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Kinetic behaviour of pancreatic lipase inhibition by wine

[1,]*Khatchapuridze, Z., [2]Ploeger, A. and [3]Gulua, L.

[1]*Agricultural University of Georgia, 240, David Aghmashenebeli Alley, Tbilisi, 0159, Georgia*
[2]*University of Kassel, Faculty of Organic Agricultural Sciences, Nordbahnhof Strasse 1 A, 37213 Witzenhausen, Germany*
University of Georgia (UG), School of Health Science, Merab Kostava St, Tbilisi, 0171 Georgia

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Abstract

This study determined the *in vitro* inhibitory effects of Mukuzani (dry red) wine and its ethyl acetate/aqueous fractions against pancreatic lipase (PL). Also, it characterises the kinetic behaviour of such inhibition. To current knowledge, no studies have been conducted to describe the kinetic behaviour of PL inhibition by wine or the combined effect on the PL activity of some individual phenolic compounds present in wine. Additionally, the influence of different winemaking methods on PL activity is unknown. Lipase activity was determined by the titrimetric assay method. The kinetic analysis was determined by using the graphical method via Lineweaver-Burk plots. The mode of inhibition (MOI) was determined by looking at the interception pattern and crossing linear lines for the reciprocal data of PL activity with and without inhibitors versus olive oil concentration. The mode of inhibition identified for the wine sample was mixed-mode inhibition. In this type of inhibition, the value of K_m (244.329±10.214 μmol·mL^{-1}) was higher, and V_{max} (80875.4±3489.754 μmol·mL^{-1}·hour^{-1}) was lower compared to the value of Km (170.901±7.544 μmol·mL^{-1}) and V_{max} (88735±4036.741 μmol·mL^{-1}·hour^{-1}) for the non-inhibited PL. This inhibitor decreased the PL activity rate and reduced the affinity between the substrate (olive oil) and enzyme (PL). Selected wine showed predominantly competitive inhibition. The value of inhibition constant K_{i1} was equal to 40.556±1.932, and K_{i2} was equal to 179.361±8.678 μmol·mL^{-1}, meaning that binding affinity to the pancreatic lipase was higher than to the enzyme-substrate complex. The liquid/liquid extraction method was used to fractionate phenolic compounds of wine into aqueous and ethyl acetate fractions. Fractionated phenolic compounds of wine un-competitively inhibited PL activity. They were considered mutually nonexclusive inhibitors, while a synergistic relationship was found among them. The kinetic parameters of apparent V_{max} and K_m decreased. Due to the uncompetitive inhibition, the same factor decreased these kinetic parameters. The results obtained herein allow one to conclude that red wine made with the Kakhetian winemaking method can inhibit pancreatic lipase in vitro and play a role in body weight management.

1. Introduction

Overweight and obesity are defined as the abnormal or excessive accumulation of body fat, fundamentally caused by the energy imbalance between the consumed and expended calories over a given time (World Health Organization, 2000). Inhibiting fat absorption is an attractive approach to reducing energy intake and developing anti-obesity agents (Kumar and Chauhan, 2021). The inhibition of pancreatic lipase (PL) is the most widely studied mechanism since it hydrolyses 50-70% of total dietary fat (Birari and Bhutani, 2007).

Only Orlistat is a clinically approved pharmacologic agent as a pancreatic lipase inhibitor (Kumar and Chauhan, 2021), which can inhibit about 30% of the daily ingested fat intake (Zhi *et al*., 1994). In addition, it is an irreversible inhibitor of pancreatic and gastric lipase (Weiner and Mason, 2019). However, the ingestion of this compound is limited by unpleasant gastrointestinal adverse reactions (oily stools, diarrhoea, oily spotting, flatulence) (Filippatos *et al*., 2008; Tak and Lee, 2021).

*Corresponding author.
Email: *zh.khachapuridze@agruni.edu.ge*

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Therefore, searching for naturally derived sources with fewer undesirable effects and potent anti-lipase is a current research topic.

