

## ASSESSMENT OF PURIFIED COLLAGENASE INHIBITION ACTIVITY FROM *STAPHYLOCOCCUS AUREUS* HN77 BY SOME LOCAL PLANTS EXTRACT

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### ABSTRACT

This study was aimed to inhibition of purified collagenase produced from *S. aureus* by local plants inhibitors (fruits and leaves). Sixty two local isolates of *S. aureus* which were isolated and identified by biochemical test subjected to the primary and secondary screening processes to select the active *S. aureus* isolate for collagenase production. Among these isolates, forty nine isolates with maximum zone of hydrolysis ability in primary screening (the ratio of Z/G more than 10 mm) were selected for secondary screening. It has been found that *S. aureus* HN77 had the highest productivity of the enzyme (49.122 U/mg protein). The selected isolate with highest level of collagenase activity was identified as *S. aureus* HN77 according to vitek test. The optimum conditions of collagenase production by selected isolate using submerged fermentation were obtained with medium (D) contain collagen as the best production medium, 2% starch as the best carbon source, 2% yeast extract/peptone as nitrogen source, temperature 37 °C and pH 7, after 48 hr. of incubation period, the specific activity was reached to 1502.15 U/mg. The enzyme was purified by gel filtration chromatography using Sephadex G-150 then concentration by sucrose. The results shown an increase in final purification folds 6.1 time with an enzyme yield of 85.2%. The purified enzyme was exhibited maximal activity and stability at pH 7.0. The optimal temperature for purified enzyme activity was 45°C and it was stable until 45°C. The highest rate of enzyme specificity found with collagen. Collagenase was inhibited with local plants extract (fruit and leaves). Fruit of black fig extract inhibit 90% of collagenase activity while the inhibition efficiency of collagenase with grape leaf was reached to 12%.

**Keywords:** bacteria, enzyme, inhibitors, plants extracts, fruit, leaves

صالح وحسين

مجلة العلوم الزراعية العراقية - 2022: 53(5): 1035-1047

تقدير الفعالية التثبيطية لانزيم الكولاجينيز المنقى من بكتريا *S. aureus* HN77 بوساطه مستخلصات بعض النباتات المحليه

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### المستخلص

هدفت الدراسة الحاليه الى تثبيط انزيم الكولاجينيز المنقى من العزله المحليه لبكتريا *S. aureus* بوساطه مستخلصات بعض النباتات المحليه (فواكه واوراق)، تم عزل 62 عزله من بكتريا *S. aureus*، شخضت هذه العزلات بوساطه الاختبارات الكيموجيويه وخضعت لعملية الغريله الاولى باستخدام الوسط الصلب لتحديد العزله الاكفا في انتاج الانزيم، شخضت 49 عزله كفاءة اعتماده على نسبه (النمو/الهاله الشفاهه) على الوسط الصلب. ادخلت هذا العزلات الى الغريله الثانويه باستخدام الوسط السائل، انتخبت العزله *S. aureus* HN77 بكونها الاكفا في انتاج الانزيم اذا بلغت الفعاليه النوعيه 49.122 وحدة / ملغم بروتين. حددت الظروف المثلى لانتاج الانزيم اذا بلغت 1502 وحدة / ملغم بروتين باستخدام الوسط (D) الحاوي على الكولاجين كوسط امثل للانتاج المجهز ب 2% نشأ كمصدر كربوني امثل و 2% مستخلص خميرة/ ببتون كمصدر نتروجيني امثل بدرجة حرارة 37م ويرقم هيدروجيني 7 بعد 48 ساعه من الحضانه. نقي انزيم الكولاجينيز باستخدام كروماتوغرافيا الترشيح الهلامي بوساطه هلام السيفاكريل ج-150 بعدها ركز باستخدام السكروز بعدد مرات تنقيه 6.1 وبحصيله انزيمييه 85.2%. اظهر الانزيم المنقى اعلى فعاليه وثبات انزيمي عند رقم هيدروجيني 7، كما اظهر الانزيم المنقى اعلى فعاليه وثباتيه عند درجة حرارة 45 م باستخدام الكولاجين كافضل مادة اساس. ثبط الانزيم المنقى باستخدام مستخلصات بعض النباتات المحليه ( فواكه واوراق) اذا بلغت اقصى نسبه تثبيط للانزيم المنقى عند معاملته مع مستخلص فاكهه التين الاسود اذا بلغت 90% كما بلغت 12% عند معاملته مع مستخلص اوراق العنب.

