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Blanching induced effect on bioactive compounds and anti-diabetic properties of bitter gourd

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Abstract
The purpose of the study was to determine how pretreatment techniques, such as blanching in hot water or alkaline solution, affected the bioactive ingredients, and anti-diabetic properties of bitter gourd. The bitter gourd was blanched in hot water and an alkaline solution (a mixture of saturated magnesium oxide and 0.1% sodium carbonate) at 98 °C for three minutes. Both pretreated bitter gourds were submerged in a 5% salt solution for 1 hour. Untreated fresh bitter gourd was used as the control. Ascorbic acid levels (mg/100g) and antioxidant activity (DPPH inhibition %) were dramatically ($p<0.05$) decreased in pretreated bitter gourd. When bitter gourd was blanched in an alkaline solution, more chlorophyll was retained, and when it was blanched in a hot water solution, the carotenoid concentration rose dramatically ($p<0.05$). The extract prepared from raw bitter gourd was found to have better inhibitory properties ($p<0.05$) against alpha-amylase and alpha-glucosidase, though all showed inhibition properties below 50%, which was low. The research concluded that raw bitter gourd is superior, however the extract percent needs to be increased to determine the IC_{50} value for the enzyme inhibition properties. Further research can be carried out by increasing the concentration of the extract for enzyme inhibition properties and by carrying out the trial in animals.

Keywords: *Alpha-amylase, Alpha-glucosidase, Ascorbic acid, Antioxidant activity, Chlorophyll*

INTRODUCTION

Bitter gourd (*Momordica charantia*), a member of the cucurbit family, also known as bitter melon or balsam pear, is one of the most popular vegetables in Southeast Asia (Satkar *et al.,* 2012). As reviewed by Gayathry and John (2022), bitter gourd is low in calories but is a good source of minerals, vitamins, polyphenols, and antioxidants, possessing several health benefits. Bitter gourd is mostly consumed as vegetable curries and pickles in Nepal. It can be also pan-fried, deep-fried, boiled, pickled, juiced, and dried to drink as tea (Myojin *et al.,* 2008). Despite being bitter due to alkaloid momordicine, this vegetable is valued for its medicinal properties, particularly for the treatment of general fever, malaria, and also diabetes (Walter and Decker, 1988).

Diabetes mellitus is a major global public health problem, in which blood sugar levels are elevated either because the pancreas does not produce enough insulin or cells do not respond to the produced insulin (Nair *et al.,* 2013). Inhibition of the major carbohydrate hydrolyzing enzymes like α-amylase and α- glucosidase are the potential targets in the development of lead compounds for the treatment of diabetes (Subramanian *et al.,* 2008). Bitter gourd is a low-cost food-based intervention that can play a vital role in the treatment and prevention of type 2 diabetes mellitus (Yang *et al.*, 2015). Charantin (a triterpenoid), polypeptide-p or plant insulin, and vicine (a glycol alkaloid) are the major compounds that have been isolated from the bitter gourd and are associated with anti- diabetic properties (Jinni and Joseph, 2013). Ee-Shian *et al.* (2015) in their research, demonstrated that bitter gourd can inhibit α- amylase and α-glucosidase and hence can be a potent tool in food as well as nutraceutical product development for the control of diabetes.

Blanching is an essential thermal treatment carried out before many preservation processes like drying, canning, and freezing, mainly to inactivate enzymes (such as polyphenol oxidases and peroxidases) (Xiao *et al.,* 2014; Lago and Norena, 2014) and preserve the green colour and maintain aesthetic appeal. Hot water blanching is the most common commercially adopted method as it is simple and easy to implement (Mukherjee and Chattopadhyay, 2007). Hot water blanching is carried out by immersing the products in hot water (at 70 to 100° C) for several minutes and the blanched samples are drained and cooled before being sent to the next process operation (Bingol *et al.,* 2014). Alkaline blanching is mainly used in green vegetables to retain the green color, and alkaline agents mainly used are magnesium hydroxide, magnesium oxide, calcium oxide, etc (Segner *et al.,* 1984). Chlorophyll, a pigment that imparts a green color, is stable only under alkaline conditions of about pH 8 (Gunawan and Barringer, 2000). These are highly susceptible during the processing of produce resulting in color change in food. Kharel and Khanal (2002) concluded that alkaline-blanched bitter gourd preserved color and texture better compared to hot water- blanched samples.