Many studies detected the class of polyphenols as one of the most important sources of potential PL inhibitors (Singh *et al.*, 2020; Lobo *et al*., 2021). Buchholz and Melzig (2015) outlined the most studied subclasses of polyphenols (including flavonoids, hydroxycinnamic acids, hydroxybenzoic acids, and lignans) with PL inhibitory effects. According to them, the increased inhibitory effect is dependent on several factors, such as the number and position of phenolic hydroxyl groups, degree of polymerisation, and elimination of glycosylation during digestion. Other researchers studied the mechanism and kinetics of lipase inhibition by different isolated polyphenols (Chen *et al*., 2017; Martinez-Gonzalez *et al*., 2017) to compare their inhibitory capacity to Orlistat. Even though individual polyphenols have been shown to possess high anti-lipase activity, some studies indicated that the intake of isolated compounds could have deleterious effects of overconsumption, and it is better to consume them from whole food (Williamson and Holst, 2008; Crowe and Francis, 2013). From this point of view, wine is known to be rich in polyphenols (Giovinazzo *et al*., 2019), and by drinking it, humans consume complex beverages, not single polyphenols. In a previously published study (Khatchapuridze *et al*., 2021), we investigated the lipase inhibitory activity of Georgian wines made from local cultivars, i.e., Saperavi (red) and Rkatsiteli (white). Results showed that most examined wine samples were characterised by noticeable or significantly high anti-lipase activity. In order to rank new chemicals regarding their inhibitory potency, it is essential to define their mode of inhibition (competitive, non-competitive, uncompetitive, or mixed) since only inhibition potency itself is a relatively uninformative quantity (Brooks *et al*., 2012).

To the best of our knowledge, no studies have been conducted to evaluate the kinetic behaviour of PL inhibition by wine. The present study aimed to determine an *in vitro* model of the inhibition mechanisms of wine towards PL by evaluating its kinetic parameters and inhibition mechanism. In order to determine the mechanism and kinetics of inhibition, a wine sample was chosen from previously obtained results, which possessed noticeably higher lipase inhibitory activity than the other samples. The well-known Kakhetian winemaking (Vigentini *et al*., 2016) was used to make this wine. The liquid/liquid extraction method (Roussis *et al*., 2005) was used to fractionate phenolic compounds of wine into aqueous and ethyl acetate fractions. Their mechanism and kinetics of inhibition towards PL were also evaluated. Since we believe that phenolic compounds cause the inhibitory effect of wine, gallic acid equivalent ($\mu mol \cdot mL^{-1}$) was used to express the inhibition constant (K_i).

2. Materials and methods

2.1 Wine sample

The wine included in this study was made by the Kakhetian method, aged in an oak barrel. It is a dry red wine of appellation-controlled origin (Mukuzani) made from the Saperavi grape variety (Granik, 2020). This style of wine is based on an extended period (up to 5 months) of maceration and fermentation of must with the usage of 100% of grape pomace (skin, seeds, stems). Fermentation is carried in a clay vessel called "qvevri," buried underground. Qvevri is then sealed, and the wine is left to mature (Capece *et al*., 2013)). During fermentation, phenolic compounds are extracted from pomace, defining the composition and essence of Kakhethian (Shalashvili *et al*., 2010). After fermentation, the wine sample was aged (for 9 months) in an oak barrel.

2.2 Liquid-liquid extraction of wine

The liquid-liquid extraction method was used to fractionate phenolic compounds of chosen wine (Roussis *et al*., 2005). For this purpose, wine dealcoholizing by the rotary evaporator at 40°C was performed (Salagoïty-Auguste and Bertrand, 1984). The pH of dealcoholized wine was then brought up to 2 and extracted by ethyl acetate. After evaporation of ethyl acetate under vacuum (30°C), the organic phase was redissolved in water (pH 7), extracted again with ethyl acetate, and subsequently followed by evaporation of ethyl acetate. With this process, aqueous and organic (ethyl acetate) phases (fractions) were obtained (Shalashvili *et al*., 2012). The aqueous phase mainly contained anthocyanins and polyphenolic compounds. The ethyl acetate fraction, which contains plenty of flavanols, and phenolic acid, was dissolved in methanol (Roussis *et al*., 2005). Obtained phenolic extracts were subjected to further analysis.