كلمات مفتاحية: بكتريا، انزيم، مثبطات، مستخلصات نباتية، فواكه، اوراق

## INTRODUCTION

Collagenase are the important virulence factors, which play a crucial role in the global degradation of the extracellular matrices of animals, and humans due to their collagen degradation ability (13). Collagenase are widely distributed and found in fungi, higher plant, bacteria and insects. Microbial collagenases (EC:3.4.24.3) possess broad substrate specificities and degrade both water-soluble/insoluble native and denatured collagens in its triple-helical conformation. Microbial collagenases can degrade each polypeptide chain of collagen at multiple sites, and production has been reported from some pathogenic bacteria and fungi (9). *Staphylococcus aureus* is one of essential bacteria that produced collagenase enzyme, the genus *S. aureus* currently comprises more than 50 species. These small, hardy bacteria are normal inhabitants of the skin and mucous membrane in many animal species, including humans. *S. aureus* is a gram-positive spherical bacterium, 0.4-1.2µm in diameter, which occurs in microscopic clusters resembling grapes (30). However, could extracted collagenase from *Staphylococcus* spp, the various parameters optimized for maximum production of collagenase includes; selection of optimized medium, optimal pH, incubation temperature, inoculum size and age, incubation time (19). The purification of microbial collagenase is complicated due to the presence of multiple forms and other proteases with similar physical and chemical characteristics, the purified form of collagenase is required to study its biochemical properties, enzyme structure, and catalytic mechanism. The purification of microbial collagenase was make by various procedures (ammonium sulfate precipitation, ultra-filtration, immobilized metal affinity chromatography, gel filtration chromatography, ion exchange chromatography, etc.). Inhibition of collagenase plays a vital role in protecting the unbalanced turnover of collagen in human inflamed skin. Affects many chemical compounds in the activity of enzymes, as some of these substances have an activating of enzyme called (activators), and some of them inhibits this enzyme called (inhibitors), like

MnCl<sub>2</sub>, CaCl<sub>2</sub>, ZnCl<sub>2</sub>, EDTA, and Cysteine (24). Plants have been widely investigated and found to have anti-collagenase activity, for instance polyphenols isolated from green tea (*Camellia sinensis*), Also isolated from persimmon (*Diospyros kaki*), rice grains (*Oryza sativa*), these plant extracts were investigated for the presence of various phytochemicals such as (alkaloids, tannins, saponins, steroids, carbohydrates, glycosides, amino acids and flavonoids etc.) (16). The goal of this study was to production, purification and characterization of collagenase produced from local isolate of *S. aureus* HN77, Then estimated the inhibition activity of this enzyme when treated with local plants fruits and leaves).

## MATERIALS AND METHODS Chemicals

Brain heart agar, Brain heart broth, Nutrient agar, Nutrient broth, Mannitol salt agar, Urease agar, Peptone water, and all other reagent grand chemicals were purchased from Hi-Media and Sigma Aldrich, India.

## Sample Collection and bacterial isolation

One hundred and seventy samples which were previously isolated from medical location, like Al-Kadhimiya hospital, Al- Yarmouk hospital / Burn Center and Al- Hariri hospitals, during periods (15 September to 15 December 2020). The provenience of these samples from skin, wound, burn, blood, urine, tonsillitis, ear. These samples were cultivated on brain heart broth then brain heart agar thereafter on mannitol salt agar medium as a selective medium for *Staphylococcus* sp. And to identified *S.aureus* some biochemical tests and Vitek 2 test were done. The identified *S. aureus* isolates were prepared for screening experiment. According to the method described by Anatoly (2), these isolates were evaluated for collagenase production.

## Screening *S. aureus* isolates for collagenase production

### Primary screening (qualitative screening):

Sixty-two *S. aureus* isolates were screened using a plate test with collagen solid medium to find the best ones that produced collagenase, this medium containing 1gm collagen, 2.0 gm of glucose, 0.5 gm (Na<sub>2</sub>HPO<sub>4</sub>), 0.5 gm (NaH<sub>2</sub>PO<sub>4</sub>), 0.05 gm (CaCl<sub>2</sub>) and 2gm of agar-agar for 100 ml of distilled water. It was mixed properly, pH was

regulated at 7.0. A single colony of bacterial isolate that had previously been activated in brain heart broth was spotted to the middle of collagen agar plate and then incubated at 37°C for 48 hrs. Clear zone hydrolysis around the colony was an indication of collagenase secretion (23).

