peel of, such as neohesperidin from same peel weakly inhibited the lipase, while narirutin and naringin did not show any activity [86]. Oolong tea polyphenols of the polyphenols identified from oolong tea, epigallocatechin 3,5-di-O-gallate (IC50 0.098 mM), prodelphinidin B-2 3,30-di-O-gallate (IC50 0.107 mM), assamicain A (IC50 0.120 mM), oolonghomobisflavan A (IC50 0.048 mM), oolonghomobisflavan B (33) (IC50 0.108 mM), theasinensin D (IC50 0.098 mM), oolongtheanin 30-O-gallate (IC50 0.068 mM), theaflavin (IC50 0.106 mM), and theaflavin 3,30-O-gallate (IC50 0.092 mM) showed the most potent PL inhibitory activities. Furthermore, detailed SAR studies suggested that functional galloyl moieties and the polymerization of flavan-3-ol were required for PL inhibition [87]. Grape seed extract (GSE) is rich in bioactive phytochemicals and has inhibitory activity on PL and lipoprotein lipase, suggesting that it may be useful as a treatment to limit dietary fat absorption and the accumulation of fat in adipose tissue. Further, GSE was shown to decrease isoproterenol-stimulated lipolysis in 3T3-L1 adipocytes. The effect of GSE on lipases might be caused by a synergistic action of several compounds within the extract, viz. flavonoids, procyanidins and their antioxidative metabolites, rather than by a single compound. The results from several studies have indicated a lack of toxicity which supports the use of proanthocyanidin-rich extracts from grape seeds in various foods [88]. Natural products identified from traditional medicinal plants and microbial sources have always presented an exciting opportunity for the development of new types of therapeutics. About half of all compounds that were successful in clinical trials during the past 20 years have, at least, been derived from natural origin. Despite this scenario, only orlistat, a semi-synthetic derivative of lipstatin (a natural product) is in clinical use and P57, an appetite suppressant, is in clinical trials for obesity. This clearly suggests that the rich potential of nature to combat obesity has not been fully explored yet and many newer leads may be obtained from the natural sources. Recent developments in understanding the pathophysiology of the disease process have opened up new avenues to identify and develop novel therapies to combat obesity, among these various enzymes involved in lipid metabolism provide interesting targets in the development of antiobesity agents. PL, the principle lipolytic enzyme, hydrolyses dietary fats in the first step of lipid metabolism. Thus, PL inhibitors may provide an answer to the ever-increasing problem of obesity. Many plants and microbial products have been screened for their PL inhibitory potential but the work has remained more of academic interest and nothing substantial has gone up to the clinical level. Thus, there is an urgent need to update the studies on the known inhibitors as well as to discover newer natural sources in detail to fully realize their potential on PL and focally develop them as new antiobesity therapeutics.

The choice of plant sources of enzymes and inhibitors of digestive enzymes, their maximum preservation during extraction, substantiation of the most rational and effective methods of concentration and stabilization of biocorrectors in the development of dietary supplements technologies, ensuring their stability during storage are key positions in the development of new technologies of dietary supplements with corrective properties to digestive processes.

The aim of the study was to isolate and investigate the physicochemical properties of pancreatic lipase inhibitor of phenolic nature from rapeseed seeds. To achieve this goal, the main objectives of the study were defined: to establish the nature of the active components of plant raw materials that are responsible for anti-lipolytic activity, to substantiate the methods of extraction

and purification of lipase inhibitor, to study the composition and physicochemical properties of pancreatic lipase inhibitor (the effect of pH and temperature on the activity and stability of the object under study).

Materials and methods

Seeds of rape variety "Galytskyi" were created in the Ivano-Frankivsk Institute of Cruciferous Crops (Ukraine) [89]. The studied variety is double-zero, i.e. does not contain erucic acid.

Determination of lipolytic activity. Lipolytic activity was determined by the titrometric method [39], which is based on the calculation of the number of fatty acids formed during the hydrolysis of the substrate. A 40 % olive oil emulsion stabilized with polyvinyl alcohol was used as a substrate. As a unit of activity was taken the number of enzyme that releases 1 μmol of oleic acid from 40 % olive oil suspension at 37 °C for 1 h under the conditions described below. In a test tube containing 8...15 mg of the sample, 1 cm^3 of water, 0.1 cm3 of 0.1 % pancreatin solution, 0.8 cm^3 of phosphate buffer (pH 7.6) were added and thermostated for 5 min at 37 °C. Then 1.0 cm^3 of 40 % suspension of olive oil with polyvinyl alcohol was added, thermostated under the same conditions. After exactly 1 h of incubation, the reaction was interrupted by adding 5 cm^3 of 96 % ethanol. In the control sample, the olive oil emulsion was added after the addition of ethanol. Three drops of phenolphthalein solution were added to the control and experimental samples and titrated with 0.05 M sodium hydroxide solution until light pink color. The level of lipase activity was estimated in conventional units by the difference in the amount of alkali used for titration of experimental and control samples during the hydrolysis of 40 % emulsion of olive oil.