2.3 Determination of total phenolic content

The total phenolic content (TPC) was determined using the Folin-Ciocalteu method (Singleton *et al*., 1999). The mixture of 1 mL of wine fractions and 5 mL of Folin-Ciocalteu reagent (1:10 v/v distilled water) were left for 8 mins to allow the reaction before 4 mL of sodium carbonate solution (7.5% (w/v)) was added to each test tube. The mixtures were mixed well, and the tubes were allowed to stand for another 60 mins at room temperature for colour development. Then their optical

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densities against the water were read at 765 nm, with a 10 mm path length cell. Diluted gallic acid (10-50 $\mu g \cdot mL^{-1}$) was used as a standard compound to plot a relationship between optical density and gallic acid concentration. The chart equation obtained was as follows $y = 0.0123x + 0.0236$, $R^2 = 0.9918$, where "y" is the optical density at 760 nm and "x" is gallic acid concentration $\mu g \cdot mL^{-1}$. The samples' total phenolic contents were expressed as $\mu g \cdot mL^{-1}$gallic acid equivalent (GAE).

2.4 Pancreatic lipase activity and inhibition reaction

Pancreatic lipase (PL) activity was studied via titrimetric assay method according to Stoytcheva *et al*. (2012), with slight modifications. Briefly, the starting blend consisted of 2.5mL of distilled water, 1 mL of Tris HCl buffer (200mM, pH 7.2), 3 mL of olive oil, and 0.5mL of Tween 80. After mixing for 15 mins on a magnetic stirrer, 110 mg of the lipase concentrate was added to the mixture. The test tubes were then incubated inside the water bath at 37°C, and after 30 mins, 3mL of ethanol (95%) was added. After that, the mixture was titrated with 50 mM NaOH until the predefined value (pH9) was reached. An automatic titrator (ZDJ-4A, NASA Scientific Instrument Co., Ltd, Anting Shanghai, China) was used for titration. Lipase activity was calculated according to the following equation:

$$\text{Lipase units/mL enzyme} = \frac{(A-B)\,(1000)\,(2)\,(DF)}{(1)} \quad (1)$$

where [A] denotes the volume of 50 mM NaOH consumed by the test sample in mL; [B] denotes the volume of 50 mM NaOH consumed by the blank sample in mL; [1000] indicates the conversion factor from milliequivalents to micro equivalents; 2 stands for time conversion factor from 30 min to 1 hour; [DF] stands for dilution factor; 1 denotes the volume (in mL) of the used enzyme.

One unit of lipase activity was defined as the amount of lipase that will release 1.0 micro equivalent of fatty acid from a triglyceride in one hour at a pH of 7.2 at 37° C. Lipase units were determined in the presence and absence of inhibitors. The inhibition percentage for each inhibitor was calculated. An aliquot (1 mL) of inhibitors was incorporated into the initial mixture.

2.5 Evaluation of pancreatic lipase inhibition kinetics

The kinetic analysis of PL activity inhibited by wine and its fractions was determined using the graphical method via double reciprocal (Lineweaver-Burk) plots (Bhagavan, 2002). The plots were set up at different olive oil concentrations varying from 0.5 to 3mL for the PL reaction with and without wine and wine fractions. The concentration of inhibitors and PL were kept constant. The mode of inhibition (MOI) was determined by looking at the interception pattern and crossing linear lines for the reciprocal data of PL activity with and without inhibitors vs olive oil concentration.