**Secondary screening (Quantitative screening):** Forty nine isolates with maximum productivity based on primary screening were selected and cultivated on collagen solid media. A 250 ml flask containing 50 ml of submerged medium composed of (1.0 gm of collagen, 2 gm of glucose, 0.5 gm of Na<sub>2</sub>HPO<sub>4</sub>, 0.5 gm of NaH<sub>2</sub>PO<sub>4</sub>, 0.05 gm CaCl<sub>2</sub>) for 100 ml distilled water with pH 7.0. was inoculated with 1.0 ml of overnight culture isolates with a bacterial concentration of 3×10<sup>9</sup> cell/ml. The flasks were incubated at 37°C in the rotary shaker incubator (120 rpm). After 48 hrs. of incubation, culture of each flask was centrifugation at 8,000 rpm for 10 min. The clear supernatant was considered as a crude enzyme and it was assayed for collagenase activity and protein concentration. The enzyme activity and protein concentration were run in duplicate.

#### **Collagenase activity**

Collagenase activity method described by (28), using collagen as a substrate, the hydrolysis of collagen was detected by measuring the absorbance at 280 nm using spectrophotometer. Protein concentration measured according to the method described by Bradford (10)

#### **Optimum conditions for collagenase production**

**Effect of fermentation media:** Four different types of media were used to determine the optimal medium for collagenase production. Including: (A) glucose 20g/L, collagen 19 g/L, CaCl<sub>2</sub> 0.05g/L, NaH<sub>2</sub>PO<sub>4</sub> 0.5g/L and K<sub>2</sub>HPO<sub>4</sub> 0.5g/L. (B) KH<sub>2</sub>PO<sub>4</sub> 2g/L, K<sub>2</sub>HPO<sub>4</sub> 7g/L, MgSO<sub>4</sub>.7H<sub>2</sub>O 0.1g/L, citrate.2H<sub>2</sub>O 0.05h/L, yeast extract 1g/L, CaCl<sub>2</sub>.2H<sub>2</sub>O 0.1g/L, and collagen 3g/L at pH 7.5 . (C) 0.1% (w/v) NH<sub>4</sub>Cl, 0.06% (w/v) MgSO<sub>4</sub>.7H<sub>2</sub>O, 0.435% (w/v) K<sub>2</sub>HPO<sub>4</sub>, 0.01% (w/v) glucose, and 1.0% (v/v) mineral solution (100 mg FeSO<sub>4</sub>. 7H<sub>2</sub>O, 100 mg MnCl<sub>2</sub>.4H<sub>2</sub>O, 100 mg ZnSO<sub>4</sub>.H<sub>2</sub>O, 100 mg CaCl<sub>2</sub>.H<sub>2</sub>O. (D) 1.0% (w/v) peptone, 0.3% (w/v) yeast extract, 2.0% (w/v) glucose,

mineral solution (100 mg FeSO<sub>4</sub>.7H<sub>2</sub>O, 100 mg MnCl<sub>2</sub>.4H<sub>2</sub>O, 100 mg ZnSO<sub>4</sub>.H<sub>2</sub>O and 100 mg CaCl<sub>2</sub>.H<sub>2</sub>O). Erlenmeyer flasks (250) ml containing 50 ml of each tested medium in duplicate were autoclaved, then inoculated with 1.0 ml of overnight culture consisting of 3×10<sup>9</sup> cell/ml of selected isolate and incubated at 37°C for 48 hrs. in shaker incubator at 120 rpm, after incubation, the culture of each flask was filtrated by centrifuge and the enzyme activity, protein concentration, and specific activity of the filtrate were all measured (17).=

#### **Optimal carbon sources**

In (250 ml) Erlenmeyer flasks, 50 mL of optimal fermentation medium was made, and the pH was adjusted to 7.0. These flasks were autoclaved for 15 minutes at 121°C., then 2% of each sugar solution (maltose, sucrose, glucose, lactose and starch) was added in each flask separately. The flasks were inoculated with 1% (3×10<sup>9</sup> cell/ml) of overnight culture of *S. aureus* isolate and incubated in shaker incubator (120 rpm) at 37°C for 48 hrs. After the incubation, the cultures were centrifuged and the filtrate was taken for the determination enzyme activity, protein concentration and specific activity (17).

#### **Optimal nitrogen sources**

The collagenase production medium was supplemented with (2%) of several nitrogen sources, including yeast extract, collagen, peptone, NH<sub>4</sub>Cl, yeast extract, and peptone, and the pH was adjusted to 7.0. After sterilization, the flasks were inoculated with 1 % (3×10<sup>9</sup> cell/ml) of the chosen isolate, and the medium was incubated at 37°C for 48 hours in a shaker incubator (120 rpm). The culture was centrifuged from each flask after incubation, and the supernatant was utilized to measure enzyme activity, protein concentration, and specific activity. (17).

