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IN VIVO, IN VITRO, AND MOLECULAR DOCKING STUDY OF RAT PANCREATIC LIPASE INHIBITION USING ISOPROPYL SALICYLATE

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Pancreatic lipase (PL) represents a significant treatment target that has been the focus of research on anti-obesity medications. Orlistat is the only regularly used prescription that has been approved for long-term use. The discovery of new compounds for anti-obesity treatment based on PL inhibition can be achieved, in particular, by structure-based virtual screening with docking software. The aim of this research was to study isopropyl salicylate (IPS) anti-hyperlipidemic activity and inhibitory effect on rat pancreatic PL in comparison with orlistat. Wistar rats were divided into four groups of 8 animals each: control; fed with a high-fat diet (HFD) for 12 weeks to produce hyperlipidemia; fed with HFD and Orlistat (10 mg/kg BW daily); fed with HFD and IPS (10.81 mg/kg BW daily). It was shown that BW gain and lipase activity in the plasma of the high-fat diet rats treated with either orlistat or isopropyl salicylate were reduced considerably compared with untreated rats. The pancreatic lipase was partially purified from the plasma of obese rats, and a kinetic study of the IPS effect identified a competitive inhibition mode with an assessed K_i of 30.53 mM. An in silico study of the interaction between IPS and rat pancreatic lipase-related protein 2 (PDB ID: 1BU8) was conducted. The binding energy value ΔG for the IPS-protein complex at the enzyme's active site was found to be -5.4 kcal/mol, while that for the orlistat-protein complex was -4.4 kcal/mol, indicating the stronger interaction of the enzyme with isopropyl salicylate than with orlistat.

K e y w o r d s: obesity, pancreatic lipase, inhibition, isopropyl salicylate, orlistat, rat pancreatic lipase-related protein 2, in silico study.

Obesity, which is characterized by an excessive accumulation of adipose tissue, has been linked to several chronic diseases, making individuals and healthcare systems more burdened [1, 2]. It is a growing threat to human health worldwide. Diabetes, cardiovascular disease, and movement disorders are major comorbidities associated with obesity [3, 4]. Additionally, there is a strong correlation between the prevalence of obesity and several neoplastic diseases [5], such as renal [6], pancreatic[7], and breast[8]. The main challenge in obesity is maintaining weight loss over the long term. This can be achieved through lifestyle modifications and pharmacological approaches [9]. After eating a high-fat meal, triglycerides (TG) are hydrolyzed by an enzyme called pancreatic lipase (PL) (EC 3.1.1.3). Since dietary fats are the main source of lipids, once absorbed in the intestines, these fat molecules are converted to TG and stored in the body as a reservoir for a major energy source[10]. It is a vital enzyme that catalyzes the breakdown of TG into monoglycerides (MG) and two molecules of free fatty acids [11, 12] as shown in Fig. 1.

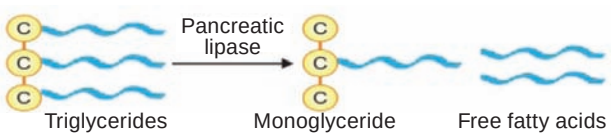

Fig. 1. Schematic illustration of pancreatic lipase action [10]