Phytonutrients in vegetables result in bitterness and are not consumed by many people (Mithen *et al.,* 2000). It has been proven that soaking the vegetables in sodium chloride solution can help to minimize the bitter taste. There have been various attempts to develop functional and diabetic beverages from bitter gourd alone or by blending with other fruits and vegetable juices (Satkar *et al.,* 2012; Din *et al.,* 2011). However, in the context of Nepal, the effect of different methods of blanching on bioactive components and anti-diabetic potential is still limited. The research objective is to determine the effects of pretreatments of bitter gourd on the bioactive properties and enzyme inhibition effect of vegetables.

MATERIALS AND METHODS

Materials

The bitter gourd used for the research was collected from the farm of the Kavrepalanchowk district, Nepal, and transported

to the laboratory in an icebox (4 °C) to minimize the changes in the properties.

Preparation of bitter gourd

Matured bitter gourd was cleaned with potable water to remove dust and other adhered materials. Cleaned bitter gourd was then cut into desired size with the help of a sharp stainless-steel knife and chopping board and seeds were removed to facilitate further processing. The cut bitter gourd was subjected to hot water blanching and alkaline blanching, where the ratio of vegetable to the weight of solution was 1:3. For alkaline blanching, the pH of the solution (saturated solution magnesium oxide and 0.1% of sodium carbonate in equal amounts) was maintained 8. For both the blanching the temperature and time combination used was 98 ℃ for 3 minutes (Kumar *et al.,* 2015; Jadev *et al.,* 2010). After blanching, it was then cooled and washed with clean water and then dipped in a 5% salt solution for 1 hour (Din *et al.,* 2011). The pretreated bitter gourd was washed with potable water 3 times and preserved in refrigerated conditions until analysis.

Experimental design and statistical analysis

The experiment was conducted thrice and was carried out in a completely randomized design with three treatments. The prepared slice was subjected to bioactive components and enzyme inhibition analysis. Results are expressed as the mean and standard error of the mean. The differences between treatments are conducted using the Student-Newman-Keuls test, executed through SPSS software version 26 (IBM, Chicago, America).

Ascorbic acid

Ascorbic acid (mg/100g) was determined by Association of Official American Chemists (AOAC) method number 967.21 (2-6- dichlorophenol-indophenol visual titration method) as described by AOAC, 2005.

Chlorophylls and Carotenoids

For chlorophylls and carotenoids, 1g sample was extracted in mortar and pestle using acetone (80% v/v) till the residue was colorless followed by filtration of acetone extract in 250 ml volumetric flask. The volume was made up using acetone (80% v/v). Fifty ml of extract was transferred into a separating funnel containing 50 ml petroleum ether. Water was added from the sides of the funnel until the water layer was free of fat-soluble pigments. The water layer was drained off while the petroleum ether layer was washed 10 times with 10 ml portions of distilled water until the ethanol layer was free of acetone. Ether extract was transferred into a 100 ml volumetric flask and ether was added to make up the volume. A three-gram of anhydrous Na_2SO_4 was added to the extract. The extraction process for chlorophylls and carotenoids was adapted from the method described by Lichtenthaler & Buschmann (2001) with some modifications. The absorbance was taken in a spectrophotometer at 470 nm, 653 nm, and 663 nm as reviewed by Saini *et al*. (2022). The calculation was done as:Chlorophyll a, (mg/100 g) = 12.7 (A663) - 2.69(A645) $100\ g(FW) * 250\ ml/50\ ml$ Chlorophyll b, (mg/100 g) = 22.9(A645) – 4.68(A663) $100\ g(FW) * 250\ ml/50\ ml$

Total chlorophyll, (mg/100 g) = Chlorophyll a + chlorophyll b

Total Carotenoids (mg/100 g) = {(1000 A_{470}-3.27C_a-104 C_b)/229} $100\ g(FW) * 250\ ml/50\ ml$

