DETERMINATION OF THE OPTIMUM CONDITIONS FOR UREASE INHIBITTION EXTRACTED FROM SOME LOCAL PLANTS

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ABSTRACT

In the current study, four types of plants commonly used namely Soybean, chickpea, bean, pea were obtained and screened for urease activity, among this plants, chickpea was chosen with maximum enzymatic activity, and it had the highest productivity of urease enzyme (1243 U/mg protein). Also sodium acetate buffer (0.2 M, pH 5.0) was chosen as a best extraction buffer with specific activity 1460 U/mg protein. The optimum extraction ratio represented by 1:8 (w:v) after 15 min, it was given 1988 U/mg protein. As well as four types of plants include garlic, red onion, green onion and cabbage were used to select the optimum plant material that inhibited urease enzyme. Cabbage was chosen, it had the highest inhibition activity of the enzyme (41%). Also tris buffer (0.2 M, pH 9) was selected as a best extraction buffer of plants inhibitor with inhibition activity 80%. The optimum extraction ratio represented by 1:8 (w:v) after 60 min, it was given 86% enzyme inhibition activity.

Keyword: chickpea, cabbage, plant inhibitor, urease enzyme, optimization

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تحديد الظروف المثلى لتثبيط اليورييز المستخلص من بعض النباتات المحليه

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المستخلص

في هذه الدراسة تم اختبار اربعه أنواع نباتية مختلفة لغرض اختيار النبات الامثل كمصدر لانزيم اليورييز شملت (فول الصويا، الحمص، الباقلاء والبزاليا). بينت النتائج ان نبات الحمص هو الأمثل من بينت النباتات المنتخبة، حيث امتلك اعلى فعالية انزيميه (1243 وحدة /ملغم بروتين)، كذلك استخدام بفر خلات الصوديوم بتركيز 0.2 مولاري وبدالة حموضة 5 كأفضل دارئ للاستخلاص بفعالية 1460 وحدة /ملغم بروتين. كما أظهرت النتائج ان نسبة 1:8 (وزن: حجم) هي أفضل نسبة لاستخلاص الانزيم من النبات المنتخب بعد 15 دقيقة اذ وصلت الفعالية الانزيميه الى 1988 وحدة /ملغم بروتين. درس ايضا تأثير بعض النباتات المحليه في تثبيط انزيم اليوريز حيث بينت النتائج ان نبات اللهانه امتلك اكبر تاثير تثبيطي للانزيم (41%) مقارنه ببقيه النباتات (البصل الابيض والبصل االاحمر والثوم واللهانه) وعند دراسه الظروف المثلى للمثبط الانزيمي وجد ان اعلى فعاليه تثبيطيه كانت 86% عند استخدام بفر الترس القاعدي بتركيز 0.2 مولاري وبداله حموضه 9 بنسبه استخلاص 1:8 بعد ساعه واحدة من الاستخلاص.

كلمات مفتاحيه: حمص ، لهانة، مثبطات نباتية، ظروف مثلى

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INTRODUCTION

Urease (EC 3.5.1.5; urea amidohydrolase) is a nickel-dependent enzyme that catalyzes the hydrolysis of urea to ammonia and carbamate; the latter decomposes spontaneously, producing another molecule of ammonia and carbon dioxide (15). It's a key enzyme benefiting bacteria *H. pylori* and *Proteus mirabilis* through making its persistence possible in the acidic environment of the stomach and as a result, cause gastrointestinal diseases, in particular gastritis, duodenal, peptic ulcer, and gastric cancer (3). Additionally, urease activity leads to other disease like urinary stones, pyelonephritis (24). Urease is produced by a wide variety of plants, fungi, bacteria and invertebrates, enabling them to utilize urea as a nitrogen source (18). Research has indicated that urease of *H. pylori* and *Proteus mirabilis* is located in the cytoplasm in freshly prepared cultures and in the outer membrane in older cultures (11). In addition to pathogenicity from these bacteria, evidence indicates that ammonia generated by urease can cause injury to the gastroduodenal mucosa (23). Specific inhibition of urease activity has been proposed as a possible strategy to inhibit this microorganism (20). Many naturally occurring compounds found in dietary and medicinal plants, herbs, and fruit extracts have been shown to possess antimicrobial activities (25). According to the literature, most of the prescribed medicines and antibiotics for the mentioned disease treatment, not only evince adverse effects but also the bacteria grow resistance against (6). Furthermore, application of some compounds and especially synthetic ones for controlling urease function has been banned due to their toxicity and low chemical and physical stability in the natural environment (4). Medicinal plants have long been applied as remedies to cure diseases which nowadays are known as viral infections. This study has focused on monitoring and evaluating the ability of 4 herbs for their possible inhibitory activity against plant urease. And determination the optimum extraction condition for both enzyme and inhibitor from some local plants.