Finding pancreatic lipase inhibitors that can effectively reduce nutrient digestion and absorption is a key goal in treating obesity and related conditions [13]. One potential strategy for obesity treatment involves inhibiting pancreatic lipase activity, which decreases fat absorption [14, 15]. Orlistat, developed as an anti-obesity medication, is the only prescription drug approved for long-term use. It works by reducing dietary fat absorption through the inhibition of intestinal lipase [16]. Chauhan et al. [17] developed and tested various rhodanine-3-acetic acid derivatives as pancreatic lipase inhibitors. *In vitro,* synthetic aurone derivatives such as 6-hydroxyaurone, 6-dialkoxyaurone, 4-hydroxyacetone, and 6-alkoxyaurone suppressed PL activity. Spectrophotometric analysis demonstrated their inhibitory effects compared to orlistat [18]. Isopropyl salicylate (prop-2-yl-2-hydroxybenzoate) is the ester of isopropanol and salicylic acid. It is a salicylate that acts as an anti-inflammatory and pain-relieving compound [19]. Its lipophilicity makes it useful as a drug intermediate in synthesis or as a structural probe in biochemical research [20]. The ability of isopropyl salicylate (IPS) can incorporate into lipid-rich environments, including enzyme active sites, increases its significance in pharmacological studies [21]. Along with its favorable safety profile, IPS may support its potential as an anti-obesity treatment, either alone or in combination with other compounds [22]. Computer-aided techniques, like structure-based virtual screening with docking software, can facilitate successful drug discovery by identifying new compounds targeting specific proteins [23, 24]. Our research aimed to isolate PL from fat rat plasma and evaluate the effectiveness of IPS as an inhibitor. Additionally, molecular docking supports this by revealing potential interactions between the inhibitor and rat pancreatic lipase-related protein (ID: 1BU8), highlighting its potential as an anti-obesity drug. The selection of IPS is based on its structural similarity to the substrate, as it contains an ester bond that the enzyme being studied can hydrolyze. Also, lipophilicity may improve its interaction with the active site of lipases and help mimic triglyceride substrates.

Material and Methods

Chemicals in this study, bovine serum albumin and p-nitrophenyl butyrate, were purchased from Merck Co. Isopropyl salicylate and carboxymethyl cellulose were obtained from Sigma-Aldrich Co. The instruments used included an Ultra–cooling Centrifuge (Heraeus–Christ GmbH Co., Germany), a UV-Visible spectrophotometer (Shemadzo Co., Japan), an electronic balance (A&D Company Ltd, Japan), and a Water Bath Gallen Kamp (B.Braun Co., Germany).

Preparation of high-fat diet. The high-fat diet (HFD) was prepared using the method described by Smine [25]. The HFD consists of typical foods subsequently saturated with melted lamb fat. After heating the fat to 100°C to liquefy it, the plugs were submerged in the hot fat for 15 min. After drying at room temperature, the HFD food was given.

Animals and experimental protocol design. We obtained male Wistar rats weighing between 190 and 200 grams from the University of Mosul's College of Veterinary Medicine's International Animal Care and Use Committee. Rats were housed in a typical pet store with free access to food and water, at 23 ± 5°C and a 12-hour light/dark cycle. The experiments on animals were carried out in compliance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes and the general ethical norms of animal experimentation. Following two weeks of acclimating, the rats were randomly grouped into four equal groups, each containing six rats, and received treatment based on Body Weight (BW) as follows [26]. Group 1: fed with standard laboratory diet and drinking water ad libitum and served as a control. Group 2: fed with a high-fat diet to produce hyperlipidemia for 12 weeks. Group 3: fed with HFD and Orlistat (10 mg/kg BW daily). Group 4: fed with HFD and IPS (10.81 mg/kg BW daily).

The Orlistat and IPS were administered orally for twelve successive weeks. The orlistat dosage was chosen based on a preceding study [27]. For dose determination, preliminary experience indicated this dose (14.4 mg/kg body weight, orally) was highly effective. Experimental subjects were freshly prepared immediately before administration, and each administration was based on the most recent recorded body weight.

Measurement of body. Each rat's body weight was measured on Day 0 and every week for the duration of the experiment using a digital balance scale. The weight difference was computed using the starting and ending body weights [28].

Collection of blood. When the experiment periods concluded, all of the rats were decapitated after fasting for 12 h continuously. To prevent clotting, each sample was put in a tube containing

ethylenediaminetetraacetic acid while being stirred. Using a centrifuge that was chilled to 4°C, the blood was centrifuged at 3000 ×g for 10 min. Following that, the plasma was extracted and stored for use in upcoming studies [26].

Estimation of protein. The concentration of total protein was measured at 540 nm using the Biuret method [29]. With bovine serum albumin serving as the reference, a calibration curve was developed.

