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IN VITRO: INHIBITION OF PARTIALLY PURIFIED PANCREATIC OVINE LIPASE BY WILLOW BARK EXTRACTS

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Abstract

Obesity has existed for a very long time. Obesity leads to improper physiological metabolism, which in turn produces a host of physiological and social issues in addition to aesthetic concerns. Individuals are working to discover anti-obesity medications and other safe and efficient treatment options. Because pancreatic lipase (PL) is essential to human fat metabolism, PL inhibitors are now used to treat obesity in clinical settings. This research involved partially purifying lipase from the ovine pancreas. One lipase isoenzyme was identified using the CM-Cellulose ion exchange chromatography technology. It had a specific activity of 2.467 U/mg protein and a recovery of 272 in comparison to the crude extract. The molecular weight was determined to be 33.8 kilodaltons, and the electrophoresis method produced only one band. Using p-nitrophenyl acetate as a substrate, the capacity of willow bark extracts to inhibit partly purified pancreatic lipase activity was investigated.

The inhibitory percentages via treating the enzyme with EthOH 70% and H_2O were found to be 27.14 and 61.43 respectively. Furthermore, the Lineweaver-Burk plots revealed the inhibition modes noncompetitive with both extracts.

KEYWORDS: Ovine Pancreatic Lipase, Willow Bark, Extracts, Purification, Inhibition.

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1. Introduction:

Obesity has become a global issue due to the sharp rise in the number of fat people. The number of chronic metabolic disorders that are readily brought on by fat has increased, drawing the attention of researchers [1]. Several chronic disorders, including diabetes[2,3], hypertension, hyperlipemia [4], and cardiovascular disease [5], are intimately linked to obesity, which is also a significant risk factor for these conditions [6-8]. An important digestive enzyme called triacylglycerol acyl hydrolase, also known as pancreatic lipase (PL) (EC 3.1.1.3), catalyzes the breakdown of triacylglycerol, a naturally occurring oil molecule, into monoglycerides and free fatty acids [9]. According to Huang et al. (2020) [10], this enzyme is actually in charge of the body's absorption of lipids, which leads to obesity as a chronic

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metabolic disorder. Pancreatic lipase is a vital enzyme in lipid absorption via entire dietary fat hydrolysis [11,12]. Pancreatic lipase inhibitors, such as orlistat (Xenical) [13], have been accepted as treatments for obesity syndrome in the United States.

In recent years, researchers have focused a great deal of attention on PL inhibitors. Natural product PL inhibitors have drawn particular attention because of their structural diversity, low toxicity, and ability to be sourced from a wide variety of sources without causing side effects. These benefits may pave the way for the development of a new class of diet pills or health care products. Polyphenols, flavonoids, saponins, terpenoids, alkaloids, and other active compounds are found in common natural sources of lipase inhibitors [1].

Numerous investigations were carried out to assess the extracts' inhibitory effect on PL. For example, Rosmarinus officinalis and Mentha spicata extracts showed inhibitory action with IC 50 values of 7.0 and 7.8 mg/mL, respectively, according to Conforti et al. [14]. With 74.7% and 53.8%, respectively, the methanolic extracts of Prunella vulgaris L. and Rheum palmatum L. had the most inhibitory efficacy against PL [15]. Additionally, PL is inhibited by around 61.2% in dichloromethane extract of Lagerstroemia indica fruits [16]. The results collected to examine the potential of acetone extract for various Alpinia zerumbet components revealed that seed extract had the strongest inhibitory impact on PL activity, with an IC50 of 5 μg/mL [17]. In vitro, Eleusine indica methanolic extracts had the most inhibitory impact on pancreatic lipase, at 31.36%. Additionally, Phyla nodiflora accounts for 18%, Melastoma candidum for 20%, Myristica fragrans for 18–20%, and Dicranopteris linearis for 14% [18]. The ethanolic extracts of Vitis vinifera L. [19], Arachis hypogaea L. [20], Punica granatum L. [21], and Mangifera indica L. [22] were used to suppress the activities of pancreatic lipase and lipoprotein lipase.