Enzyme kinetics can be governed by the following Michaelis-Menten equation (Equation 2), where K_m represents the Michaelis-Menten constant and indicates the substrate-binding affinity. V_{max} stands for the maximum reaction rate, also called enzyme activity. These kinetic parameters were calculated using the Lineweaver-Burk equation (Equation 3), which is the reciprocal of the Michaelis-Menten Equation.

The Lineweaver-Burk equation can be compared with the equation for a straight line: $y = ax + b$, in which "b" (the y-intercept) is equal to the $1/V_{max}$ and "a" (the slope) is equal to the value of K_m/V_{max} (Roskoski, 2015). The value of K_m/V_{max} is also known as specificity time (Cornish-Bowden, 2012). K_m and V_{max} in Equation3 refer to the K_m and V_{max} value of inhibition, which in the inhibition study can also be denoted as apparent (app) values - $K_{m,\,app}$, and $V_{max,\,app.}$ The inhibition constants (Ki) were calculated according to the MOI.

$$V = \frac{V_{max}[s]}{K_M + [s]} \quad (2)$$

$$\frac{1}{V} = \frac{K_m}{V_{max}}\frac{1}{[s]} + \frac{1}{V_{max}} \quad (3)$$

where [V] stands for the reaction velocity, [V_{max}] – the maximum reaction velocity, [K_m] – Michaelis-Menten constant [S] substrate concentration.

2.6 Statistical analysis

All statistical analyses were performed with Microsoft Excel (Microsoft 365 MSO, Version 2112, statistical functions, Microsoft Corp., Redmond, WA, USA). Presented data are the mean of a minimum of three replicates±standard deviation (SD). The LINEST function was used to fit known data points to the straight line by the least square method and to return the statistics of that. One-way analysis of variance (ANOVA) was done to analyse the significance of the variation of means between the control and the experimental samples. Tukey's honestly significant difference test (Tukey's HSD) was used to test differences among sample means for significance. All the analyses were done using PHStat (Microsoft Excel version 16.0, Pearson Education Inc.).

3. Results and discussion

The characteristics of wine studied herein are shown in Table 1. This wine belonged to the appellation of controlled origin (AOC) dry red wine produced from Saperavi, grown in the Mukuzani micro-viticulture area

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Table 1. Wine characteristics and composition.

Name of the bottle	Nekresi Estate - Mukuzani
Producer	Nekresi winery
Vintage	2016
Grape variety	Saperavi
Type	Dry Red
Alcoholic strength %	13
Winemaking method	Kakhetian (aged in oak barrel)
Titratable Acidity g·L^{-1}	6.625±0.002
Total dry extract g·L^{-1}	30.70±0.01
Total Polyphenol content µg·mL^{-1} gallic acid equivalent	2965.312±67.152
Antioxidant activity µg·mL^{-1} vitamin C equivalent	3494.381±94.199

of the Kakheti region (41.8089° N, 45.7310° E). Saperavi is the most widely planted grape variety in Georgia.

Mukuzani wine contained 2965.312±67.152 µg·mL^{-1} GAE. The TPC was also measured for aqueous and ethyl -acetate fractions, which were 2078±89.75 and 791.22±31.659 µg·mL^{-1} GAE. An acceptable margin of error indicated a successful fractionation process. The mode of PL inhibition exhibited by the Lineweaver-Burk plot of wine was found to be mixed-inhibition (Figure 1), which means that wine was able to bind to the enzyme (PL) and also to the complex formed between the enzyme and the substrate (PL-Olive oil complex) (Kenakin, 2017). Based on MOI, the overall inhibition reaction mechanism of mixed inhibition featured by wine is shown in the Figure 1). Based on this reaction mechanism, the inhibitor can bind to both the free enzyme and the enzyme-substrate complex, where K_{i1} is the inhibition constant for binding wine to the PL and K_{i2} is the inhibition constant for binding wine to the PL - Olive Oil complex. This interaction promotes a decrease of V_{max} (maximum velocity of the enzyme) and an increase in K_m in the presence of an inhibitor (Kenakin, 2017).