Assay of PL. Pancreatic lipase activity was estimated using the method of Lee et al. [30]. The lipase catalyzes the hydrolysis of (10 mM) p-nitrophenyl butyrate (p-NPB) as a substrate. The produced p-nitrophenol was measured at 405 nm. Total enzyme activity (U): expressed in µmol/min (U), where 1 unit (U) is defined as the amount of lipase that hydrolyzes p-nitrophenyl butyrate (p-NPB) to release 1 µmol of p-nitrophenol per minute. Specific enzyme activity (U/mg): defined as total enzyme activity per milligram of total protein, used as an indicator of enzyme purity.

Purification of PL. Dialysis. About 6.3 ml of previously obtained plasma from HFD rats (Group 2) was collected and placed in a dialysis tube. The dialyzed was done against 10 mM phosphate buffer (pH 7.2) at 4°C four times, while buffer change was performed to partially purify the enzyme [31].

Ion exchange chromatography. The dialyzed solution was placed onto a 25×2.5 cm CM-Cellulose column, and a 10 mM phosphate buffer (pH 7.2) was added. The protein was eluted using a flow rate of 1 ml per minute. The protein was identified by monitoring the absorbance at 280 nm [32]. Following the assessment of the enzyme activity of the fractions, they were collected and lyophilized in preparation for their further application in inhibitory investigations [33].

Preparation of IPS. The stock solution of IPS was prepared at a concentration of 1 M.

Inhibition of PL. It was developed to inhibit the activity of the purified enzyme by incubating it with IPS for 15 minutes at 25 °C. The activity was measured at 405 nm with p-NPB as the substrate. The Lineweaver-Burk plot was utilized to examine the inhibitory mechanisms at substrate doses ranging from 0.5 to 3 mM.

The equation, as follows, was used in calculating the percentage of PL inhibition (I%):

$$I\% = [(A_o - A)/A_o] \times 100,$$

where A_o – absorbance of the control; A – absorbance of the sample.

Molecular docking study. The AutoDock Vina 1.1.2 program for molecular docking studies was applied for molecular docking investigations. The 3D structure of rat pancreatic lipase-related protein 2 has been imported directly from the RCSB Protein Data Bank (https://www.rcsb.org) and obtained at a resolution of 1.8 angstroms. Before performing docking calculations for the chemical under study, all H_2O water molecules and the ligand were excluded. The ligand was constructed using ChemAxon Marvin Sketch 5.3.735, and its 3D structure was energy-minimized to produce a single, low-energy conformation, which was stored in mol2 format [34, 35]. Using Gaussian 09, the ligand structures were optimized and their energy was minimized. The protein and ligand were prepared using Auto Dock Tools (ADT) 1.5.6. Rat pancreatic lipase-related protein 2 was used to investigate the binding affinities of isopropyl salicylate and orlistat through simulating their molecular interactions with the target enzyme, rather than directly calculating binding energies.

For each ligand, a flexible docking mode was automatically generated and applied to carry out the docking simulation. To investigate the interactions between the ligand and the targeted proteins, Discovery Studio Visualizer (BIOVIA, Discovery Studio, v4.0.100.13345) was utilized. To investigate the interactions between the ligand and the targeted proteins, Discovery Studio Visualizer (BIOVIA, Discovery Studio, v4.0.100.13345) was utilized [36].

Statistical analysis. All the values were expressed as mean ± SEM (standard error of the mean). Duncan's test compared the means using the SAS program (Proc. GLM, SAS program, version 9.3). The differences at $P \leq 0.05$ were considered statistically significant.

Result and Discussion

Effect of Orlistat and IPS on body weight. We were able to create representative curves of the development of BW by regularly monitoring the evolution of BW in each of the four groups of rats over 12 weeks. According to our findings, the animals' BW significantly increases from the first to the last day of the HFD diet (Fig. 2). Indeed, BW improved in control rats from 195.54 ± 4.37 to 308.86 ± 3.94 g and in HFD diet-receiving rats from 207.12 ± 2.36 to 411.70 ± 5.33 g.