#### **Effect of pH value**

To determine the impact of initial pH value of the culture medium on enzyme production, after selected carbon and nitrogen source that gives the maximum productivity of collagenase A 250 ml Erlenmeyer flasks containing 50 ml of the selected medium were adjusted to different pH values (4.5, 5, 6, 6.5, 7, 7.5, 8, and 9), then the culture medium was inoculated with culture bacterial isolate at a concentration of 3×10<sup>9</sup> cell/ml and incubated

at 37°C for 48 hours in a shaker incubator (120 rpm). After incubation, the enzyme activity, protein concentration, and specific activity were determined in the supernatant from each flask. (17).

#### **Optimum incubation period**

Different incubation times were examined to determine the suitable incubation period for collagenase production. The selected medium was at optimum pH, inoculated with overnight culture of bacterial isolate ( $3 \times 10^9$  cell/ml) and incubated at 37°C for different incubation time; 24, 48, 72, 96 hrs. Enzyme activity, protein concentration, specific activity were measured for each times (17).

#### **Optimal temperature**

Different temperature was determined for suitable collagenase production. The selected medium was at optimum pH, inoculated with 1% of overnight culture from bacterial isolate ( $3 \times 10^9$ ) cell/ml and incubated at different temperature included 30, 37, 40, 45, and 50 for 48 hrs. After enzyme extraction with centrifugation, the enzyme activity, protein concentration and specific activity was measured for collagenase productivity (17).

#### **Extraction of crude collagenase**

After growing the selected isolate in the above-mentioned optimum production medium, the cells were separated by centrifugation at 8000 rpm for 10 minutes. The supernatant was then utilized to determine enzyme activity and protein concentration, as well as purification experiments.

#### **Purification of collagenase**

The collagenase was purified from *S.aureus* isolate (HN77) by gel filtration chromatography using sephadex G-150 followed concentration with sucrose.

#### **Separation of enzyme through sephadex G-150 column**

The column was attended to and packed according to the manufacturing company's directions (Pharmacia-Sweden). The crude enzyme was passed over a sephadex G-150 column, and the elution step was performed with sodium phosphate buffer solution 0.2 M, pH 7.0, flow rate 20 ml/h, 3 ml for each fraction. The enzyme activity of these fractions was determined after the protein fractions were assessed at 280 nm in each fraction. The volume of effective fractions was

measured, and subsequently activity and protein concentration were estimated. The volume was condensed and distributed in tubes before being frozen for a period of time.(12).

#### **Enzyme concentration by sucrose**

The purified enzyme solution was concentrated with sucrose in dialysis tubes, and the enzyme activity, protein concentration, and specific activity were all measured.

#### **Characterization of partial purified collagenase**

**Effect of pH on collagenase activity:** The effect of pH on activity of the partial purified collagenase was determined by preparing collagen as a substrate in different buffer solutions include 0.02 M sodium acetate buffer (pH 4, 5, 6). 0.02M sodium phosphate buffer (pH 7.0) and 0.02M tris-base buffer (pH 8, 9, 10). The activity was measured and the relation between an enzyme activities towered pH values were plotted to determine the optimum pH of collagenase activity.

**Effect of different pH on stability of partial purified collagenase:** Equal volume from partially purified enzyme was mixed with the buffers at different range of pH (4-10) at ratio of (1:1) the mixture was incubated in a water bath at 37°C for 15 min., then the samples were transferred directly to an ice bath. The enzyme activity was assayed, stop the reaction and estimate the remaining enzymatic activity (%), then the reaction between remaining activity % toward pH values was plotted to determine the optimum pH of collagenase stability.

**Effect of temperature on collagenase activity:** Partial purified collagenase activity was estimated at different range of temperature include (25, 30, 37, 40, 45, 50, 55 and 60) °C, and the relation between enzyme activates and temperature was evaluated to determine the optimal temperature of enzyme activity.

**Effect of temperature on collagenase stability:** Partially purified collagenase was incubated at different temperature (25, 30, 37, 40, 45, 50, 55 and 60°C) for 15 min. followed by incubation in optimal degree for collagenase activity (45°C). Then remaining activity (%) was estimated.

**Collagenase specificity:** The effect of different substrates on collagenase activity was examined by using different substrate include

(collagen, gelatin, casein) these substrate solutions were prepared by dissolving 1 gm from each substrate above in 100 ml of 0.02 M of sodium phosphate buffer at pH 7.0. The relation between enzyme activities toward types substrates was evaluated to determine the optimum substrates of collagenase activity.

**Effect of some chemical compounds on collagenase activity:** The impact of chemical compounds include (CaCl<sub>2</sub>, MgCl<sub>2</sub>, NaCl, NH<sub>4</sub>Cl, HgCl<sub>2</sub>, MnSO<sub>4</sub>, FeSO<sub>4</sub>, NiSO<sub>4</sub>, EDTA, and cysteine, on purified collagenase activity was study. Solution for each one was prepare at concentration 1mM and 5mM by dissolving in 0.2 M of phosphate buffer. Enzyme solution was incubated with metal ions solution at a ratio of 1:1 (v/v) for 15 min at 37 °C, then the enzyme activity was assayed and compared with control that represents the untreated enzyme, thereafter remaining activity % was calculated.