With a widespread worldwide distribution and species native to every continent save Antarctica, the genus Salix (willow) has long been a very advantageous temperate plant for humans [23]. The Willow bark (*Salix* spp.) from the *Salicaceae* family is utilized in phytopharmaceutical products for the treatment of pain, headaches, fever, and inflammatory processes [24,25]. The salix bark extracts are high in phenolic glycoside concentration [26]. Salicylates are the major group of phenolic glycosides, as well as salicin, which has been previously described as an active constituent of bark extract [27 - 29]. In this research, we aimed to purify the ovine pancreatic lipase, before treating it *in vitro* with willow bark extracts as natural inhibitors[30].

Experimental:

2.1 Plant collection:

A willow tree's bark (*salix alba*, Collected May 2022) was obtained from Al-Qasr village in Nineveh Governorate in Iraq. It has been dried in the dark before being peeled and chopped via a blender for use in extracting methods.

2.2 Tissue used (pancreas):

Pancreatic tissue was freshly obtained from the Nineveh Slaughterhouse in (Mosul, Iraq), in November 2022. The pancreas of ovine was collected and it was free from diseases, pregnancy, or childbearing and under the supervision of specialized veterinarians. The tissues were wrapped with aluminum foil and placed at a temperature of -20 °C until they were used in subsequent experiments.

2.3 Preparation of Pancreatic crude extract:

Weighed 17 g after removing fatty pieces, washed, and cut. Phosphate buffer 10 mM, pH 7.2 (containing 150 mM sodium chloride, 1.3 mM calcium chloride, and 2.5% DMSO) in a ratio of (1:2) w/v was added. The pancreas was homogenized in mortar surrounded by an ice bath

for 15 minutes [31]. Then frozen by liquid nitrogen and centrifuged at 2871.36 xg g for 10 min, the supernatant was collected and stored to purify.

2.4 Determination of ovine pancreas protein:

Total protein conc. was estimated at 540 nm by the Biuret method [32] using a calibration curve with bovine serum albumin as a standard.

2.5 Lipase Assay:

The technique of [33] was used to estimate lipase activity. The hydrolysis of (1.5 mM) p-nitrophenyl acetate (p-NPA), a substrate for p-nitrophenol, is catalyzed by the lipase and detected at 410 nm.

Enzyme activity (µmol/min/ml) = 10 x [p-nitrophenol] /139.11 x time (Equation 1)

2.6 Purification of Lipase:

2.6.1 Precipitation by Ammonium Sulfate:

The crude extract was progressively supplemented with 70% ammonium sulfate, and stirred slowly by a magnetic stirrer surrounded by an ice bath. Leave the solution overnight in the refrigerator. The solution was centrifuged for 10 minutes at 2817 xg. The supernatant was poured and the pellet obtained was dissolved in a small volume of buffer [34].

2.6.2. Dialysis:

Dialysis was carried out by putting the resulting enzyme produced from the previous step in a dialysis tube and stirring against phosphate buffer (10mM, pH 7.2) at 4˚C with four-time buffer changes.

2.6.3 Ion Exchange Chromatography:

The dialyzed solution was loaded over a column containing CM-cellulose, followed by a 10 mM phosphate buffer (pH 7.2). The protein was eluted at a flow rate of 1ml / min. The protein was identified by following the absorbance at 280 nm. Lipase activity was measured in the fractions, then collected and lyophilized [35].

2.6.4 Determination of Molecular Weight:

Using the method of [36], sodium dodecyl sulfate-polyacrylamide gel electrophoresis was accomplished on a 4% polyacrylamide stacking gel and an 18% polyacrylamide-resolving gel. The Coomassie Blue staining method was used to see the protein bands. The reference proteins have a mass between 14 and 220 kDa.

2.7 Preparation of willow bark extracts:

Twenty grams of willow bark powder was weighed and placed in a beaker, then 200 ml of water, hexane, ethanol 70%, and ethanol absolute were added separately. It was continuously stirred for three hours using a magnetic stirrer at laboratory temperature and left for 24 hours. After that, a centrifugation at 1500 xg for 10 minutes filtration process was performed, where the precipitate was isolated and the filtrate was taken and evaporated using a rotary evaporator device under the influence of vacuum pressure to concentrate the extracts [37].

2.7.1 Lipase inhibition:

Purified pancreatic lipase activity was inhibited by incubating it with each extract separately for five minutes at 25 °C. At 410 nm, an activity was measured with p-NPA as the substrate. Using different substrate concentrations ranging from 1 to 5 mM, the inhibitory ways were illustrated.