MATERIALS AND METHODS

Plants: The plants used throughout this study were locally available in the market. *Glycine max* (Soybean), *Cicer arietinum* (chickpea), *Vicia faba* (bean), *Pisum sativum* (pea) were used as a source of material to screen for urease enzyme activity and *Allium sativum* (garlic), *Allium cepa* (red onion), *Allium cepa* (green onion) and *Brassica oleracea var. capitata* (Cabbage) were used as a source of material to screen for urease inhibitor activity.

Extraction and recovery of urease enzyme

One gm of each plant were homogenizing separately with 8 ml of 0.2 M of phosphate buffer pH 7.0, the mixing was done by using mortar for 15min at room temperature. The slurry was centrifuged at 10000 rpm for 10 min and filtered through a whatman No.1 filter paper for removing any cell debris that remains in the preparation (12). The clear supernatant obtained represented the crude extract, and was assayed for urease enzyme.

Estimation the standard curve of NH_4Cl

In order to prepare NH_4Cl standard curve for the urease assay, serial concentrations (100-500 µM) from stock solution of NH_4Cl (0.5 mM) were prepared in triplicate. The standard curve of NH_4Cl was plotted between the ammonium chloride concentration (µM) and the corresponding absorbance of standard ammonium chloride at 625 nm as shown in (Fig. 1).

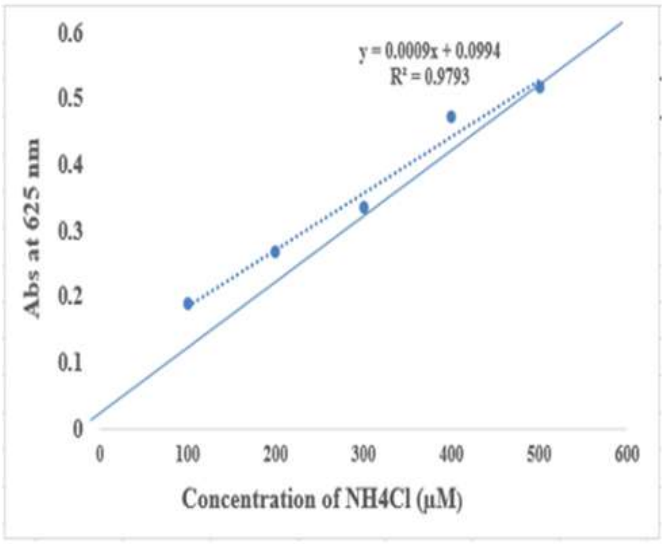

Figure 1. Ammonium Chloride Standard Curve

Determination urease assay

The activity of urease enzyme was measured by modified Berthelot reaction (14) which depend on standard curve of NH_4Cl

throughout liberated of ammonia by the enzyme. All glassware sterilized should be washed with warm dilute hydrochloric acid and rinsed thoroughly with distilled and de-ionized water. The reaction mixture (11 ml) contained 1 ml of crude enzyme with 10 ml of substrate [5 ml from 0.01 M of reagent A (5gm of phenol and 25 mg of sodium nitropruside) with 5 ml from 0.01 M of reagent B (2.5gm of sodium hydroxide with 8.4 ml of sodium hypochloride)] in 500 ml distilled water for each, for 1 hr. in water bath at 37 °C, reaction was stopped with heating at 80 ^{o}C for 5 min. Urease activity was detected by measuring the absorbance increase at 625 nm. Enzymatic activity unit known as the amount of enzyme liberated 1 µmole from ammonia in one minute under optimum conditions as follows:

$$\textbf{Urease Activity} = \frac{\textbf{Ab}}{\textbf{Slope}} \times \textbf{60} \times \textbf{14}$$