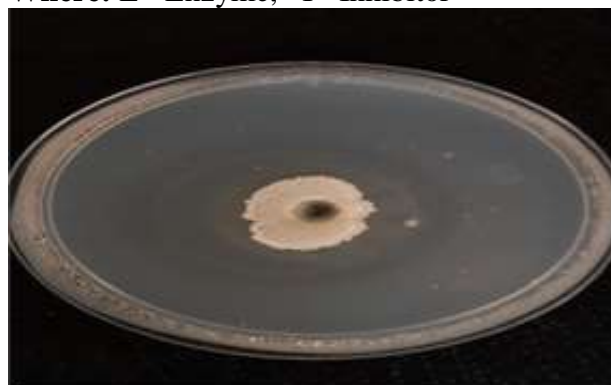
**Effect some plants sources on collagenase activity :** Plants were used through this study were locally available in a market, include: Grapes pomace (*vitis vinifera*), Pomegranate (*punica granatum*), green tea (*Camellia Sinensis*), Fig tree (*figus Carica*), Celery (*Apium graveolens*). Fruit and leaves were used as the source of inhibition material. Inhibitors extracts from fruits of grape and pomegranate were extracted with gauze, while inhibitors extracts from black and yellow fig fruits were extracted by blender with 0.02 M of phosphate buffer, then extracts of all fruits plants were centrifuged at 1000 rpm for 15 min. Also inhibitor from leave plants in addition of celery seeds were extracted by homogenizing one gm of each plants leaves with 10 ml of 0.02M of phosphate buffer, the mixing was crushed by mortar for 15 min at room temperature. The mixture was centrifuge at 10000 rpm for 15 min. The clear supernatant obtained from (fruits and leaves) represented as the crude extract and used to assay collagenase inhibitor activity, by incubated each plants extracted separately (fruit and leave) with known volume of enzyme by (1:1) ratio for 30 min at room temperature, thereafter collagenase activity was estimated. Control represented 100% of enzyme activity was conducted in same manner replacing. The inhibition activity was

estimated according to the following equation (27)

#### Collagenase inhibition activity

$$= \frac{E \text{ without I} - E \text{ with I}}{E \text{ without I}} \times 100$$

Where: E= Enzyme, I= Inhibitor



**Figure 1. Collagenase activity of *S. aureus* on solid medium at 37°C for 48 hr**

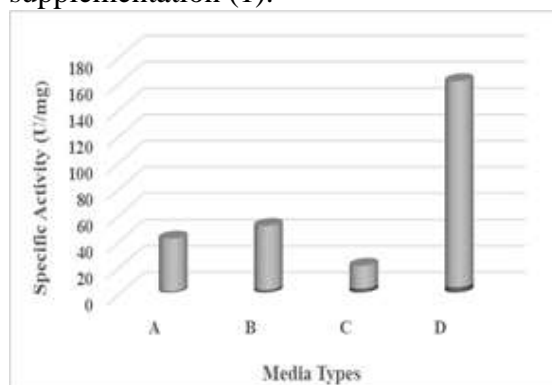
**RESULTS AND DISCUSSION Primary screening of collagenase (qualitative screening using collagen plate agar medium) :** Sixty-two bacterial isolates were subjected for qualitative screening method using collagen plate agar medium and clear hydrolysis zone assay was done. By calculating the ratio between the colorless zone diameter around the bacterial culture (Z) and the growing culture diameter (G), the efficiency of bacterial isolates was compared. Among all *S. aureus* isolates, Forty-nine isolates were collagenase producer whenever a clear zone of hydrolysis was screened around colony of isolate in the collagen plate agar (Fig. 1). The clear zone ratio of isolates was ranged from (10-40). These isolates were chosen for further secondary screening (quantitative screening). The diameter of the clear zone was different from one isolate to another. This result agreement with (26), when he examined the ability of *Pseudomonas* sp. and *clostridium* sp. to produce collagenase on solid plate medium by make wells, he notes that a variable in diameter of clear zone around wells, this depend on type of bacteria species and type of media.