Lipase activity was calculated by the formula

$$LA = \frac{(A - A_1)}{B} \cdot 100, \text{ U/g}$$

where A is the number of 0.05 M NaOH used for titration of the experimental sample, cm^3;
A_1 – number of 0.05 M NaOH used for titration of the control sample, cm^3;
B – number of enzyme in the reaction mixture, milligram;
100 – coefficient for conversion to micromoles.

IA was determined by the difference of lipolytic activities of intact lipase and lipase in the presence of inhibitor and expressed in inhibitory units per 1 g of inhibitor (U/g).

Hydrolysable polysaccharides (HP) were determined by the reducing ability of solutions obtained after hydrolysis with 2 % HCL for 4 hours. Hard-to-hydrolyze polysaccharides (HHP) were determined by the reducing ability of the hydrolysates of the residue after removal of HP. Lignin was determined as the residue after removal of HP and HHP without ash and protein. The total content of of phenolic compounds was determined by spectrophotometric method (Folin-Dennis method). The content of condensed and hydrolyzable tannins was determined by the Leventhal method (permanganatometry). Lipid composition was studied by column and thin layer chromatography. Ash was determined by burning with subsequent calcination of the mineral residue at 500...600 °C. The isolation and study of the lipid component of rapeseed was carried out according to the scheme (Fig. 1) [89].

Free lipid complex was isolated by exhaustive extraction of raw material with diethyl ether. The solvent was distilled off, the residue - free lipid complex, was dissolved in chloroform.

The free lipid complex was separated on a silica gel column (91.5×1.5 cm). The following organic solvents were used as eluents: chloroform (175 cm^3) – for the isolation of chlorophylls, carotenoids, glycerides and free fatty acids; acetone (700 cm^3) – phosphatidic acid; methanol (175 cm^3) – phospho- and glycolipids. The content of carotenoids and chlorophylls was determined in the chloroform extract spectrophotometrically at a wavelength of 440, 663 and 645 nm, respectively.

Glycerides were separated on a silica gel column. Elution of triglycerides was carried out with a mixture of hexane-ether (95 : 5), diglycerides - with a mixture of hexane-ether (85 : 15), monoglycerides - with diethyl ether with subsequent gravimetric determination.

The number of free higher fatty acids was determined by acid number [31].

Bound lipids were isolated from the residue after removal of free lipids. The extraction was carried out with chloroform-ethanol (2 : 1) solvent system, the extract was evacuated at 40 °C and a complex of bound lipids was obtained. Carbohydrates were removed from it by settling at +4 °C for 1...1.5 h and subsequent decanting, and "crude phospholipids" were obtained. "Crude phospholipids" were dissolved in chloroform and treated with acetone in a separating funnel (HM (hydromodulus, solid:solution ratio) 20 (1 : 20), 14...16 h, t = –10 °C) to remove neutral lipids. The acetone extract was removed. The remaining phospholipids were dried at 45 °C.

Phospholipids analysis by thin layer chromatography (TLC). Phospholipids were

separated by one- and two-dimensional thin-layer chromatography on Sorbfil plates with a fixed layer of silica gel STX-1A (grain size 5...17 μm, layer thickness 110 μm, silica gel binder, PETF substrate) in the solvent system: chloroform-methanol-water (65 : 25 : 4), chloroform-acetone-methanol-acetic acid-water (50 : 20 : 10 : 10 : 5). The sample of purified phospholipids in the form of 0.1 % chloroform solution was applied with a thin capillary to the starting line of the plate in the amount of 2.6 μdm^3. After separation of the investigated mixture of substances, the chromatograms were placed in a fume hood and then in a thermostat for 5 min at 110 °C to remove solvents. Identification of fractions was carried out by standards and acquired Rf values. Quantitative analysis of phospholipids was performed by the Rosenthal method [33]. Phospholipid spots were scraped off from chromatographic plates. To the lipid sample containing 0.5...10 μg of phosphorus was added 0.4 cm^3 of 72 % chloric acid; the mixture was heated for 1 min, cooled, 4.2 cm^3 of water, 0.2 cm^3 of amidol reagent, 0.2 cm^3 of ammonium molybdate solution were added, and stirred. Then it was heated in a water bath for 7 min, cooled and after 15 min the absorbance of the stable blue solution at 830 nm was measured in a cuvette with a cuvette thickness of l = 1 cm. The number of phosphorus was determined by a calibration straight line constructed from aliquots of standard phosphate solution containing 1...4 μg of phosphorus.

Isolation of phenolic compounds complex. The complex of low molecular weight phenolic compounds was isolated from defatted seeds by extraction with 95 % ethanol in the volume ratio of solid phase – extractant 10 : 1 using a homogenizer (5000 rpm). The extract was centrifuged (10 min, 6000 g), the supernatants were combined, evaporated at 40 °C under vacuum until the solvent was completely removed. The complex of phenolic compounds was obtained [35].

Separation of low and high molecular weight phenolic compounds was carried out by gel chromatography on Sephadex LH-20. Without passing through the molecular sieve, polyphenols remained in the upper lobe of the column.