Where: Ab/ slope: Concentration of ammonia, **60:** Time of reaction, **14:** Constant. Protein concentration was measured according to the method described by Bradford (1)

Determination of optimum condition for extraction urease enzyme

Type of plant material

Soybean, chickpea, bean and pea were extracted by using 0.2 M of phosphate buffer pH 7.0. One gm of each plant was mixing separately with 8 ml (1:8) of buffer solution using mortar for 15 min at room temperature, then centrifugation at 8000 rpm for 10 min and filtered through a whatman No.1 filter paper. The filtrate was taken for determination the enzyme activity, protein concentration and specific activity.

Type of extraction buffer

Chickpea was homogenized with different types of buffers for 15 min at 30^{o}C for urease extraction. These buffers are 0.2 M sodium acetate buffer (pH 4 and 5), 0.2 M sodium-phosphate buffer (pH 6 and 7) and 0.2 M Tris-base buffer (pH 8, and 9). The enzyme activity, protein concentration and specific activity were assayed in each experiment.

Extraction ratio

In order to investigate the best chickpea ratio, different ratios of buffers were selected to extract the enzyme included 1:4, 1:8, 1:12, 1:15 and 1:20 (w:v) by mixing 1gm of chickpea with each extraction ratio for 15 min, then centrifugation at 10000 rpm for 10 min and filtered through a whatman No.1 filter paper. The enzyme activity, protein concentration and specific activity were determined.

Extraction time

In order to determine the optimum extraction time for urease enzyme, the time course for extraction was (5, 15, 30 and 60) min by mortar, then centrifugation at 10000 rpm for 10 min and filtered through a whatman No.1 filter paper. The filtrate was taken for the determination the enzyme activity, protein concentration and specific activity.

Extraction and recovery of urease inhibitor

One gm of each plant were homogenizing separately with 8 ml of 0.2 M of phosphate buffer pH 7.0, the mixing was done by using mortar for 15min at room temperature. The slurry was centrifuged at 10000 rpm for 10 min and filtered through a whatman No.1 filter paper for removing any cell debris that remains in the preparation. The clear supernatant obtained represented the crude extract, and was assayed for urease inhibitor activity (13).

Determination of urease inhibitory activity

A crude extract of plant was incubated with a known volume of urease enzyme (chickpea) by 1:1 ratio for 30 min at room temperature, thereafter the urease activity was estimated, the control representing 100% enzyme activity were conducted in the same manner replacing the plant extract with distilled water. The remaining activity is the present inhibitory activity of the enzyme with respect to the present enzyme activity without inhibitor according to the following equation:=

$$\textit{Remaining enzyme activity}\ (\%) = \frac{\textit{Enzyme without inhibitor} - \textit{enzyme with inhibitor}}{\textit{Enzyme without inhibitor}} \times \textbf{100}$$

Optimum conditions of urease inhibitor

Type plant material: Different types of plants include (garlic, red onion, green onion and cabbage) were used in this study to select the optimum plant material that inhibited urease enzyme. One gm of each plant was homogenizing with 8 ml (1:8) of 0.2M of phosphate buffer, the mixing was crushed by mortar for 15 min at room temperature. The mixture was centrifuge at 10000 rpm for 10 min. The clear supernatant obtained

represented the crude extract and was assayed for urease inhibitor activity.

Type of extraction buffer

Cabbage was homogenized with different types of buffers for 15 min at 30°C for urease inhibitor extraction. These buffers are 0.2 M sodium acetate buffer (pH 4 and 5), 0.2 M sodium-phosphate buffer (pH 6 and 7) and 0.2 M Tris-base buffer (pH 8, and 9). The clear supernatant obtained represented the crude extract and was assayed for urease inhibitor activity.