When compared to the control group, the BW gain was considerably reduced by treatment with

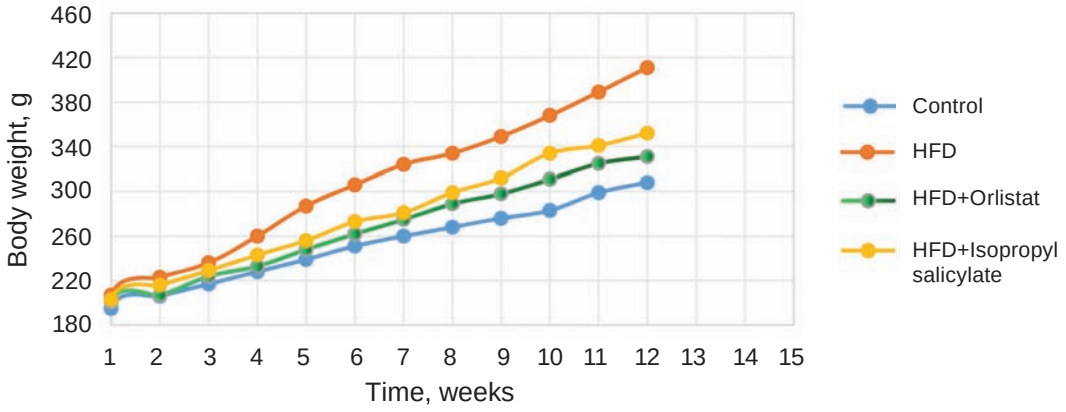

Fig. 2. Effects of Orlistat and isopropyl salicylate on body weights

Table 1. Effect of IPS doses on PL activity in rats

Dose of IPS, mg/kg BW	Control	3.60	7.20	10.81	14.41	18.02
Lipase activity, U/ml	0.371 ± 0.064^{a}	0.315 ± 0.049^{b}	0.292 ± 0.024^{bc}	0.253 ± 0.040^{d}	0.262 ± 0.056^{d}	0.270 ± 0.058^{cd}

Note. Values are given as means ± standard error (n = 5). Superscript letters indicate significant differences between groups horizontally. Values that do not share the same letter are significantly different ($P \leq 0.05$)

orlistat and IPS. BW changed from 201 ± 2.91 g to 331.30 ± 9.62 g and from 203 ± 1.75 to 352 ± 8.24, respectively. This indicates that whereas regular ingestion of HFD causes obesity and its associated difficulties, normal food consumption has no detrimental consequences on animal health. The BW is correlated with the food's composition rather than how much is eaten each day.

Effective dose determination in healthy rats. Table 1 illustrates the dose-dependent effect of IPS on PL activity in healthy rats. It turned out that all the doses used led to a significant decrease in the enzyme activity. The most potent of the tested doses was 10.81 mg/kg body weight, which lowered lipase activity significantly to 0.253 ± 0.04^{d} U/ml, while that of the control was 0.371 ± 0.064^{a} U/ml. This is an expression of statistically significant PL activity inhibition.

High-fat diet treatment increased significantly the activity of lipase by 0.491 ± 0.083^{a} U/ml in comparison to the control group, 0.337 ± 0.079^{c} U/ml. Conversely, the activity of lipase decreased significantly to 0.391 ± 0.063^{b} and 0.414 ± 0.034^{b} after treatment with orlistat and IPS, respectively, compared with the high-fat diet rats group (Table 2). Both treatments had a close effect and there was no significant difference between them, but Orlistat showed a slightly stronger impact than IPS.

Purification of PL. Table 3 presents data indicating that the specific activity increased to 3.24 U/mg protein following the dialysis process, in comparison to the crude enzyme with a purification fold of 3.02. Following the dialyzed enzyme run through a CM-cellulose column, a single isoenzyme (Fig. 3) with a purification fold of 30.09 and a specific activity value of 32.20 U/mg protein was produced. Most animal tissues contain lipases. It was isolated from rat liver by Claycomb and Kilsheimer [37]. Gastric lipase and PL are the two primary enzymes in mammals that break down neutral fats [38]. Also, PL has been purified and characterized according to its chemical stability and activity at pH and temperature [39]. Using cation exchange resin, a single peak of lipase was extracted from adult pancreatic juice [40].