Antioxidant activity

The methanolic extract of bitter gourd was prepared as described by Ojha *et al*. (2019) with some modifications. For this 5 g of bitter gourd was extracted with 30 ml of absolute methanol for 20 minutes, filtered and the process was repeated twice, and the final volume was made 100 ml by absolute methanol. The antioxidant activity of bitter gourd was determined by the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method as described by Walvekar and Kaimal (2014) with some modifications. For this, a 3 mL bitter gourd extract was combined with 3 mL of a 0.004% solution of DPPH (2,2-diphenyl-1-picrylhydrazyl). This mixture was then left to incubate in darkness for 30 minutes. After incubation, the absorbance of the solution was measured at 517 nm using a UV-Vis spectrophotometer (GENESYS^{TM} 10S Vis Spectrophotometer, Thermo ScientificTM, Germany). The absolute methanol was used as a reference (blank).

The scavenging activity of the extract against the stable DPPH was calculated using the following equation,

Scavenging activity (%) = (A – B) / A x 100s

Where,

A is the absorbance of DPPH

B is the absorbance of DPPH and extract combination

The calculated value was given by 50000 ppm (5 g bitter gourd in 100 ml methanol) of bitter gourd extract.

Extract preparation for enzyme assay

The washed bitter gourd pieces were dried at 50 °C for about 5 hours. Then the 2 g dried sample was grounded, and extraction was done in 100 ml methanol by using a rotary vacuum evaporator. The extract was then kept in a vile at refrigerated condition at 4 ℃ for further analysis.

Porcine pancreatic α- Amylase (PPA) inhibitory activity assay

PPA inhibitory activity was determined by following the method described by Hansawasdi *et al.* (2000) with a slight modification. Starch azure (2 mg), which was used as a substrate, was suspended in 0.5 M Tris–HCl buffer (pH 6.9) containing 0.01 M $CaCl_2$ and soaked in boiling water for 5 min. Then, the starch azure solution was pre-incubated at 37℃ for 5 min. The test samples (0.2 mL) in 50% dimethyl sulfoxide (DMSO) and 0.1 mL of PPA solution (2.189 U/mL, amylase from Porcine Pancreases) were added to each assay sample. Whereas 0.1 mL 0.5 M Tris–HCl buffer was used in place of the plant extract for the blank sample. After thoroughly mixing, both the sample and the blank test tubes were incubated at 37℃ for 10 min and the reaction stopped by adding 0.1 mL of 50% acetic acid. The reaction mixture was then centrifuged (3000 rpm, 4℃) for 5 min. The absorbance of the supernatant, at 595 nm in UV-Vis spectrophotometer (GENESYS^{TM} 10S Vis Spectrophotometer, Thermo Scientific^{TM}, Germany), was measured and the inhibitory activity was calculated using the following formula:

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Inhibitory activity (%) = ($OD_{control}$- $OD_{test\ sample}$)/$OD_{control}$×100

Assay for α- Glucosidase Inhibition

α- glucosidase Inhibition activity was examined according to the previous protocol of Zhang et al. (2023). 50 mM sodium phosphate buffer (pH 8) was used for assay and α- α-glucosidase (0.2u/ml) activity was assayed with the substrate p-nitrophenyl-α-D-glucopyranoside. In each experiment the concentration of α- glucosidase was 0.2 U/ml. The enzyme (20 µL) along with 100 µL of phosphate buffer, saline and 60 µL of various concentrations of the sample was pre-incubated at 37°C. Substrate (0.7 mM, 20 µL) was added after the pre- incubation of 15 min, and the reaction was carried out at 37 °C for 30 minutes. Enzymatic activity was calculated by measuring the absorbance of p-nitrophenol at 405nm on a microtiter plate spectrophotometer (Biotech Epoch 2, 96-well plate spectrophotometer, USA). Acarbose was used as the positive control. The percent inhibition of p-nitrophenol formation in the test sample versus control was calculated for each compound by using the following formula.

Inhibitory activity (%) = (A_O– A_T)/A_O ×100 A_O = Absorbance of control

A_T = Absorbance of sample

RESULTS AND DISCUSSION

Effect of pretreatment methods on ascorbic acid content

Both alkaline blanching and hot water blanching significantly ($p<0.05$) reduced the ascorbic acid concentration with significant ($p<0.05$) retention in hot water blanching compared to alkaline blanching (**Figure 1**).