Fractionation of low molecular weight phenolic compounds was carried out by chromatography on a stationary column (91.5×1.5 cm) with Sephadex LH-20. Ethanol 95 % was used as mobile phase [35]. Portions of 8 cm^3 were collected. The absorbance of the eluates was measured at 326 nm, the chromatogram contour was determined based on the absorbance of individual fractions compared to methanol on a spectrophotometer SF-26. Further, for the identification of low molecular weight phenolic compounds, the eluates were concentrated and applied to Sephadex LH-20 TLC plates. A mixture of benzene-methanol-acetic acid (90 : 16 : 9) was used as mobile phase. Phenolic components were detected by spraying the chromatograms with an aqueous solution of ferric chloride and potassium ferrocyanide. Individual phenolic compounds were identified by their Rf values and reference substances. The amount of phenolic compounds was measured in mg/g SAE (synaptic acid equivalent), because synaptic acid and its derivatives are the predominant substances of phenolic compounds in rapeseed.

Fractionation of polyphenolic compounds (tannins) was carried out by means of precipitation reactions, allowing them to be separated into condensable and hydrolyzable. Pre-defatted rapeseed was treated with ethyl acetate to remove the bulk of low molecular weight phenolic compounds. The residue was dried by vacuum at 40 °C, and tannins were extracted from it with 95 % ethanol. By precipitation with a solution of lead acetate in acetic acid medium, hydrolyzable tannins were obtained, condensable tannins remain in solution; condensable tannins precipitate when bromide water is added. Total, hydrolyzable and condensable tannins were quantitatively determined by the Leventhal method (permanganatometry), which is based on the ability of tannins to be oxidized by potassium permanganate in a slightly acidic medium in the presence of indigo sulfonic acid indicator.

Study of lipase inhibitor properties. Determination of pH stability. The inhibitor samples of equal activity were incubated at different pH values (2.5...8.0) for 0...360 min, then the pH of the solution was adjusted to pH 7.0 and the inhibitory activity was determined. In the comparative study of the pH stability of immobilized forms of lipase inhibitors of phenolic nature, 0.1 M citrate buffer (pH 1.1...4.5); 1/15 M Na-phosphate buffer (pH 4.5...8.0); 0.05 M tetraborate buffer (pH 8.0...9.0) were used.

Determination of thermostability. The inhibitor samples of equal activity in buffer pH 7.6 were incubated at 20, 37, 55 and 65 °C for 0...360 min, then the temperature was brought to 37 °C and the inhibitor activity was determined.

Infrared spectroscopy. IR absorption spectra were recorded on a Specord 75 R

spectrophotometer in the range 4000...400 cm-1. Tablets were used for the analysis, which were prepared by pressing with excess KBr, pre-dried at 150 °C for 24 hours. The mass of the filler was 150 mg, the test sample – 1.5 mg. The mixture of the drug under study was subjected to grinding. The resulting powdered mass (100 mg) was used to prepare the tablet. Then the drug was vacuumed in a mold under a pressure of 150 kg/cm^2 [89].

Results and discussion

The analysis of literature sources and our previous studies [89] showed that rapeseed can inhibit lipase activity. In the studies, varieties with both high and low content of erucic acid, as well as erucic acid-free two-zero varieties were considered. The level of antilipolytic activity (ALA) of raw materials was evaluated by the ability to inhibit lipase activity. The results of determining the anti lipolytic activity identified the most promising objects (Table 1).

The data obtained indicate that the content of erucic acid and the value of inhibitory activity are not interdependent. This made it possible to justify the choice of the object of further research - seeds of erucic acid-free two-zero rapeseed variety, which has the most pronounced antilipolytic activity.

Table 1

Antilipolytic activity of rapeseed (n = 3; p ≥ 0.95)

The variety of rapeseed seeds	Mass fraction of erucic acid, %	ALA, U/g
Ordinary rape (spring)	47.2±0.14	35.6±0.39
Ordinary rape (winter)	45.6±0.14	35.1±0.14
Mykytynetskyi (ravine)	1.1±0.12	40.7±0.19
Galician (winter)	0.0±0	44.4±0.25

The results of determining the chemical composition of rape seeds of the variety "Galitsky" are given in Table 2.

Table 2

Chemical composition of seeds of rape variety "Halytskyi" (*n* = 3; *p* ≥ 0,95)

Component	Mass fraction, % dry weight
Lipids (free)	45.6±0.09
Protein	18.5±0.09
Ash	2.4±0.12
HP	13.1±0.94
HHP	7.5±0.14
Klason lignin	5.9±0.09

A significant proportion of rapeseed is lipids and protein. Their share is more than 64 % of the dry weight of raw materials.

The fatty acid composition of lipids of seeds of rape variety "Galytskyi", which was determined using gas-liquid chromatography, is given in Table 4.

Table 4

Fatty acid composition of lipids of rapeseed seeds of "Galytskyi" variety (*n* = 3; *p* ≥ 0,95)

Fatty acid	Mass fraction, % free lipids
Palmitic acid $C_{16:0}$	6.21±0.06
Stearic $C_{18:0}$	0.52±0.01
Oleic $C_{18:1}$	50.35±0.09
Linoleic $C_{18:2}$	26.45±0.09
Linolenic $C_{18:3}$	12.43±0.06

The composition of rapeseed oil in a significant amount includes oleic and linoleic acids. Palmito-oleic ($C_{16:1}$), behenic ($C_{22:0}$), arachidonic ($C_{20:4}$), lignoceric ($C_{24:0}$), selacholic ($C_{24:1}$), paric ($C_{18:4}$) acids are present in small amounts. Undesirable for the human body erucic acid ($C_{22:1}$) is absent, that is, the studied variety of rapeseed "Galytskyi" belongs to erucic acid-free varieties – Canola, which allows its use for food purposes.