Extraction ratio

In order to investigate the best cabbage extraction ratio, different ratios of buffers were selected to extract the inhibitor included 1:4, 1:8, 1:12, 1:15 and 1:20 (w:v) by mixing 1 gm of cabbage with each extraction ratio separately for 15 min, then centrifugation at 10000 rpm for 10 min and filtered through a whatman No.1 filter paper. The clear supernatant obtained represented the crude extract and was assayed for urease inhibitor activity.

Extraction time

In order to determine the optimum extraction time for urease inhibitor, the time course for extraction was (5, 15, 30 and 60) min by mortar, then centrifugation at 10000 rpm for 10 min and filtered through a whatman No.1 filter paper. The clear supernatant obtained represented the crude extract and was assayed for urease inhibitor activity.

RESULTS AND DISCUSSION

Optimum conditions for urease extraction

Different bioprocess conditions that effect on urease extraction from some plants were optimized for maximum enzyme production, a large number of factors affect the extraction of urease such as types of plant material, types of buffer, extraction ratio and extraction time etc. Hence, optimization of these conditions helps to reduce extraction cost and to obtain a high yield of urease enzyme.

Type of plant material

The influence of plant type on the enzyme extraction was determined by four types of plants commonly used soybean, chickpea, bean and pea by using 0.2 M of phosphate buffer pH 7.0, it was observed from the results that urease extraction was found to be maximum in chickpea with specific activity of 1243 U/mg, while the specific activity of urease in bean, pea and soybean were 32.6, 505 and 595 U/mg respectively (Fig. 2). El-Hefnawy *et al.,* (5) founded the specific activity of urease extracted from *Pisum Sativum* L. seeds was reached to 454.5 U/mg protein.

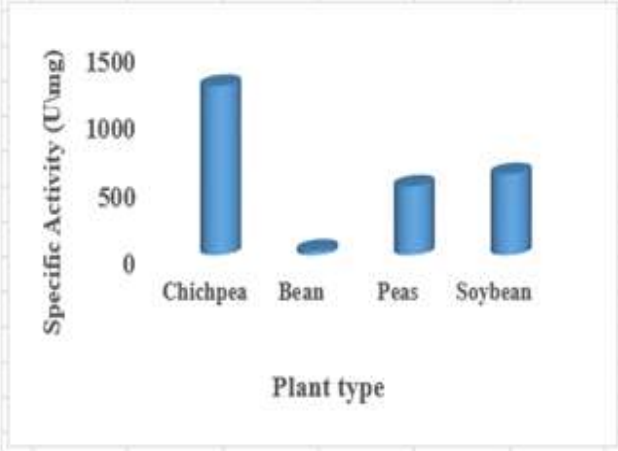

Figure 2. Effect the types of plant on urease extraction using phosphate buffer pH 7 at 30°C and for 15 min

Type of extraction buffer

The specific activity of urease was estimated after extraction using different buffers, and the results were illustrated in (Fig. 3). These results show that sodium acetate buffer (0.2 M, pH 5.0) was best extraction buffer with specific activity 1460 U/mg protein. While other buffer with different pH were given low specific activity. pH effected of enzymatic extraction also activity and stability by the fact that protein structure of an enzyme molecule is influenced by the acidity and alkalinity of the solution because of the differences in ionization state of various amino acid residues through changing the charge state of the solute. If the pH of the solution is such that a particular molecule carries no net electric charge, the solute often has minimal solubility and precipitates out of the solution (19).The pH of enzyme environment affects the activity of the enzyme in several ways. Firstly each enzyme has its own optimum pH, at which the maximum enzyme activity, but the enzyme is stable within certain limits under and above the optimum. Secondly, enzyme stability is influenced by environmental pH, at extremes acidity or alkalinity the enzyme may be denatured. Thirdly, the reaction mixture pH may effects on association of substrate with the enzyme (9).There are many studies that

used different buffers with different pH values for urease extraction from different sources, Narjis (21) pointed to extraction of urease from *Proteus mirabilis* by phosphate buffer (20 Mm, pH 7.5).