#### Secondary screening (quantitative screening) of collagenase production

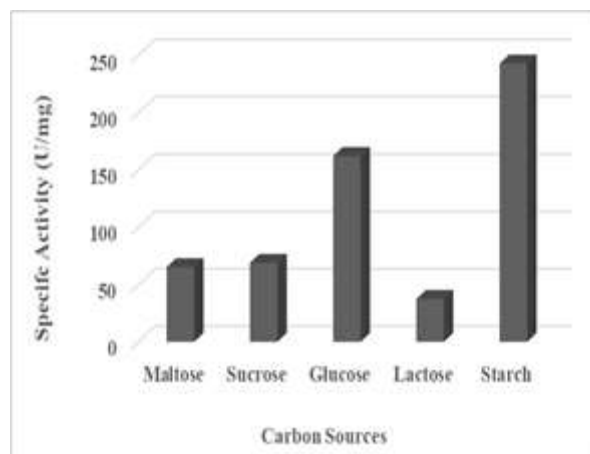
For more detection and selection, the 49 isolates of *S. aureus* with maximum zone of hydrolysis in primary screening, were screening again for their enzymatic activity using submerged fermentation method.

Among 49 isolates, *S. aureus* HN77 gave the highest enzymatic activity, collagenase specific activity in crude supernatant was 49.122 U/mg protein. Whereas the collagenase specific activity for other isolates was ranged between 47.17 and 5.97 U/mg fig (2), the *S. aureus* HN77 isolate which had the higher specific activity was selected for further studies. The variation between members of the same species in ability for collagenase production may be due to the genetic variation, type and the source of isolates and cultivation condition such as a media component's, temperature, pH, aeration and stirring, which help to increase the ability of (HN77) isolate to production the enzyme in a liquid media (18).

**Optimum condition for collagenase production: Effect of fermentation medium:** The effect of fermentation media on collagenase production was investigated using the isolate *S. aureus* HN77, which was grown in four different media. According to particular activities, medium (D) was shown to be the best medium for collagenase production among the four media studied. The greatest specific activity of collagenase detected was 156.66 U/mg, while the specific activity of collagenase utilizing medium A, B, and C was reduced to (40.66, 48.40, and 17.30) U/mg protein, respectively (Fig. 2). As a result, this medium was chosen to maximize the activity of the other fermentation parameters. Collagenase production was found to be highly dependent on the medium, with improved enzyme production ascribed to the presence of a complete pool of amino acids, a carbon and nitrogen supply, and additional supplementation (1).



**Figure 2. Production of collagenase in different liquid media by *S. aureus* HN77 using submerged culture using shaker incubator 120 rpm at 37°C for 48 hrs**



**Figure 3. Effect of carbon sources on collagenase production from *S. aureus* HN77 using submerged culture pH 7.0 at 37°C for 48 hrs**

#### Optimum carbon source

The production of collagenase was investigated in the presence of various carbon sources added into the production medium at a concentration of 2% (w/v). Starch was found to enable good collagenase production among five distinct carbon sources (Fig.3). The specific activity of collagenase was increased to 242.7 U/mg, compared to 37.37 U/mg with the maltose-containing medium. Carbon is an essential element required for organism growth, and most microbes prefer it as a carbon source. As a result, it is largely used as a significant carbon source for microbial growth and metabolite production (6).

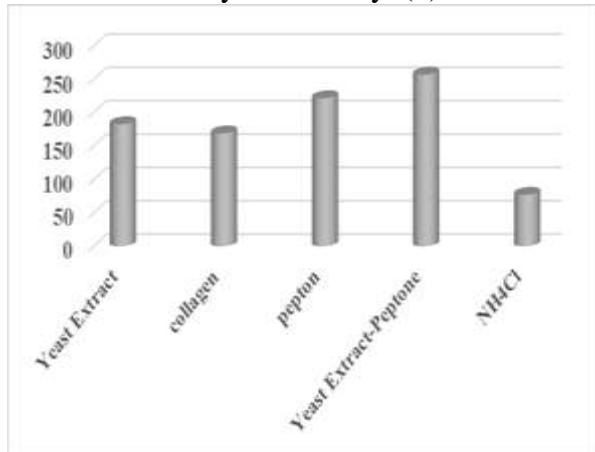
#### Optimum nitrogen source

At the optimum carbon source, the impact of several nitrogen sources was assessed. When compared to alternative nitrogen sources, yeast extract-peptone was the greatest source of nitrogen for supporting *S. aureus* HN77 growth and enzyme production, increasing collagenase specific activity to 257.5 U/mg (Fig.4). The nature and concentration of nitrogen in the culture medium used to grow the organism are critical for collagenase synthesis. Collagenase activity was greatest in the medium containing yeast extract-peptone. yeast extract is a simple source of protein that the microbe may quickly absorb. (11).