The effect of the IPS compound on the lipase activity. Table 4 shows the inhibitory effect of purified PL activity by using different concentrations of IPS. It was observed that the increase in the inhibi-

Table 2. Effects of IPS optimal dose (10.81 mg/kg BW) on PL activity in rats fed HDF

Rat groups	Control	HFD	HFD+ Orlistat	HFD+ IPS
Lipase activity, U/ml	0.337 ± 0.079^{c}	0.491 ± 0.083^{a}	0.391 ± 0.063^{b}	0.414 ± 0.034^{b}

Note. Values are given as means ± standard error ($n = 5$). Superscript letters indicate significant differences between groups horizontally. Values that do not share the same letter are significantly different ($P \leq 0.05$)

Table 3. Purification steps of PL from plasma of obese rats

Purification steps	Total volume, ml	Total protein, mg	Total activity, U*	Specific activity (U/ mg protein) $\times 10^{-3}$	Yield, %	Purification fold
Crude	6.3	364.1	0.390	1.07	100	–
Dialysis	6.9	220.8	0.717	3.24	183.84	3.02
Ion exchange	23.2	28.07	0.904	32.20	231.79	30.09

Note. U* – unit refers to an amount of lipase that hydrolyzes p-nitrophenyl butyrate to release one micromole of p-nitrophenol per minute

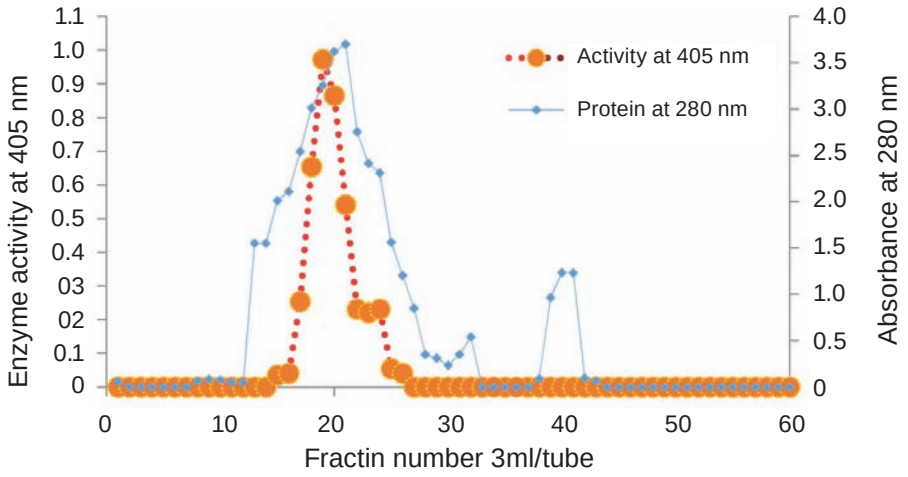

Fig. 3. Partial purification of rat PL by CM-cellulose resin

Table 4. Effect of IPS on the activity of lipase

Inhibitor, mM	Activity at 405 nm	Inhibitory effect, %
Control	0.76	0.00
20	0.58	23.69
40	0.50	34.03
60	0.41	46.20
80	0.23	69.50
100	0.20	73.29

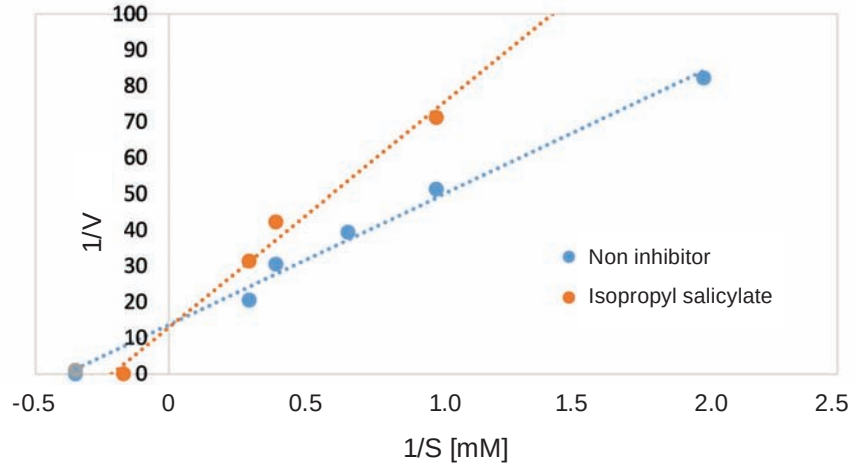

Fig. 4. Inhibition mode of purified PL by isopropyl salicylate

tory effect was directly proportional to the concentration of the inhibitor and was 73.29 at 100 mM.