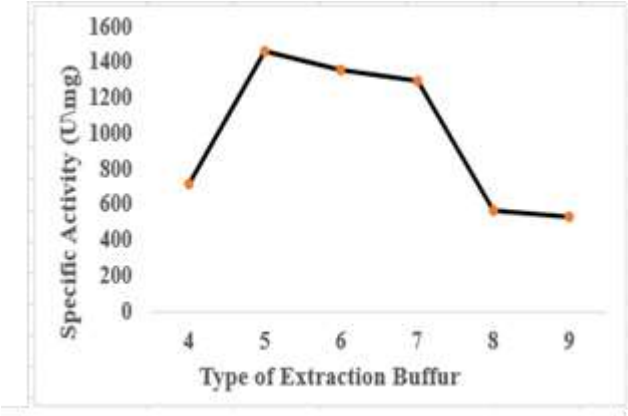

Figure 3. Effect the types of buffers on urease extraction from Chickpea at 30˚C for 15 min

Extraction ratio

Five extraction ratio were chosen 1:4, 1:8, 1:12, 1:15 and 1:20 (w:v) to determine the best extraction ratio of urease by using sodium acetate (0.2 M, pH 5.0). The highest specific activity was measured for crude extract at 1:8 ratio, it was reached to 1864 U/mg protein, while other ratio were gave the following specific activities 1089.6, 1759, 1210 and 856.7 U/mg protein respectively (Fig. 4). Variation in native extract ratio can result in a variable amount of herbal material used in an extract. In some instances, variation in the equivalent dry weight of a herb used in herbal preparation. Where a large proportion of extractable material is obtained from a herbal material, the native extract ratio will be low. For example, a low native extract ratio of 2:1 indicates that 50 percent of the extractable matter obtained from the herb is represented in the final extract. However, when only a small amount of extractable material is obtained using a particular extraction profile, the native extract ratio will be high e.g. a native extract ratio of 20:1 indicates that only 5 percent of extractable components are obtained (2).

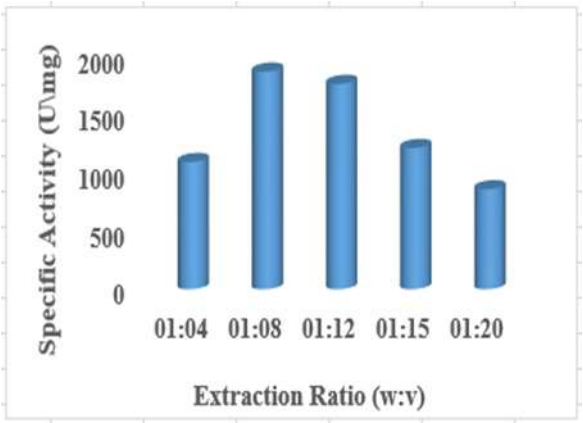

Figure 4. Effect of extraction ratio on urease extraction from Chickpea at 30˚C for 15 min

Extraction time

Four extraction periods were chosen (5, 15, 30 and 60) min to determine the best extraction time of urease by using sodium acetate (0.2 M, pH 5.0). The highest specific activity was measured for crude extract after 15 min, it was reached to 1988.5 U/mg protein, compared to the lower of specific activity after 5, 30 and 60 min 710, 926 and 900 U/mg protein respectively (Fig. 5). Granick (8) found that the extraction time for plant urease was varies between 10 and 15 minutes.