In the context of this work, the prospect of this type of raw material is due not only to its high ALA, but also to the planned expansion of its crops [89]. Having clarified the nature of the components responsible for the inhibitory activity of rapeseed seeds, we carried out a step-by-step isolation of individual components of raw materials and determined their ALA (Table 4). Based on the information presented in the literature [27], PLI of plant origin are substances of lipid, protein or phenolic nature.

Table 4

Comparative characteristics ALA of rapeseed components and Orlistat (n = 3; p ≥ 0.95)

Substances Rapeseed seeds	Extract Agent	Mass fraction in raw materials	ALA, U/g
Rapeseed seeds			43±0.94
Free lipids, %	Diethyl ether	45.5±0.48	60±0.94
Residue after removal of free lipids, %		54.7±0.89	71±0.94
Bound lipids, %	Chloroform-ethanol (2 : 1)	4.3±0.14	65±0.94
Residue after removal of bound lipids, %		50.1±0.42	absent
Tightly bound lipids, %	Alkaline hydrolysis followed by extraction with diethyl ether	0.86±0.005	absent
Phenolic compounds, mg%	Ethanol 95 %	0.113±0.002	8872.7±6.4
Orlistat			9705.7±5.7

It was found that the substances of lipid and phenolic nature are responsible for the manifestation of ALA of raw materials: the residue of raw materials after their removal does not show inhibitory activity, which implies that the protein does not have inhibitory activity against pancreatic lipase.

In parallel, the dependence of the ALA value on the mass ratio of inhibitor-enzyme was determined (Table 5).

Table 5

Effect of inhibitor:enzyme ratio on the preservation of lipolytic activity, % (n = 3; p ≥ 0.95)

Sample	Mass ratio of sample:enzyme						
	1:1	5:1	10:1	20:1	30:1	40:1	50:1
Complex of phenolic compounds	74.8±0.14	76.1±0.14	77.1±0.82	77.1±1.0	77.5±0.68	-	-
Orlistat	81.1±0.14	82.7±0.23	83.2±0.14	83.2±0.21	83.4±0.25		

In the experiments as a standard of comparison was used "Orlistat" – a pharmaceutical drug, which is a lipase inhibitor of synthetic origin, widely used in medical practice for the treatment and prevention of obesity and related diseases.

The results of the experiment indicate that the inhibitory activity of phenolic compounds is much higher than that of lipids and is comparable to ALA "Orlistat", reaching 95.5% of its activity. This determines the potential possibility of using the phenolic complex of rape seeds as an alternative to anti-lipolytic drugs of synthetic origin.

From the data given in Table 6, it follows that for phenolic compounds and Orlistat the most rational ratio is 1:1, since further increase of the ratio practically does not affect their inhibitory activity. The maximum antilypolytic activity of the phospholipid complex was 50.6 % of that of Orlistat at a mass ratio of sample-enzyme 10:1, which is significantly inferior to the ALA of the phenolic complex.

Deepening the research in this direction, further fractionation of the components of raw materials with anti lipolytic activity was carried out.

Isolation and fractionation of phenolic complex. The study of phenolic compounds was carried out in several stages:

1. isolation of phenolic compounds complex;
2. separation into fractions and individual components;
3. evaluation of antilipolytic activity.

Phenolic compounds from rapeseed were extracted with ethanol [33]. To determine its rational concentration, which ensures the completeness of extraction along with the maximum inhibitory activity, a number of experiments were conducted, the results of which are shown in Table 6.

During ethanol extraction in the concentration range from 50 to 100 % the number of extracted phenolic compounds varies. However, their inhibitory activity increases with increasing ethanol concentration and reaches a maximum when 95 % ethanol is used as an extractant. At the same time there is a more complete extraction of the complex of phenolic compounds.

Table 6

Antilipolytic activity of phenolic compounds (n = 3; p ≥ 0.95)

Extracting agent	50 % ethanol	70 % ethanol	80 % ethanol	95 % ethanol	Absolutized ethanol (≈100 %)
Amount of phenolic compounds, mg/g ESC*	10712±6.96	10893.3±4.66	11011.67±10.86	11214.33±16.63	11137.33±32.24
Inhibitory activity, U/g	6708.33±6.96	7203.33±4.66	7563±4.11	8873.66±4.46	8863.67±3.81

* ESC - synaptic acid equivalent.

In Fig. 1 shows the scheme of fractionation of phenolic compounds.

It is developed on the basis of literature data and includes the following main steps:

- fractionation of the extracted complex of phenolic compounds on Sephadex with further separation of the obtained fractions by TLC and identification of individual low molecular weight phenolic compounds;

- isolation of polyphenolic compounds and their further separation into hydrolyzable and condensable ones.