3 Result and Discussion:

3.1 Lipase purification from ovine pancreas

The specific activity of lipase following sedimentation was 0.071 units/mg protein, indicating a 3.737-fold increase, based on the data

shown in Table 1. Recovery of an enzyme was 106.05 when compared to a crude extract. Following that, a dialysis process was applied to remove the ammonium sulfate residue, amino acids, peptides, and ions from the previous step. Lipase sp. activity was 0.01 U/mg protein, which has been multiplied by 5.263 when compared with the crude extract. Dialyzied enzyme passed through the CM-cellulose column[38], and we obtained a single lipase isoenzyme (Figure 1) with specific activity values of 2.467 U/mg protein with recovery 272 compared to the crude extract.

Several studies indicated the purification of this enzyme from animal, plant, and microorganism sources. Lipase was isolated from porcine pancreas and found to have a single protein chain containing 449 amino acids with a molecular weight of approximately 50 kDa, and its molecular weight rises to 52 kDa due to the associated glycosides [39]. Also, the gastric lipase was purified from goats, sheep, and calves. These enzymes prefer to catalyze the decomposition of short fatty acids in milk fat [40]. Gastric lipase was likewise isolated from the stomach of other mammals, such as humans and dogs, and it was found that the human gastric lipase is a glycoprotein with a molecular weight of 50 kDa [41]. In plants, lipase was partially purified from pecan nut kernel [42], and sunflower seeds [43]. On the other hand, one peak of lipase was observed after isolating from *Pseudomonas Heineken* bacteria [44].

Table 1: Purification steps of lipase from Ovine pancreas

Purification steps	Total volume (ml)	Total protein (mg)	Total activity (U)	Sp. Activity (U/mg protein)	Purification Fold	Recovery (%)
Crude extract	31	117.8	2.294	0.19	-	100%
Ammonium sulfate (70%)	15.3	34.272	2.433	0.071	3.737	106.05
Dialysis	14.16	18.98	1.913	0.10	5.263	83.39
Ion exchange CM-Cellulose	48	2.592	6.24	2.467	129.84	272.01

U: unit refers to the amount of lipase enzyme that releases one micromole of p-nitrophenol per minute.