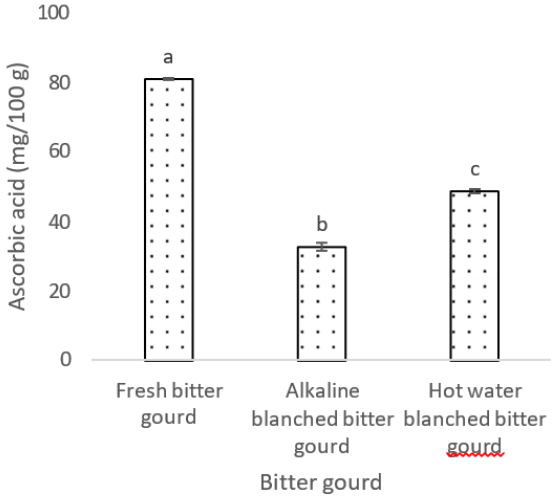

Figure 1: Effect of pretreatments on ascorbic acid of bitter gourd

Note:

*Vertical line plus bars indicates means ± standard error (n=3)

*Different letters in each bar indicate values are significantly different

The ascorbic acid content of the untreated bitter gourd was similar to the value published by Sharma and Thakur (2018), which was 82 mg/100 g. The loss of ascorbic acid in the bitter gourd subjected to alkaline blanching is due to the instability of ascorbic acid in alkaline conditions and is changed to dehydroascorbic acid (Yin *et al*., 2022). As reported by Vasquez- Parra *et al*. (2014), a drop was observed in the Vitamin C content of gooseberries from 0.55 mg/g untreated fruit to 0.26-0.46 mg/g alkaline dipped fruit. Kharel and Khanal (2002) also reported 54.3% retention of ascorbic acid in hot water blanched bitter gourd compared to 41% retention in alkaline blanched bitter gourd.

Effect of pretreatment methods on chlorophyll content

Both alkaline blanching and hot water blanching significantly ($p<0.05$) reduced the chlorophyll content of bitter gourd, with significant ($p<0.05$) retention in alkaline blanching compared to hot water blanching (**Figure 2**). As reported by Segner *et al.,* 1984, the retention of chlorophyll content in fruits and vegetables highly depends on the pH to which it is exposed. As reported by Minguez- Mosquera and Gandul-Rojas (1995), the basic structure of the chromophore group is intact in alkaline conditions and is linked with the porphyrin ring via magnesium, not significantly affecting the color. Whereas in acidic conditions, a magnesium ion in the porphyrin ring is replaced by hydrogen ions and color is changed from bright green to olive green. The reduction in chlorophyll content in alkaline blanched bitter gourd might be due to the activation of the chlorophyllase enzyme, that affects the isocyclic rings of chlorophyll. As per Koca *et al*. (2007), the chlorophyll retention of green peas was high at pH 7.5 compared to pH below 7.0. As reported by Schwartz & Lorenzo (1990), heat treatment converts the chlorophyll to pheophytins and pyropheophytins, changing the color from light green to olive green. Sicari *et al*. (2021) published a high reduction in chlorophyll in blanched Asteraceae vegetables compared to fresh ones. Ampofo-Asiama *et al*. (2021) reported that the reduction of chlorophyll in hot water blanching is also a function of time.

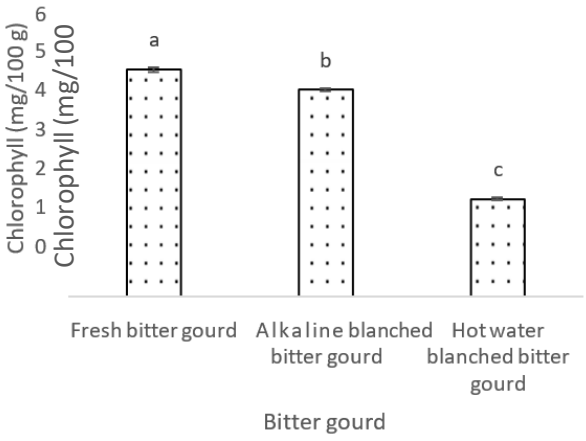

Figure 2: Effect of pretreatments on Chlorophyll Content of bitter gourd

Note:

*Vertical line plus bars indicates means ± standard error (n=3)

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*Different letters in each bar indicate values are significantly differentEffect of pretreatment methods on carotenoid content The carotenoid concentration of the fresh bitter gourd was significantly ($p<0.05$) higher compared to hot water blanching, while significantly lower ($p<0.05$) than alkaline blanched bitter gourd juice (**Figure 3**).