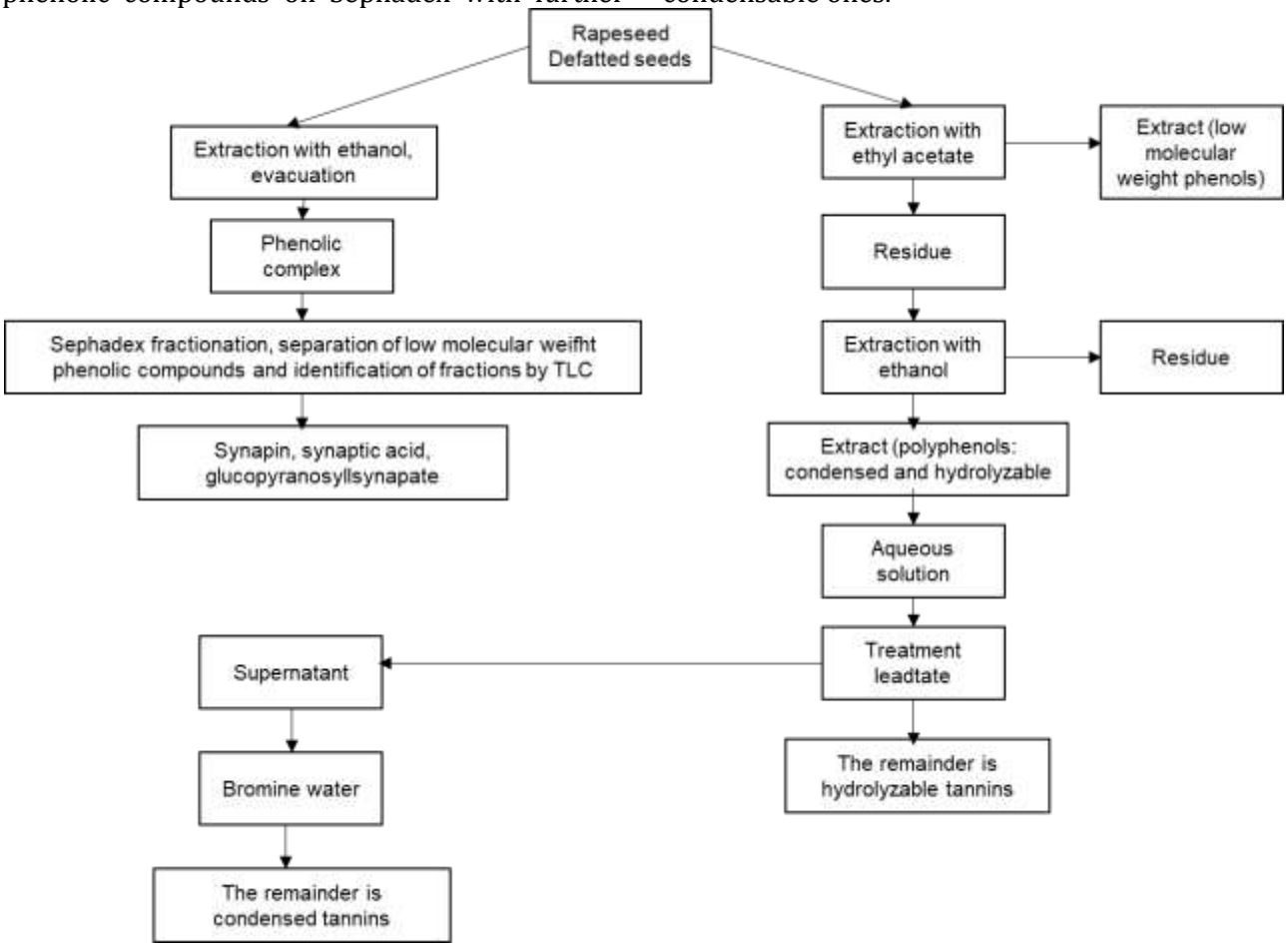

Fig. 1. Scheme of fractionation of phenolic compounds.

The predominant component of the phenolic complex is low molecular weight phenolic compounds; polyphenolic compounds are almost equally represented by tannins – condensed and hydrolyzable.

Low molecular weight phenolic compounds were fractionated by column chromatography on Sephadex LH-20 (Fig. 2). The initial elution curve of low molecular weight phenolic compounds is characterized by the presence of six peaks. Identification of individual components was carried out by TLC (Fig. 3).