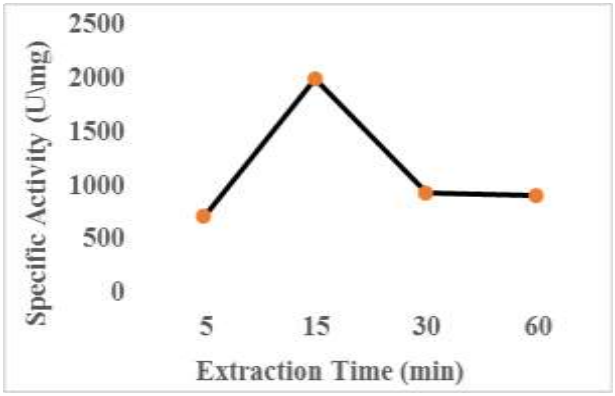

Figure 5. Effect of extraction time on urease extraction from Chickpea at 30˚C for 15 min.

Optimum conditions for urease inhibitor extraction

Type of plant material

Four types of plants, commonly used in this study, namely (garlic, red onion, green onion and cabbage) were obtained and screened for their enzymatic inhibition activity. Herein, urease inhibitory activity of 4 natural extracts were evaluated among which 2 extracts were elucidated as the most potent ones including cabbage and red onion. As it is presented in (fig. 6). Natural therapy has recently absorbed many attentions to itself. Although, herbs have always been applied for the treatment of a vast

variety of diseases throughout the history, but the drawbacks of the synthetized medicines, especially the side effects coming along their consumption, has again arisen a significant interest among scientists to more precisely monitor and extract herbal active compounds pharmacological properties from these appropriate sources of active chemicals to be used as templates for designing and/or developing more effective compounds, preferably with less side effects (10). Gastrointestinal disorders, particularly gastritis, duodenal, peptic ulcer, and gastric cancer are mainly caused as a result of *H. pylori* infection. This bacterium agitates human pathogenic state and causes diseases from which the most common ones are urinary stone formation, peptic ulcer, pyelonephritis, and hepatic coma. *H. pylori* habitance in the acidic medium of the stomach is highly dependence on the urease enzyme activity. A unique feature of *H. pylori* infection is its persistence as a result of urease enzyme buffering activity. The enzyme changes the stomach medium to an endurable environment for the bacteria via neutralizing gastric acid through hydrolysis of urea to form carbon dioxide (CO_2) and ammonia (NH_3) (16).

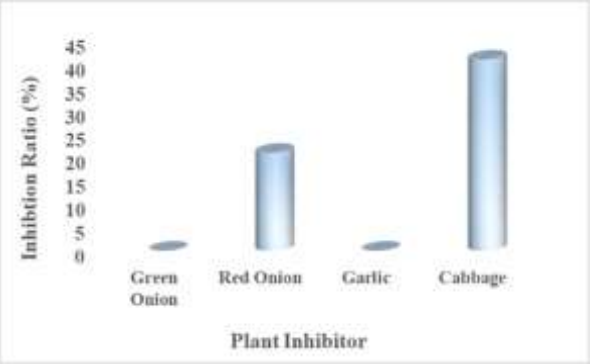

Figure 6. Effect the types of plants materials on urease inhibition using phosphate buffer pH 7 at 30˚C and for 15 min

Type of extraction buffer

The inhibition activity of urease enzyme was estimated after extraction, using different buffer, and the results were illustrated in (Fig 7).The higher urease inhibition value was obtained at acidic and alkalinatic buffering solution with maximum inhibitory effect (80%) at pH 9 using 0.2 M of tris buffer. While in neutral pH the urease inhibitor activity was reached to minimum value (50 and 41) %. Shi with coworkers (22) extracted 12 chines herbs as sources of urease inhibitor by using 95% ethanol or water (400 mL each). Ten grams of different ratios of oregano/cranberry powder mixture using as urease inhibitor were extracted by 90 ml of water to make concentrated stock extracts and stored at 4°C (17).