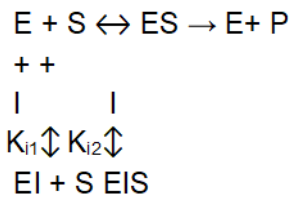

Figure 1. Scheme of a reversible linear mixed inhibition. [E]: enzyme; [S]: substrate; [I]: inhibitor; [P]: product. Wine as the inhibitor (I) can interact with the free enzyme (E) to form the complex enzyme/inhibitor (EI), as well as with the enzyme/ substrate complex (ES).

Overall, PL inhibition reaction by wine is characterised by reciprocal data equation (Equation 4) (Bhagavan, 2002), which shows that the slope of K_m/ V_{max} was decreased by a factor of {1+[I]/K_{ia}}, due to the inhibitory effect given by wine.

$$\frac{1}{V} = \left(1 + \frac{[I]}{K_{i1}}\right)\frac{K_M}{V_{max}}\frac{1}{[S]} + \frac{\left(1+\frac{[I]}{K_{i2}}\right)}{V_{max}} \quad (4)$$

By comparing of Equations 3 and 4 it can be concluded that:

$$V_{max,app} = \frac{V_{max}}{1+\frac{[I]}{K_{i2}}} \quad (5)$$

$$K_{m,app} = K_m \frac{1+\frac{[I]}{K_{i1}}}{1+\frac{[I]}{K_{i2}}} \quad (6)$$

The values of Ki1 and Ki2 were calculated using Equations 5 and 6, where [I] refers to the concentration of gallic acid equivalent µmol·mL^{-1} in wine.

Calculated values of kinetic parameters are shown in Table 2. As seen from Table 2, the kinetic parameters of the reaction without the inhibitor itself were as follows: Michaelis-Menten constant (K_m) was equal to 170.901±7.544 µmol·mL^{-1}, and the maximum reaction rate (V_{max}) was equal to 88735±4036.741 µmol·mL^{-1}·hour^{-1}. In the presence of wine, the value of V_{max} decreased and was equal to 80875.4±3489.754 µmol·mL^{-1}·hour^{-1}. This meant that wine was proficient in preventing catalysis regardless of whether the inhibitor was attached to the free enzyme or to the enzyme-substrate complex (Figure 2). The value of K_m increased up to 244.329± 10.214 µmol·mL^{-1}. A larger value of K_m showed a weak binding of a substrate to an enzyme (Buchholz *et al*., 2012).

The results found that the value of K_{i1} was smaller than the value of K_{i2}, 40.556±1.932 and 179.361±8.678 µmol·mL^{-1}, respectively. This indicated that the affinity of wine to bind to the free enzyme (PL) was higher than the binding affinity to enzyme-substrate complex, which makes the inhibitory effect stronger. Therefore, according to the Nomenclature Committee of the International Union of Biochemistry (NC-IUB), we can entitle this case as predominantly competitive inhibition since $K_{i1} < K_{i2}$ (Buchholz *et al*., 2012).

A similar study was conducted by Gu *et al*. (2011).

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Table 2. Kinetic parameters of inhibition reaction by wine and wine fractions.

Sample	Linear Equation	R^2	K_m μmol·mL^{-1}	V_m μmol · mL^{-1} · hour^{-1}	$K_{i\,(1)}$ μmol·mL^{-1}	$K_{i\,(2)}$ μmol·mL^{-1}
No inhibitor (Control)	y = 0.0019259707x + 0.0000112695	0.9879	170.901±7.544^{b}	88735.08±4036.741^{a}	N/A	N/A
Wine	y = 0.0030210571x + 0.0000123647	0.9637	244.329±10.214^{a}	80875.4±3489.754^{b}	40.556±1.932	179.361±8.678
Ethyl acetate fraction	y = 0.0020081973x + 0.0000238395	0.9186	84.238±3.455^{c}	41947.19±2001.395^{c}	4.521±0.209	N/A
Aqueous fraction	y = 0.0018547013x + 0.0000254384	0.9111	72.91±3.333^{d}	39310.65±1687.53^{c}	1.978±0.086	N/A

Values are presented as mean±SD. Values with different superscripts within the same column are statistically significantly different (P<0.05).