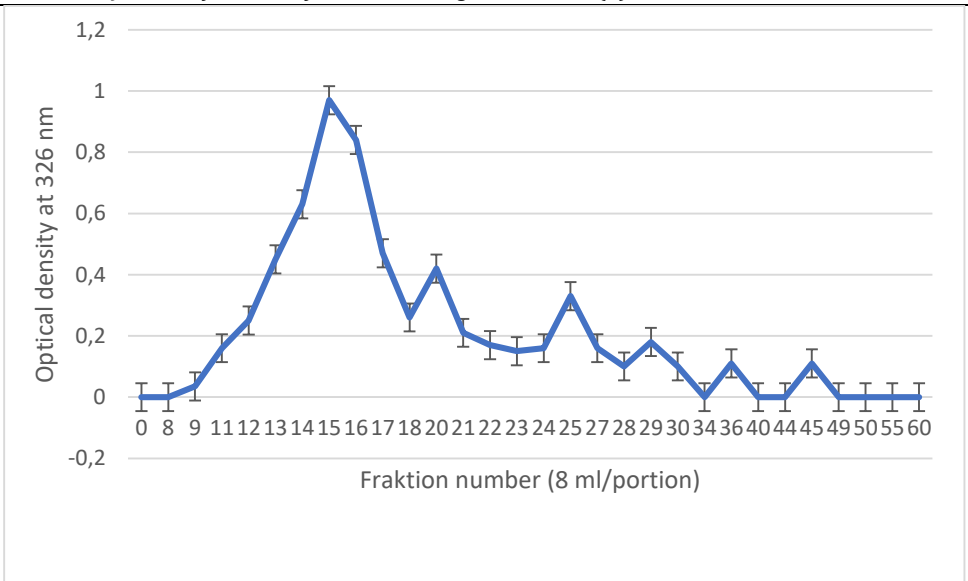

Fig. 2. Output fractionation curve of phenolic compounds on Sephadex.

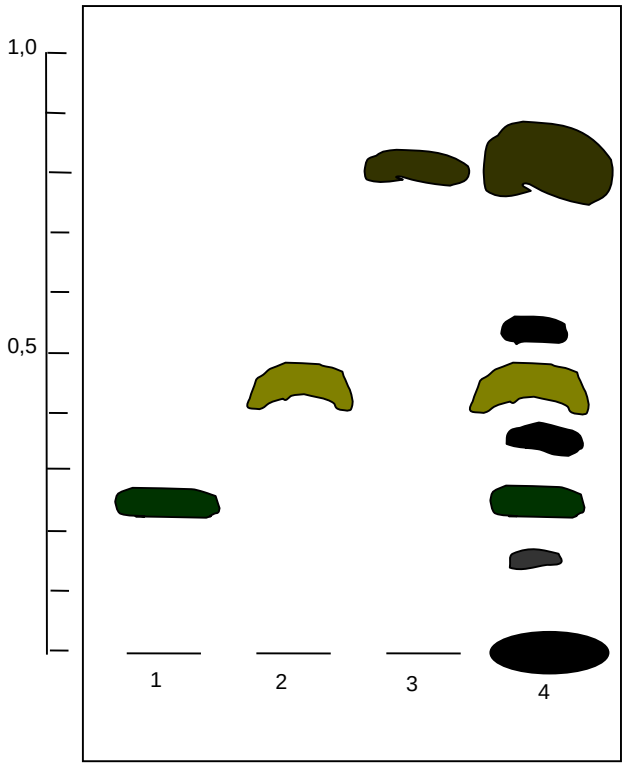

Fig. 3. Distribution of phenolic components of rapeseed on the TLC plate. 1 - glucopyranosylsinapate, 2 - sinapic acid, 3 - sinapin, 4 - extract of phenolic compounds of rapeseed.

The number of spots detected on the plates corresponds to the number of peaks of the original curve. According to TLC data, the main components of low molecular weight phenols are glucopyranosylsinapate, sinapic acid and sinapine (Fig. 4). In addition, three unidentified phenolic compounds were found, the amount of which is insignificant, and no lipase inhibitory activity was detected.

Inhibitory activity was shown by fractions corresponding to sinapine and sinapic acid, others were indifferent to lipase.

Thus, from the data shown in Fig. 4, it can be concluded that among the phenolic compounds of rapeseed seeds, sinapin and hydrolyzable tannins have the highest anti lipolytic activity against lipase. Their inhibitory activity is 18811.9 U/g and 18541.4 U/g, respectively.

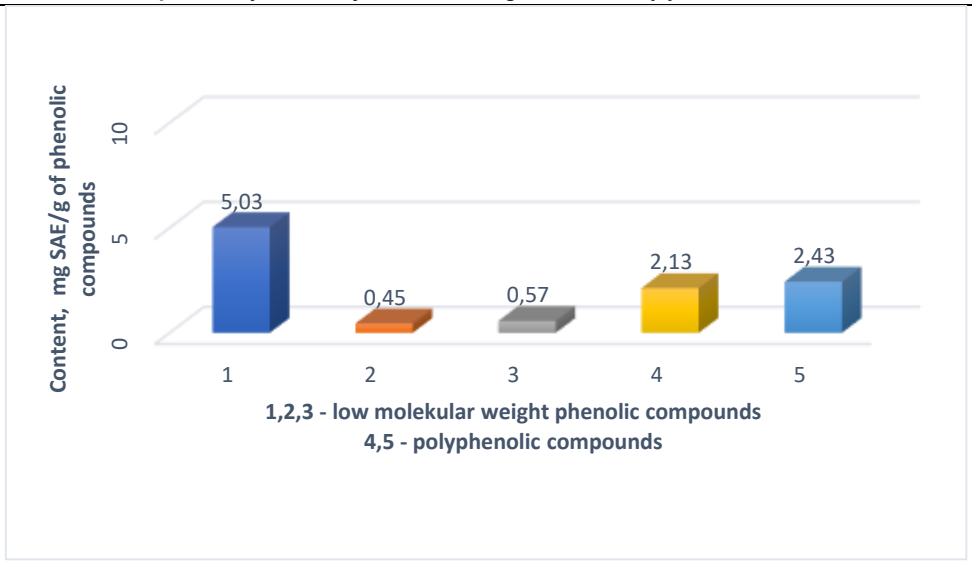

Fig. 4 a. Phenolic compounds content (n = 3; p ≥ 0.95): 1 - synapin; 2 - synaptic acid; 3 - glycopyranosylsynapate; 4 - hydrolyzable tannins; 5 - condensed tannins