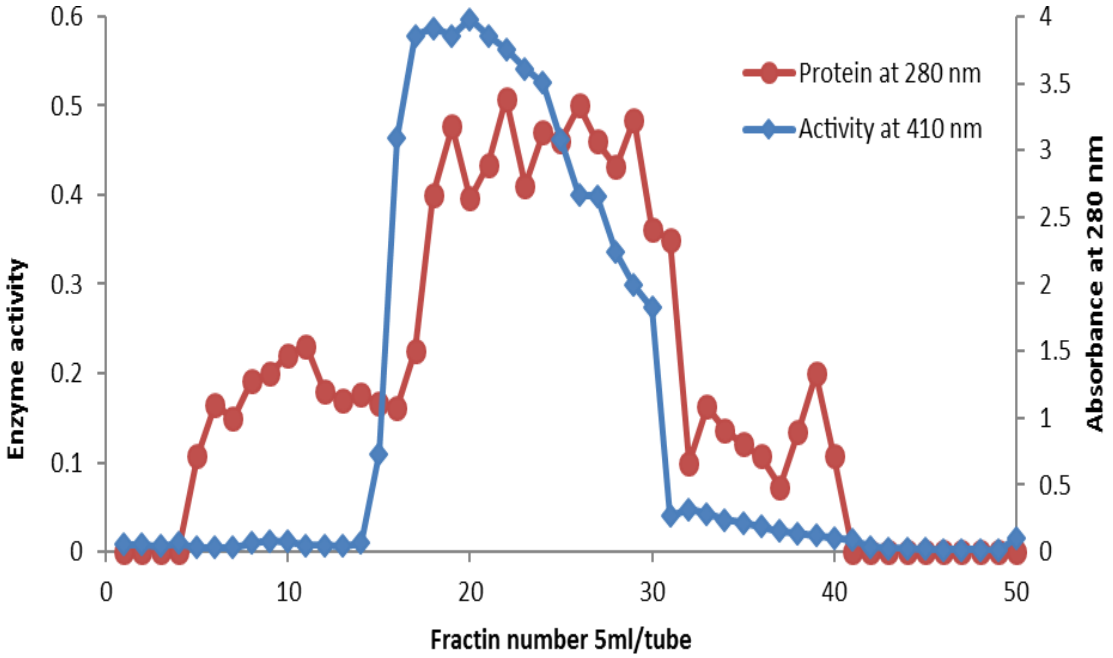

Figure 1: Partial purification of lipase by CM-Cellulose chromatography

3.2 Molecular weight

The findings of the SDS-PAGE electrophoresis method showed that the purified lipase from the ovine pancreas only showed one peak as a single, distinct band. According to Figure 2, the molecular weight was calculated to be 33.8 kilodaltons. Palm lipase has a molecular weight of 35 kilodaltons, which is comparable to the molecular weight mentioned above [45]. In contrast, it was smaller than the pure enzyme from the pistachio khinjuke kernel (43.5 KD) [46] and pecan kernel (42 KD) [38]. Following ion exchange chromatography, the isolated enzyme from *Pseudomonas reinekei* was found to be 50 kilodaltons [44].

3.3 Effect of Willow Bark Extracts

The effect of willow bark extracts using water, hexane, 70% ethanol, and absolute ethanol on the activity of the lipase was studied. Table (2) showed that the highest percentage of inhibition among the extracts was the aqueous extract, as it reached 61.43%, while the lowest percentage of inhibition was for absolute ethanol and hexane extracts, as their effect was very weak and amounted to 3.57 and 0.714%, respectively. Therefore, they were excluded from subsequent experiments.

3.4 Effect of 70% ethanol willow bark extract on lipase activity

Lipase activity was investigated using 70% ethanol of willow bark extracts, by using different volumes of extract. Table 3 revealed the inhibitory effect on the activity of lipase. The highest inhibition percentage was discovered at a concentration of 2.5 mg / 50 µl at 62.03%, but the lowest inhibition percent was 9% beginning at a concentration of 3 mg / 60 µl.

Using orlistat as a synthetic positive control, Sahib et al. [47] assessed the ethanolic extract of *Centella Asiatica*, *Morinda citrifolia*, and *Momordica character's* in vitro anti-pancreatic lipase activity at different doses. The ethanolic extract of *Cudrania tricuspidata* leaves was used to examine the PL inhibitory action in vivo. Although these effects were not as strong as those of orlistat, the acquired data demonstrated significantly delayed lipid absorption and lowered plasma triacylglycerol levels [48].

Moringa stenopetala leaf ethanolic extract was utilized by Toma et al. [49] to suppress PL with an IC 50 value greater than 5 mg/mL. In comparison to orlistat, the ethanolic extracts of Cornus officinalis fruit and Salicis Radicis bark demonstrated a considerable inhibition of porcine pancreatic lipase, with 34.8% and 31.4%, respectively [50]. With an inhibition percentage of 46.15%, the green pepper ethanolic extract showed the most potent inhibitory impact on pancreatic lipase [51]. In contrast, human pancreatic lipase was inhibited in vitro by the aqueous ethanol extract of Bergenia crassifolia rhizomes, with an IC value of 3.4 μg/mL [52].

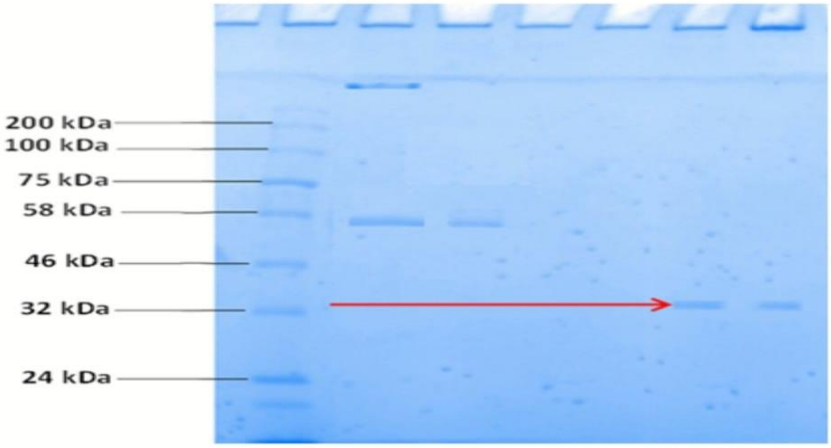

Figure 2: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of ovine pancreatic lipase isoenzyme.