Addis *et al.* (2009) found that blanching in boiling water increased the carotenoid content of both ivy gourd and fenugreek leaves. Mayer- Miebach & Spieb (2003) also reported that blanching treatment at 90 ℃ can improve the yield of carotenoids as blanching treatment softens the bitter gourd tissue and increases the permeability of bitter gourd cells. As reported by Shivhare *et al.*, (2009), the blanching treatment can also inhibit a variety of enzymes especially oxidases thereby improving the stability of carotenoids. As reviewed by Addis *et al*. (2009), a carotenoid may be increased in hot water blanched bitter gourd due to better extraction in softened tissue and inactivation of the carotenoid degrading enzyme. Bell *et al.* (2016) reported a greater reduction of carotenoids in fruit juice at alkaline pH and an increase in the case of acidic pH. The greater reduction of carotenoids in alkaline blanching might be due to the instability of the pigment at pH greater than 7.0 (Yadav and Prabha, 2014).

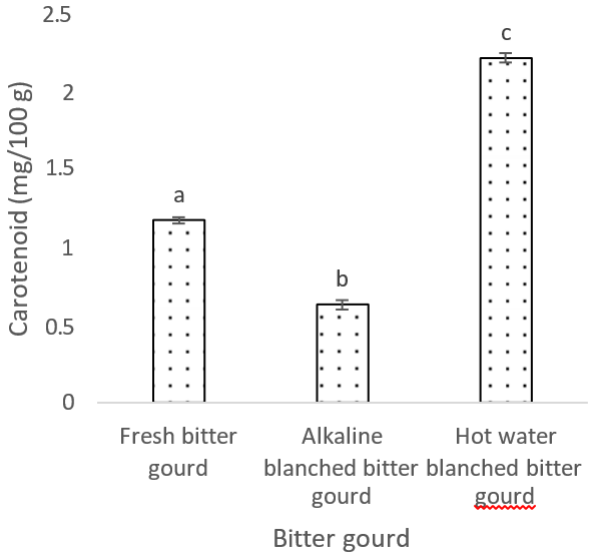

Figure 3: Effect of pretreatments on carotenoid content of bitter gourd

Note:
*Vertical line plus bars indicates means ± standard error (n=3)
*Different letters in each bar indicate values are significantly different

Effect of pretreatment methods on antioxidant activity

The antioxidant activity as % DPPH of the bitter gourd after pretreatment methods was decreased significantly ($p<0.05$), whereas a significant difference in antioxidant activity between hot water blanched and alkaline blanched bitter gourd was not observed (**Figure 4**). Myojin *et al.* (2008) reported a significant decrease in the radical scavenging activity of bitter gourd by blanching, however, retention was 80%. However, in our result, the retention was about 90%. The retention in blanched juice might be due to more extraction of bioactive components, inactivation of polyphenolase, and release of bound phenolic components (Howard *et al*. 1999; Vega-Galvez *et al*. 2009). Oboh *et al.* (2012) also reported a significant decrease in the radical scavenging activity of green leafy vegetables by hot water blanching. Various authors have reported the reduction in phenolic compounds and ascorbic acid during heat treatment of blanching results in a decrease in antioxidant activity (Oms-oliu *et al.,* 2012; Klimczak *et al.,* 2007*).* The reduction in antioxidant activity is also attributed to the types of vegetables, the size of vegetables employed for blanching, and processing time (Roy *et al.* 2009; Sikora *et al.* 2008).