They assessed in vitro inhibitory effects of cocoa extracts and procyanidins against digestive enzymes. This study showed that the regular cocoa extract, procyanidin pentamer, and decamer reduced the V_{max} and increased K_m of PL, by that means suggesting a mixed-type inhibition. Another study evaluated the inhibitory effect of some plant extracts on PL, and a similar inhibition mode was observed in *Levisticum officinale* methanolic extract against PL (Gholamhoseinian *et al*., 2010).

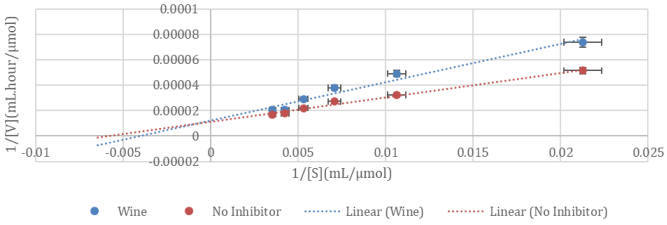

Figure 2. Lineweaver-Burk plots of pancreatic lipase activity with and without wine.

The Lineweaver-Burk plots of aqueous and ethyl acetate fractions (Figures 3 and 4, respectively) showed a pattern of parallel lines, indicating an uncompetitive inhibition mechanism. Results indicated that wine fractions were able to bind to the complex formed between the enzyme and the substrate (PL - olive oil complex) (Figure 5). As seen from Table 2 here, the values of the apparent $V_{max,app,}$ and $K_{m,app}$ decreased. $K_{m,app}$ of ethyl acetate fraction was equal to 84.238±3.455.238 μmol mL^{-1} and $V_{max,app}$ was equal to 41947.19±2001.395 μmol mL^{-1}·hour^{-1}. K_m value for aqueous fraction was found to be 72.91±3.33 μmol·mL^{-1} and $V_{max,app}$ - 39310.65±1687.53 μmol·mL^{-1}·hour^{-1}. The inhibition constant for ethyl acetate and aqueous fractions were 4.521±0.209 and 1.978±0.086 μmol·mL^{-1}, respectively. A small value of K_i indicates a stronger inhibitory effect because the inhibitor bounds tightly (Cornish-Bowden, 2012). Due to the uncompetitive inhibition, the same factor decreased these kinetic parameters.

Moreno-Córdova *et al*. (2020) evaluated the inhibitory activity of selected phenolic compounds and showed that Gallotannins are uncompetitive inhibitors of pancreatic lipase activity. However, due to the vast number of molecular structures of phenolic compounds, the specific mechanism of action varies significantly, even between related compounds (Wang *et al*., 2014; Glisan *et al*., 2017).

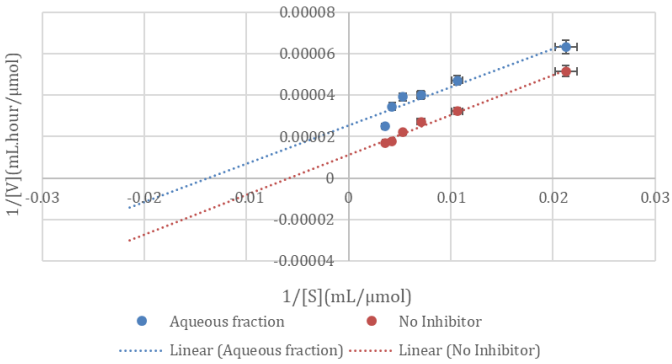

Figure 3. Lineweaver-Burk plots of pancreatic lipase activity with and without aqueous fraction.