#### Optimum Ph

*S. aureus* HN77 was cultured in the production medium with various pH values to investigate the influence of the initial pH on collagenase production (4.5, 5, 6, 6.5, 7, 7.5, 8 and 9). The maximum collagenase specific activity

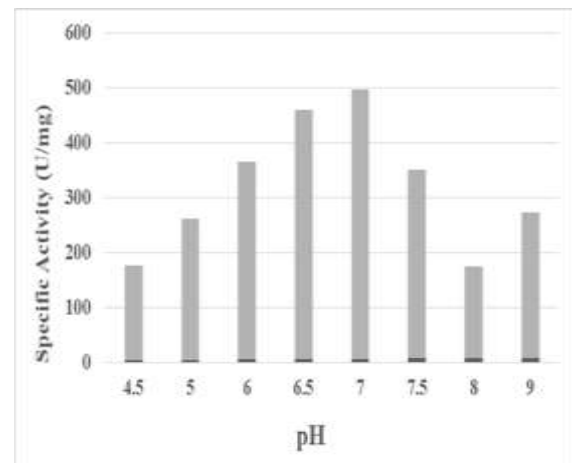
(491.04) U/mg was found at pH 7.0, as shown in Fig. (5); however, increasing or decreasing the pH value above or below 7.0 resulted in a drop in enzyme activity. The importance of pH in enzyme production is generally linked to its influence on the solubility of medium dietary components, its affect on substrate ionization and availability for the microbe, as well as its influence on enzyme stability. (7).



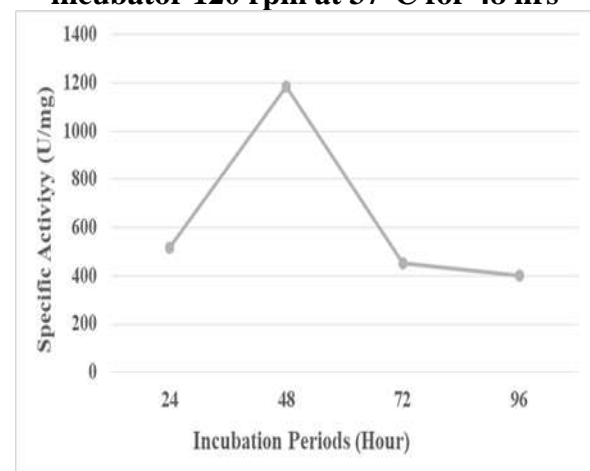
**Figure 4. Effect of nitrogen sources on collagenase production from *S. aureus* HN77 at 37°C in shaker incubator 120 rpm pH 7.0 for 48 hrs.**

#### Effect of incubation periods on collagenase production

The optimum incubation period was studied to detect the periods of bacterial growth and enzyme production. The finding in Fig. (6), show that 48 hr. was better incubation period for collagenase production from *S. aureus* HN77, the specific activity was reached to 1183.33 U/mg protein the specific activity was decreased with increasing the incubation time, this may be due to the change in the conditions of culture along this periods such as diminishing of oxygen, nutrients and accumulating of toxic metabolites which inhibit the bacterial growth. The incubation time plays an important role in the growth of microorganisms and enzyme secretion. Some researcher indicated that the production of enzyme start in the early stages and follow some hours of microbial growth. Yasuko *et al*, (31) found similar results, an increased activity was observed at 24 hrs and 48 hrs respectively. After 96 hrs. the enzyme production was completely inhibited.

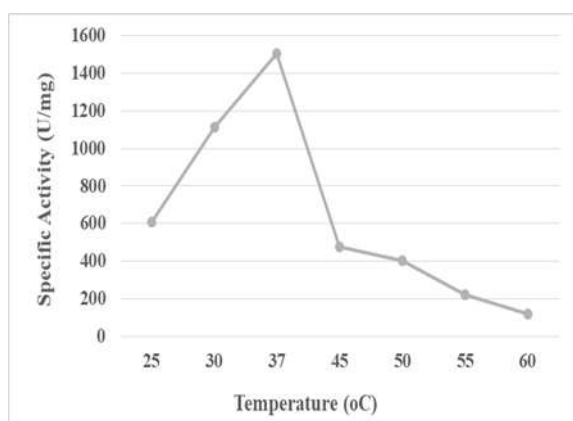


**Figure 5. Effect of initial pH on collagenase production from *S. aureus* HN77 in shaker incubator 120 rpm at 37°C for 48 hrs**