The value of IC_{50} was calculated by the equation: IC_{50} = 50-*b*/*a* by using the equation of the straight line, where *a* and *b* represent the slope and intercept values, respectively, and was found to be 62 mM.

Mode of inhibition. The Lineweaver-Burk plot demonstrates that enzyme activity was inhibited by 62 mM of IPS as an inhibitor. The results indicated that the inhibition mode was competitive (Fig. 4). The V_{max} value was calculated to be stable at 0.08 U/ml, while the K_m value increased from 2.85 to 5.88 mM. The inhibition constant K_i was calculated to be 30.53 mM.

A compound IPS is an ester compound. In this way, it is similar to the substrate para-nitrophenyl butyrate structure. This rationale explains why this compound is inhibited competitively, and this agrees with the inhibition of purified pecan kernel lipase activity by 1,4-diacetoxybenzene [11]. Also, competitive inhibition of PL was noted by saponin, platycodin with a K_i of 0.18 mM [41] and carnosic acid with a K_i of 5.4 µg/ml [42]. On the other hand, a polyphenol called licochalcone A demonstrated inhibition of PL at 35 µg/ml reversibly and non-competitively, exhibiting a K_i value of 11.2 µg/ml [43]. At concentrations ranging from 1.25 to 100 µg/ml, cyanidin, epicatechin, petunidin, and peonidin all decreased PL activity *in vitro*. All of these drugs were found to have an uncompetitive inhibitory mechanism [44].

Molecular docking analysis. Molecular docking studies identified the binding mechanisms of IPS with rat pancreatic lipase-related protein 2. Fig. 5 (*A-C*) elucidates the formation of the IPS ligand-protein complex in active site of rat pancreatic lipase-related protein (ID: 1BU8), with a $\Delta G_{binding\ energy}$ of -5.4 kcal/mol. The produced complex became stable by five hydrogen bonds (green dotted lines) via amino acids, SER A:53,53, ALA A:54, and THR A:55, 55. Different colored dotted lines show diverse types of bonding interactions (like pi-alkyl with PHE A:38,38, Van der Waals, and Donor-Donor).

The Fig. 6 (*A-C*) shows the formation of compound orlistat ligand-protein complex in the active site of pancreatic lipase enzyme (ID: 1BU8) with $\Delta G_{binding\ energy}$ of -4.4 kcal/mol. The produced complex was become stable by five hydrogen bonds (green dotted lines) via four amino acids, ARGA:39, 39, TYR A:49, HIS A:75, and ASP A:83. Along altered kinds of bonding interactions (like pi-alkyl with LEUA:86, 39, 87, CYS A:103, TYR A:41, and Van der Waals) are shown by different color dotted lines.

Docking experiments using AutoDock Vina software showed that quercetin binds to the active site of pancreatic lipase. The resulting complexes showed good overlap of the flavonoid positions within the enzyme's active site. The phenyl rings of the flavonoids established stacking interactions with the hydrophobic aromatic active site residues (Phe 78, Pro 181, Tyr 115, and Phe 216). However, myricetin was found to bind near the catalytic serine (Ser 153) through hydrogen bonds [45, 46]. The most powerful binding energy against the PL protein was demonstrated by epicatechin, peonidin, and cyanidin in a prior molecular docking study. The protein target comprising residues Glu 102, Ser 129, Ile 97, and Trp 270 hotspots for protein binding showed the greatest