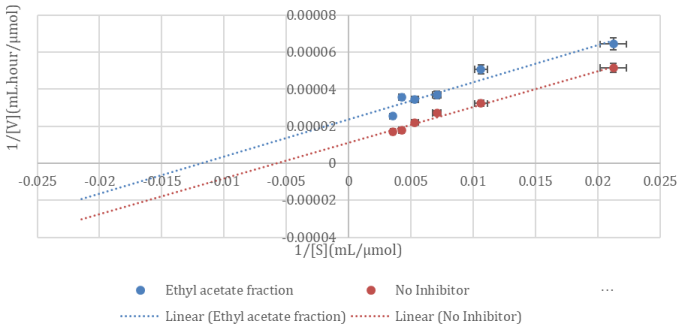

Figure 4. Lineweaver-Burk plots of pancreatic lipase activity with and without ethyl acetate fraction.

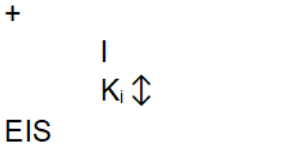

Figure 5. Scheme of uncompetitive inhibition. [E]: enzyme; [S]: substrate; [I]: inhibitor; [P]: product. Wine fractions as inhibitors (I) can interact with the enzyme/substrate complex (ES) to form the complex (EIS).

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In order to find the combined effect of two inhibitors on PL, we also wanted to find the relationship among them. In the presence of two inhibitors, the combined effect may lead to synergism, summation, or antagonism (Chou and Talaly, 1977). The following equations can be used to determine the combined effect of two different inhibitors:

Summation:

$$\frac{1}{v_{1,2}} = \frac{1}{v_1} + \frac{1}{v_2} - \frac{1}{v_0} \quad (7)$$

Synergism:

$$\frac{1}{v_{1,2}} > \frac{1}{v_1} + \frac{1}{v_2} - \frac{1}{v_0} \quad (8)$$

Antagonism

$$\frac{1}{v_{1,2}} < \frac{1}{v_1} + \frac{1}{v_2} - \frac{1}{v_0} \quad (9)$$

Where $v_{1.2} = v_1v_2/v_0$ and v_0 denotes the velocity in the absence of inhibitor, v_1 is the velocity in the presence of inhibitor 1, v_2 is the velocity in the presence of inhibitor 2.

In our case, we found that the value of

$\frac{1}{v_{1,2}}$ (0.0000538±0. 0.0000024 mL·hour·μmol^{-1})

was greater than the value of

$\frac{1}{v_1} + \frac{1}{v_2} - \frac{1}{v_0}$ (0.0000380±0.00000271 mL·hour·μmol^{-1}).

This data indicated that the ethyl acetate and aqueous fractions had a synergistic effect on the activity of PL. These inhibitors can also be called mutually nonexclusive inhibitors (Chou and Talaly, 1977).

The present study provided the first evidence that red wine is a potent pancreatic lipase inhibitor with mixed-type inhibitory activity. In addition, this is the first report on the kinetics of inhibition of PL by Georgian wine. The inhibitor activity and kinetic parameters determined from wine are expected to benefit in controlling obesity and problems associated with excess weight.

4. Conclusion

This study investigated the inhibitory potential of Mukuzani wine and its phenolic fractions against pancreatic lipase and their mode of inhibition. Kinetic analysis showed that wine produced according to the Kakhetian method inhibited PL activity in a mixed-mode. Results demonstrate that the binding affinity of wine to the free enzyme (PL) was higher than to the enzyme-substrate complex, which indicates a stronger inhibitory effect. Wine phenolic extracts un-competitively inhibited PL activity. The ethyl acetate and aqueous fraction had synergistic effects on a single target enzyme (PL) and were considered mutually nonexclusive inhibitors. This study demonstrated that red wine made with the Kakhetian winemaking method can inhibit pancreatic lipase in vitro and play a role in body weight management.

Conflict of interest

All authors declare no competing financial interest.

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