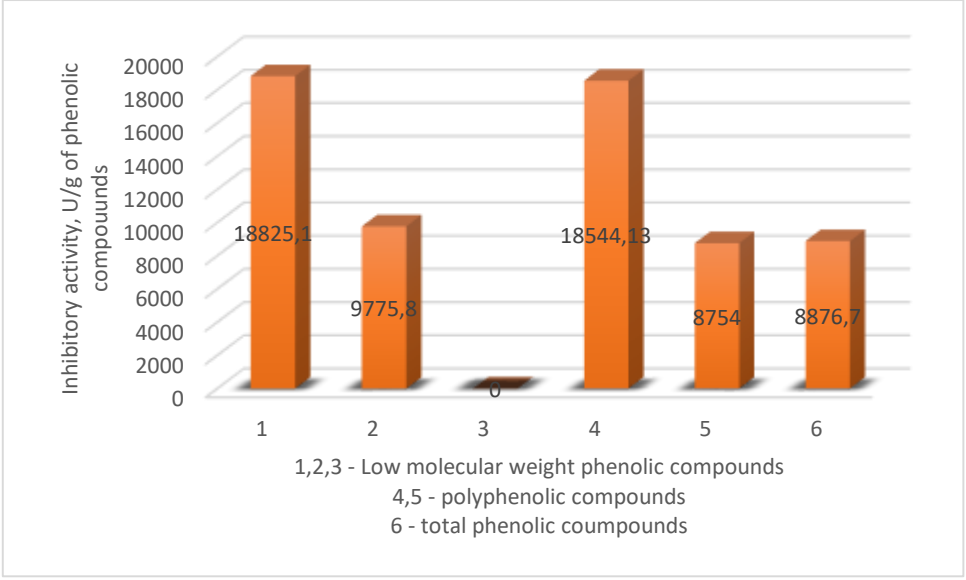

Figure 4 b. Effect of phenolic compounds of rapeseed on pancreatic lipase activity (n = 3; p ≥ 0.95): 1 - synapin; 2 - synaptic acid; 3 - glycopyranosylsynapate; 4 - hydrolyzable tannins; 5 - condensed tannins; 6 - total phenolic compounds.

That is, a significant ability to inhibit the action of pancreatic lipase is characterized by both low molecular weight and high molecular weight phenolic compounds of rapeseed. However, given that the mass fraction of sinapine in the phenolic complex is twice as high, it is obvious that its contribution to the inhibitory activity of the complex is predominant. It should be noted that the phenolic complex of rape seeds as a whole has a significant inhibitory activity against pancreatic lipase, comparable to that of the pharmacopoeial drug "Orlistat" (9700 IU/g) and its activity is much higher than that of phospholipids.

The above allows us to consider the prospects of using phenolic compounds of rapeseed seeds as effective inhibitors of pancreatic lipase - components of dietary supplements and functional foods.

Physico-chemical properties of the inhibitor. To predict the action of the inhibitor in the human gastrointestinal tract, to develop the technology of extraction and production of dietary supplements based on it, it is necessary to know what factors and how affect its activity. In this case, the most important are pH-environment and temperature.

The inhibitor orally administered into the human gastrointestinal tract is exposed to environments with different pH values from acidic (pH ~ 2.5) in the stomach to alkaline (pH ~ 8.0) in the small intestine, where the interaction of the inhibitor with lipase actually occurs. Therefore, the pH-stability of the inhibitor is an important

indicator characterizing its resistance to the pH of the environment.

pH-Stability of the investigated inhibitor of phenolic nature was studied at different pH values of the medium corresponding to physiological values and at a temperature of (37±2) °C (Fig. 5).

From the results of the study presented in Fig. 5, it follows that the inhibitor of phenolic nature retains 100 % of the initial inhibitory activity after two hours of exposure at the studied pH values.

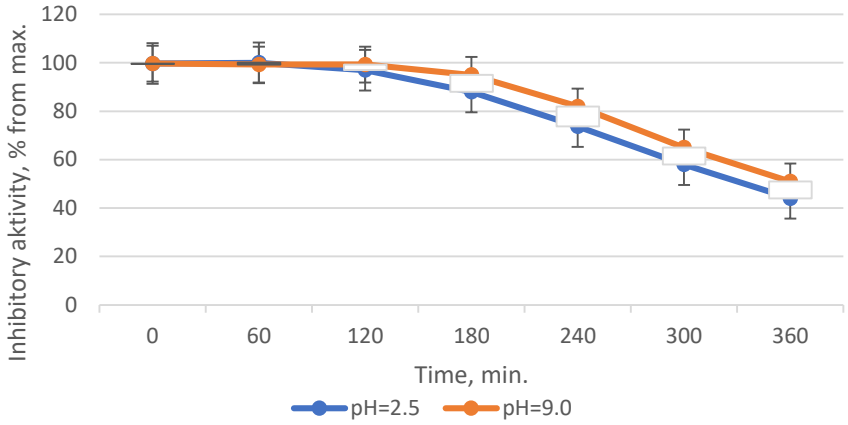

Fig. 5. pH-Stability of phenolic compounds at t = 37 °C: 1 - pH = 2.5; 2 - pH = 9.0.