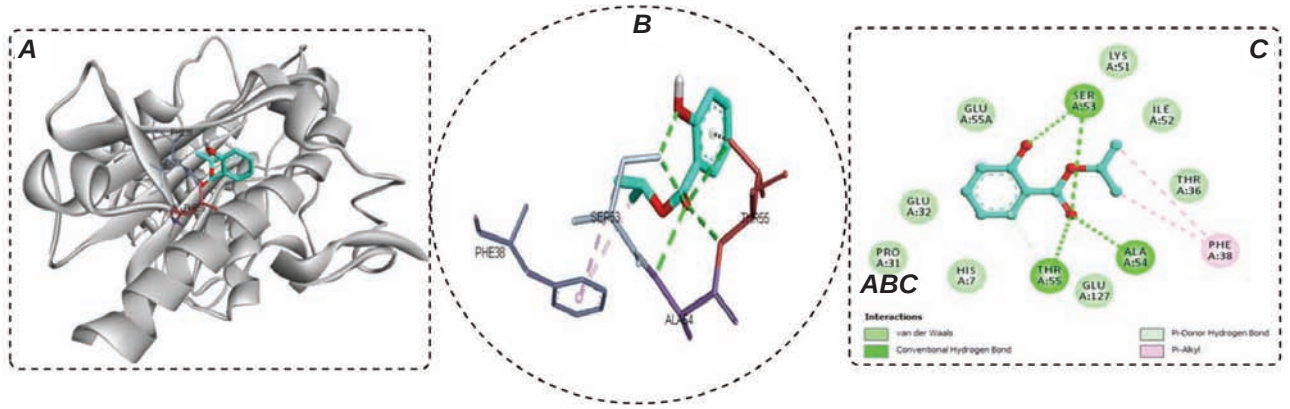

Fig. 5. ***A*** *– Illustrates the interactions between the pancreatic lipase enzyme (ID: 1BU8) in a 3D ribbon form and Isopropyl salicylate, shown in a stick model with amino acids of the enzyme (ID: 1BU8).* ***B*** *– Depicts specific amino acid residue interactions with Isopropyl salicylate in a 3D model.* ***C*** *– Shows 2D interactions of Isopropyl salicylate with specific amino acids*

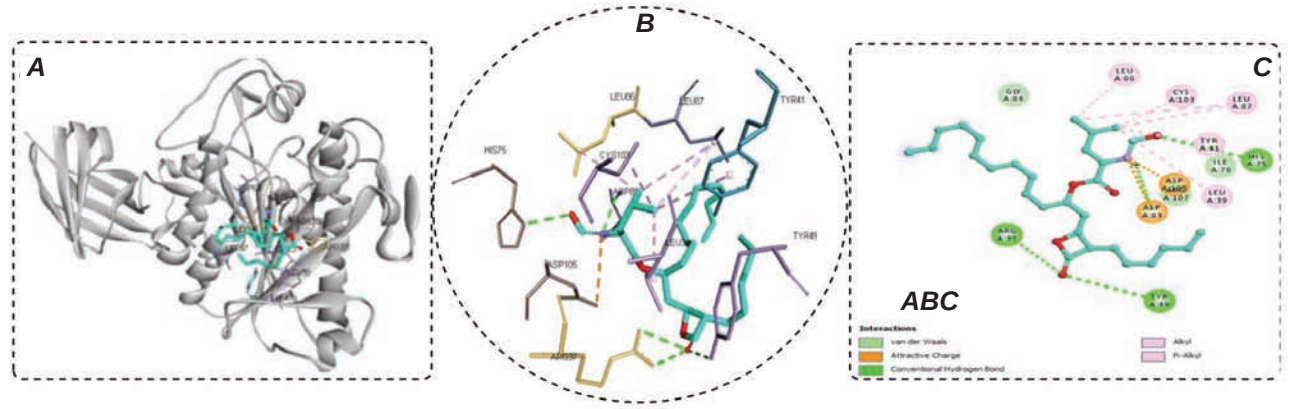

Fig. 6. ***A*** *– Illustrates the interactions between the pancreatic lipase enzyme (ID: 1BU8) in a 3D ribbon form and orlistat, shown in a stick model with amino acids of the enzyme (ID: 1BU8).* ***B*** *– Depicts specific amino acid residue interactions with orlistat in a 3D model.* ***C*** *– Shows 2D interactions of orlistat with specific amino acids*

binding [44]. Orlistat has previously been shown to have reversible covalent inhibition on an active site of Ser 152, and its long hydrophobic chains bind to residues in the lid domain, particularly Gly 76 to Phe 80 and Leu 213 to Met 217 of PL [47]. Notably, Ser152 is a crucial amino acid in the catalytic triad (Ser 152- Asp 176- His 263) on an enzyme, while Gly 76, Phe 77, Ile 78, Asp 79, and Phe 215 are residues found in a lid domain [48].