Table (2): Effect of willow bark extracts on lipase activity

Inhibitors	Activity at 410 nm	Inhibitory effect %
Control	1.4	0
Hexane	1.41	0.714
EthOH absolute	1.35	3.57
H_2O	0.54	61.43
EthOH 70%	1.02	27.14

Table 3: Effect of 70% ethanol extract on the activity of lipase

Volume extract (µl)	Activity at 410 nm	Inhibitory effect%
Control	0.677	0
30	0.266	60.71
40	0.294	56.57
50	0.257	62.03
60	0.612	9
70	0.495	26.88

3.5 Mode of Inhibition

The activity inhibition of ovine pancreatic lipase was examined in the occurrence of 1.8 mg/40µl of 70% ethanol extract as an inhibitor by drawing a Lineweaver-Burk plot. The findings show that the inhibition mode proved non-competitive (Figure 3). Calculations revealed that Vmax decreased from 4.16 to 2.08 U/ml /min, while Km remained constant at 11.11 mM.

3.6 The effect of aqueous extract on lipase activity

Table (4) shows the inhibitory effect of different volumes of aqueous extract on lipase activity. The highest percentage of inhibition, 54.35%, was discovered to be present at a concentration of 2.1mg /30 µl, while the lowest inhibition percentage was 11.37%. at a concentration of 3.5 mg / 50 µl.

Using orlistat as a positive control, Adisakwattana et al. [53] found encouraging inhibitory effects of Ginkgo biloba and Morus alba aqueous extracts on pancreatic lipase in vitro. Using in vivo tests, Ono et al. [54] evaluated the aqueous extract of Nelumbo nucifera leaves' capacity to suppress PL. After giving rats a lipid emulsion orally, they observed that the plasma triacylglycerol level increased dramatically, whereas it decreased significantly in the extract-treated group. When compared to other plant components, the aqueous extract of Juniperus communis bark had a strong inhibitory impact on PL [55,56]. A hot water-soluble extract of Salacia reticulata roots was used both in vitro and in vivo to inhibit the lipoprotein lipase from rat adipose tissue [57].

3.7 Mode of Inhibition

Ovine pancreatic lipase activity was tested for inhibition in the absence of 2.1 mg/ µl of aqueous extract as an inhibitor by drawing a Lineweaver-Burk plot. According to the findings, noncompetitive inhibition was observed (Figure 4). Km was calculated to be 5 mM and Vmax decreased from 2.63 to 0.9 U/ml.

The inhibition mode of PL was studied and found to be noncompetitive with persimmon tannin [58] Kuding tea [59] and Black tea [60] extracts. An earlier investigation utilizing quercetin rutinoside and melatonin found that the inhibiting type for a lipase extracted via the pistachio khinjuke kernel was noncompetitive [61]. A kinetic study of PL revealed that the *Araucaria angustifolia* extract is an effective inhibitor, and the inhibition type was non-competitive [62].

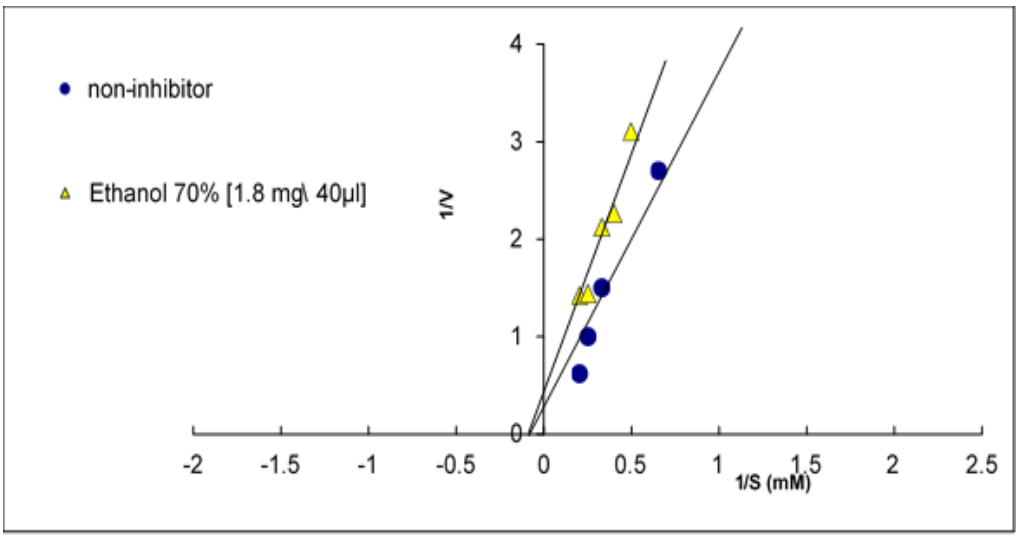

Figure 3 The mode of inhibition of purified lipase by ethanol extract 70%

Table 4: The aqueous extract effect on the activity of lipase

Volume extract (µl)	Activity at 410 nm	Inhibitory effect %
Control	0.677	0
30	0.309	54.35
40	0.793	17.13
50	0.600	11.37
60	0.597	11.81
70	0.422	37.77