The inhibitor is least stable in an acidic environment. Thus, incubation for six hours at pH 2.5 leads to a decrease in its inhibitory activity to 40 %. At pH 7 and 8 phenolic compounds retain 100 % of the initial inhibitory activity for 3 hours, after 6 hours of incubation the inhibitory activity decreases by 40 %. The most favorable for the functioning of the inhibitor is the pH value of 7.5, at which the inhibitor retains 75 % of the initial activity after 6 hours of functioning under these conditions.

Thus, at acidic pH values of the medium, the inhibitory activity of phenolic compounds in relation to pancreatic lipase decreases, which justifies the need to stabilize the inhibitor in order to prolong its action and preserve its anti lipolytic activity in digestion.

Inhibitors, like enzymes, are subject to various changes under the influence of temperature. In particular, it is known that phenolic compounds are thermolabile and can be oxidized even with short-term heating. Changes that occur with the inhibitor as a result of temperature can affect the activity of inhibitors, so it is necessary to study the thermal stability of each inhibitor, to determine the maximum temperature at which the inhibitor can be relatively long without significant inactivation. These studies are especially relevant for determining the permissible temperature intervals of technological processes of inhibitor extraction and production of dietary supplements based on it.

The effect of temperature on the anti lipolytic activity of the inhibitor of phenolic nature from rapeseed seeds at the following values was studied:

- (20±2) °C – possible storage temperature;
- (37±2) °C – temperature at which the inhibitor interacts with the enzyme in the body;
- (40±2), (55±2) and (65±2) °C – possible drying temperature of the product (Fig. 6).

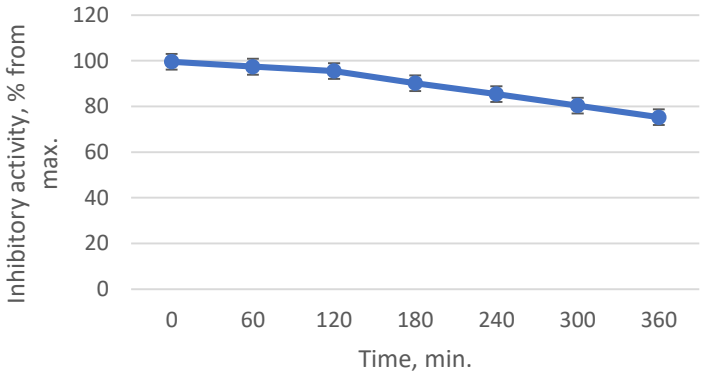

Fig. 6. Thermal stability of phenolic compounds (t = 37°C).

According to the results of studies, lipase inhibitor is most stable at a temperature of (37±2) °C – during 90 min of incubation. With increasing temperature, its anti lipolytic activity decreases markedly, which imposes restrictions on the temperature regimes used in the technology of obtaining the inhibitor and production of dietary supplements based on it. Thus, the maximum permissible temperature of technological processes is not more than 40 °C.

The results of studies of physicochemical properties of lipase inhibitor from rapeseed show that the range of its maximum activity is within the limits corresponding to the values of human body temperature. This determines the prospects of using the inhibitor as a component of dietary supplements with inhibitory effect on pancreatic lipase.

Conclusion

Phenolic complex of rape seeds of Galytskyi variety shows antilypolytic activity, which is comparable to ALA "Orlistat". This determines the potential possibility of using the phenolic complex of rapeseed as an alternative to antilypolytic drugs of synthetic origin.

Extraction of the complex of phenolic compounds of rapeseed seeds increases with increasing concentration of ethanol and reaches a maximum when using 95 % ethanol as an extractant.

According to the fractionation and identification of phenolic compounds, the main components of low molecular weight phenols are glucopyranosylsynapate, synaptic acid and synapin. Synapin and hydrolyzing tannins have the greatest anti lipolytic activity against lipase, significant ability to inhibit the action of pancreatic lipase is characterized by both low and high molecular weight phenolic compounds of rapeseed, the contribution of synapin to the inhibitory activity of the complex is predominant.

The above allows us to consider promising the use of phenolic compounds of rapeseed as effective inhibitors of pancreatic lipase - components of dietary supplements and functional foods.

The range of maximum activity of lipase inhibitor is within the limits corresponding to the values of human body temperature. This determines the prospects of using the inhibitor as a component of dietary supplements with inhibitory effect on pancreatic lipase. The above allows us to consider promising the use of phenolic compounds of rapeseed as effective inhibitors of pancreatic lipase – components of dietary supplements and functional foods.

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