Conclusion. Obesity is a major issue of public health worldwide that is alarmingly growing worse each passing day, and decreasing calorie intake through intestinal absorption-related techniques. As a result, the PL breaks down dietary lipids that are ingested during meals. The structure of lipase's natural substrate serves as the basis for the usual tactic employed in PL inhibitors. *In vivo,* the compound isopropyl salicylate exhibited good anti-hyperlipidemic activity and an inhibitory effect on PL activity when compared to orlistat. PL was purified and then *in vitro* inhibited by IPS. The kinetics data display a competitive inhibition with a K_i of 30.53 mM. As a supportive study and to detect the interactions between this inhibitor and rat pancreatic lipase-related protein 2 (PDB ID: 1BU8). *In silico* study was conducted. The IPS ligand-protein complex at the enzyme's active site had a binding energy value ΔG of -5.4 kcal/mol. However, the $\Delta G_{binding\ energy}$ value of

the orlistat-protein complex was -4.4 kcal/mol. This explains the stronger binding and interaction of isopropyl salicylate with the enzyme than with Orlistat.

Conflct of interest. Authors have completed the Unified Conflicts of Interest form at http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

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IN VIVO, IN VITRO ТА МОЛЕКУЛЯРНИЙ ДОКІНГ ДОСЛІДЖЕННЯ ІНГІБУВАННЯ ПАНКРЕАТИЧНОЇ ЛІПАЗИ ЩУРІВ ІЗОПРОПІЛСАЛІЦИЛАТОМ

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Панкреатична ліпаза (ПЛ) є важливою мішенню для лікування і знаходиться в центрі уваги досліджень з розробки препаратів проти ожиріння. Орлістат – єдиний рецептурний препарат, який регулярно використовується і схвалений для тривалого застосування. Пошук нових сполук для лікування ожиріння на основі інгібування ПЛ може бути досягнуто, зокрема, за допомогою структурного докінг-скринінгу. Метою даного дослідження було вивчення антигіперліпідемічної активності ізопропілсаліцилату (ІПС) і його інгібуючої дії на панкреатичну ліпазу щурів у порівнянні з орлістатом. Щурів лінії Вістар було розподілено на чотири групи по 8 тварин у кожній: контрольна група; група, яка отримувала дієту з високим вмістом жирів (ЖД) протягом 12 тижнів для формування гіперліпідемії; група, яка отримувала ЖД і орлістат (10 мг/кг маси тіла на день); група, яка отримувала ЖД і ІПС (10,81 мг/кг маси тіла на день). Було показано, що приріст маси тіла і активність ліпази в плазмі крові щурів, які отримували ЖД і орлістат або ізопропілсаліцилат, значно знижувалися в порівнянні з щурами, які не отримували лікування. Панкреатична ліпаза була частково очищена з плазми крові щурів з ожирінням, і кінетичне дослідження ефекту ІПС виявило конкурентний характер інгібування зі значенням K_i 30,53 мМ. Було проведено *in silico* дослідження взаємодії між ІПС і протеїном 2 панкреатичної ліпази щурів (PDB ID: 1BU8). Значення енергії зв'язування ΔG для комплексу ІПС-протеїн в активному центрі ензиму становило -5,4 ккал/моль, в той час як для комплексу орлістат-протеїн ΔG становило -4,4 ккал/моль, що вказує на більш сильну взаємодію ензиму з ізопропілсаліцилатом, ніж з орлістатом.

Ключові слова: ожиріння, панкреатична ліпаза, інгібування, ізопропілсаліцилат, орлістат, протеїн 2 панкреатичної ліпазои щурів, *in silico* дослідження.

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