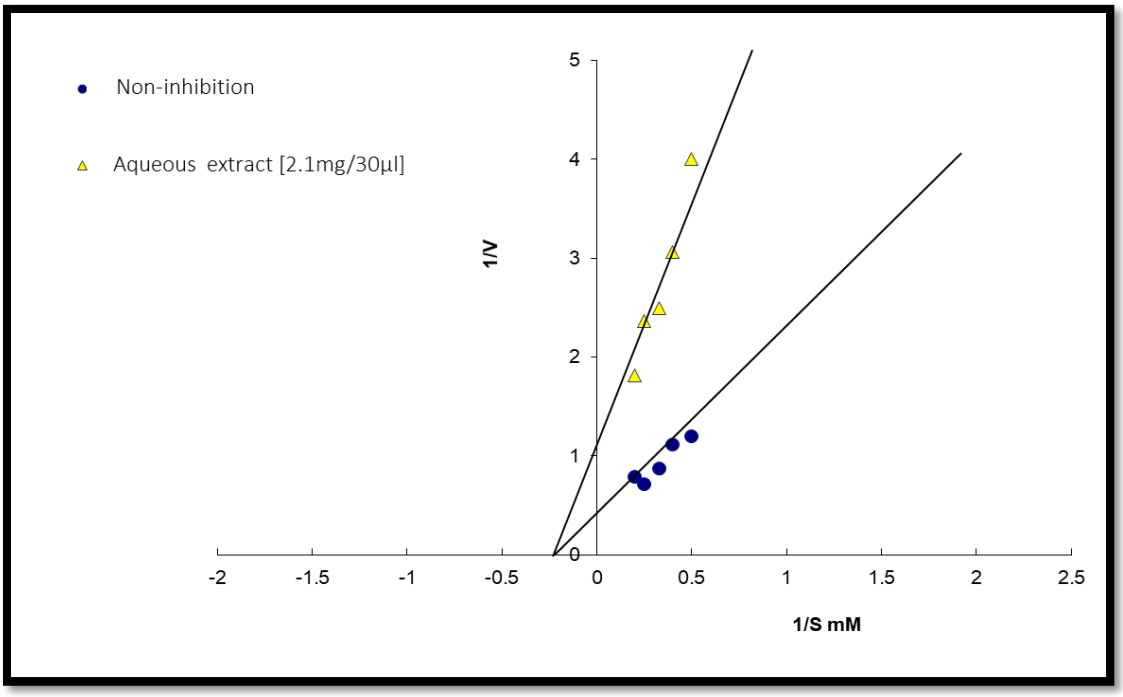

Figure 4: The mode of inhibition of purified lipase by aqueous extract

Conclusions

Fast food consumption is causing obesity, one of the main lifestyle problems, to rise alarmingly, particularly in emerging nations. Additionally, it is linked to a wide range of illnesses. Because it hydrolyzes all ingested fat, pancreatic lipase is an essential enzyme for fat absorption. The process of inhibiting this enzyme is very important for treating obesity syndrome. To date, effective synthetic drugs for this purpose are associated with relatively serious side effects. Therefore, the use of natural products has been widely used to determine their potential effectiveness as anti-obesity agents. In this work, we purified and characterized one peak of ovine pancreatic lipase with a molecular mass of 33.8 kDa. We observed that the aqueous and 70% ethanol extracts of willow bark are potent inhibitors of PL activity. Additionally, the results of kinetic analysis obeyed the noncompetitive mode by both extracts. This inhibitory effect is most presumably attributed to its phytochemical contents such as alkaloids and polyphenols. More research is required to purify and identify the specific ingredients of these extracts that are responsible for anti-lipase activity.

Conflict of Interest

There is no conflict of interest.

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