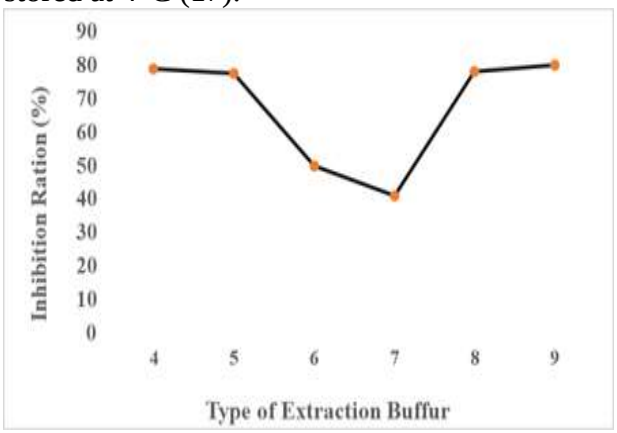

Figure 7. Effect the types of buffer on urease inhibitor extraction from Cabbage at 30˚C for 15 min

Extraction ratio

Five extraction ratio were chosen 1:4, 1:8, 1:12, 1:15 and 1:20 (w:v) to determine the best extraction ratio on extraction the inhibitor material from cabbage by using 0.2M tris buffer pH 9. The best result was obtained at extract ratio 1:8 in rate of 85%, While the other gave (83, 82.5, 80 and 79.5) % for 1:4, 1:12, 1:15 and 1:20 respectively (fig. 8). Eighty grams of peat was extracted with 800 ml of alkaline potassium hydroxide (KOH) at a concentration of 0.1 mol L-1, determining a mass/ volume (m/v) ratio of 1:10 for urease inhibitor extraction. The material was left for a two-hour rest and then centrifuged, extraction was performed at room temperature (25-30°C) (7).

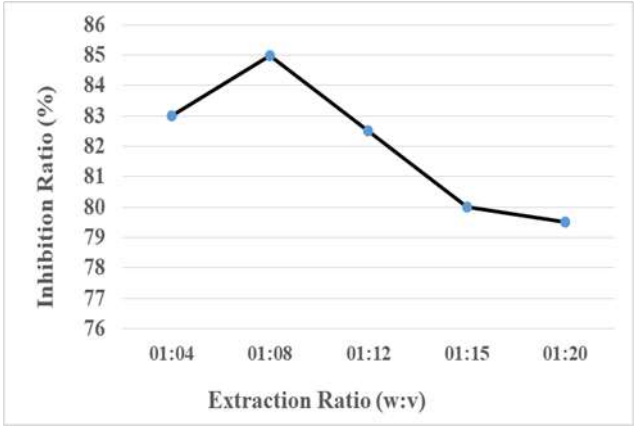

Figure 8. Effect of extraction ratio on urease inhibitor from Cabbage using tris buffer pH 9 at 30˚C for 15 min

Extraction Time

Four extraction periods were chosen (5, 15, 30 and 60) min to determine the best extraction time on extraction the urease inhibitor by using tris buffer (0.2 M, pH 9). The highest inhibition activity of cabbage was measured in 60 min; it was reached to 86 %, compared to the lower of inhibition activity in 5, 15 and 30 min (68, 70 and 73) % respectively (Fig. 9). Shi with coworkers (22) extracted 12 chines herbs as sources of urease inhibitor by using 95% ethanol or water (400 mL each) by refluxing for 4 h.

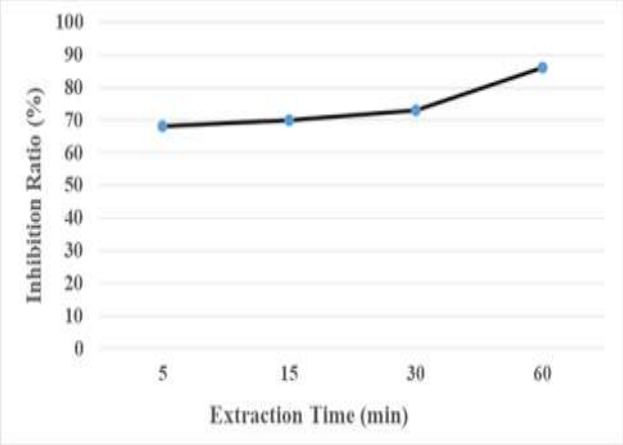

Figure 9. Effect of the extraction time on urease inhibitor from Cabbage using tris buffer pH 9 at 30˚C

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