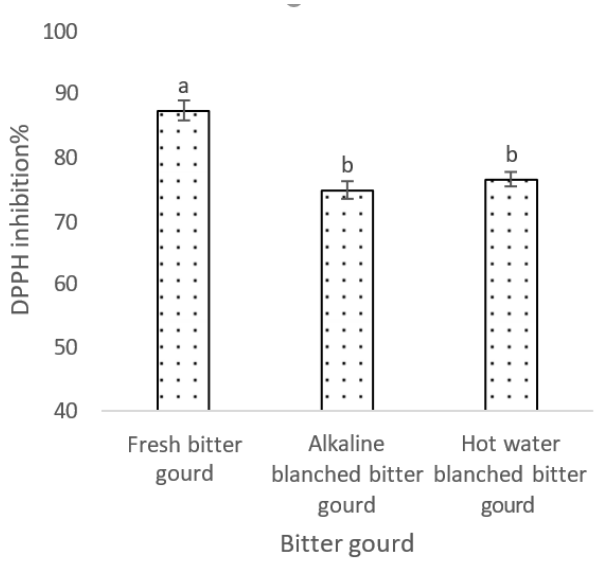

Figure 4 Effect of pretreatments on the Antioxidant activity of bitter gourd
Note:
*Vertical line plus bars indicates means ± standard error (n=3)
*Different letters in each bar indicate values are significantly different

Effect of pretreatment methods on enzyme inhibition of alpha-glucosidase and alpha-amylase

The α-amylase and α-glucosidase inhibition properties of hot water blanched bitter gourd extract were significantly lower ($p<0.05$) than fresh bitter gourd extract and alkaline blanched bitter gourd extract, whereas no difference in these properties was observed between fresh bitter gourd extract and alkaline blanched bitter gourd extract. The results are shown in **Figure 5**. Eh-Shian *et al*. (2015) also reported the enzyme inhibition potentiality of ethanolic extract of raw matured bitter gourd fruit, and the obtained value was similar to the anti-diabetic drug acarbose. However, the percentage inhibition is lower than fifty percent inhibition so further analysis for IC_{50} was not done. The reason behind the low percentage inhibition might be associated with the fact that the longer the storage

time of the extract and, the low extract concentration, the performance of the extract regarding its functional property is decreased with the decrease in its phenolic compounds (Iwona and Marta, 2007). Irondi *et al.* (2017) also reported that hot water blanching significantly decreased the alpha-amylase and alpha-glucosidase inhibition properties of *Adansonia digitate* leaves, which was in contrast to our observations. Oboh *et al.* (2012) found that enzyme inhibition properties of fluted pumpkin leaves decreased by blanching. The authors reviewed that blanching results in the loss of phenolic compounds, resulting in reduced enzyme inhibition properties. The instability of ascorbic acid, phenolic compounds, and other bioactive compounds in alkaline conditions (Friedman and Jurgenes, 2000), might decrease the enzyme inhibition properties in case of alkaline blanching.

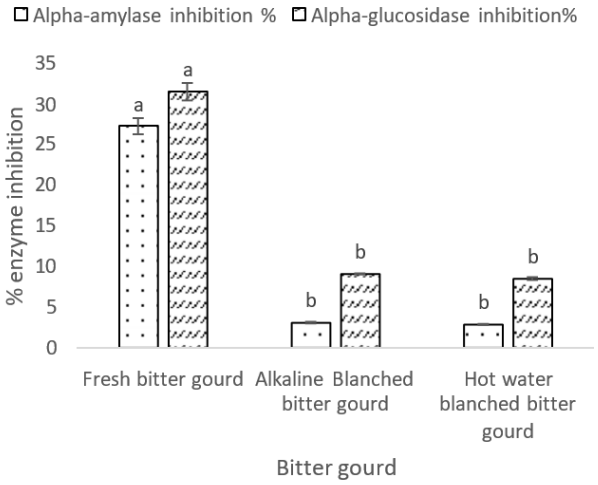

Figure 5: Effect of pretreatments on alpha-amylase and alpha-glucosidase inhibition percentage
Note:
*Vertical line plus bars indicates means ± standard error (n=3)
*Different letters in similar pattern bars indicate values are significantly different

CONCLUSIONS

The inference from the results can be drawn that hot water blanching increased the carotenoid content whereas chlorophyll retention was higher in alkaline blanched bitter gourd. However, ascorbic acid, anti-oxidant activity, alpha-amylase inhibition%, and alpha- glucosidase inhibition% were reduced in bitter gourd by both alkali blanching and hot water blanching significantly. Raw bitter gourd has better anti-diabetic properties compared to blanched bitter gourd. Since the enzyme inhibition percentage was lower than 50%, extract concentration can be further increased to identify the IC_{50} value for enzyme inhibition properties.

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