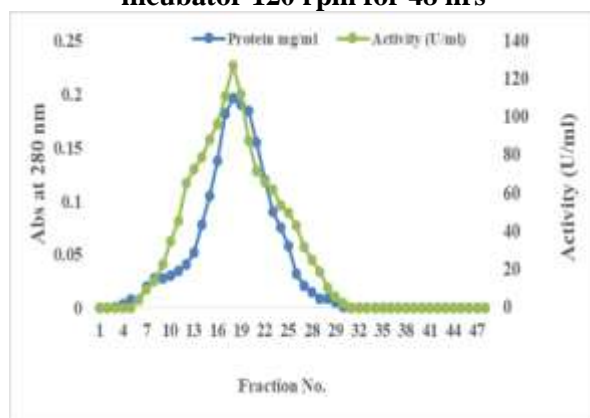


**Figure 6. Effect of incubation period on collagenase production from *S. aureus* HN77 in shaker incubator 120 rpm at 37°C**

**Effect of temperatures on collagenase production :** The results in Fig (7) showed the capability of isolate *S. aureus* HN77 to grow and produce collagenase at wide range of temperatures include 30, 37, 40 45 and 50°C, collagenase production was found to be maximum at 37 °C with specific activity of 1502.15 U/mg. Lower yields obtained at lower and higher temperature 25, 45 which was 606.30, 477.22 U/mg protein. Temperature plays a vital role in the growth and metabolism of any microbe. This results are comparable with others (21) who also reported the maximum enzyme production at the same temperature but the enzyme production by bacteria was inhibited at 50°C.



**Figure 7. Effect of temperature on collagenase production from *S. aureus* HN77 in shaker incubator 120 rpm for 48 hrs**



**Figure 8. Gel Filtration chromatography for collagenase purification from *S. aureus* HN77 by using Sephadex G150 column (1.5 x 30) cm equilibrated and eluted with phosphate buffer (0.2 M, pH 7), in flow rate 20ml/hr. 3ml for each Fraction**

### Purification of collagenase

*S. aureus* HN77 was cultivated under the optimum conditions for optimal collagenase production, enzyme extracted by centrifuge at 8000 rpm for 10 min. The crude collagenase was purified from the unwanted proteins and other components using gel filtration by (Sephadex – G150) then concentration with sucrose as follows:

### Gel filtration chromatography

**Table 1. The purification steps of collagenase from *S. aureus* HN77**

Sample	Volume (ml)	Activity (U/ml)	Protein (mg/ml)	Specific Activity (U/ml)	Total Activity (U)	Purification fold	Yield (%)
Crude Enzyme	50	294	0.2	1470	14700	1	100
Gel Filtration with Sephadex G-150	60	119	0.045	2644.4	7140	1.79	48.57
Concentration with Sucrose	30	210	0.06	3500	6300	2.38	42.85

The crude enzyme solution was passed through a Sephadex - G150 gel filtration column with diameters of (1.5×30) cm, and equilibrium with 0.2 M of phosphate buffer pH 7. The specific activity reached 2644.4 U/mg protein with a purification fold of 1.79 and yield reached 48.75 percent as shown in Fig. (8), with two peaks of protein in the eluted fractions from the column in fractions (7-27) and one peak of collagenase activity (table 1). Collagenase was purified from Mackerel, *Scomber japonicas* by DEAE Sephadex A-50 using 50 mM Tris-HCl buffer (pH 7.5) that contained 5 mM CaCl<sub>2</sub> (buffer A). Then by Sephadex G-100 using a same buffer, specific activity was reached to 384.9 U/mg protein with purification fold 23.3 and yield 0.7% (25).

### Enzyme concentration by sucrose

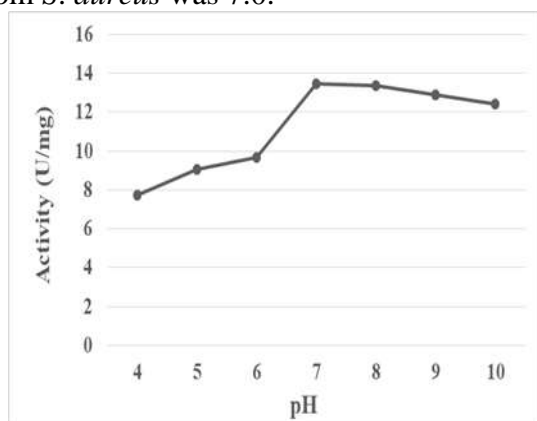
The purified enzyme from the gel filtering phase was concentrated using sucrose, and the findings revealed that 42.85% of collagenase enzyme was obtained with a purification fold of 2.38, as shown in (table 1). Sucrose was once commonly employed for protein concentration, nearly as an affordable technique of precipitating and concentrating a protein extract, sucrose impacting the enzyme as a nucleophilic effector (activator). According to Bajaj et al. (4), the procedure for concentration of enzymes involves lowering the volume of the active solution by dialyzing against a concentrated sucrose solution and withdrawing water molecules from the enzymatic solution.

### Characterization of Collagenase

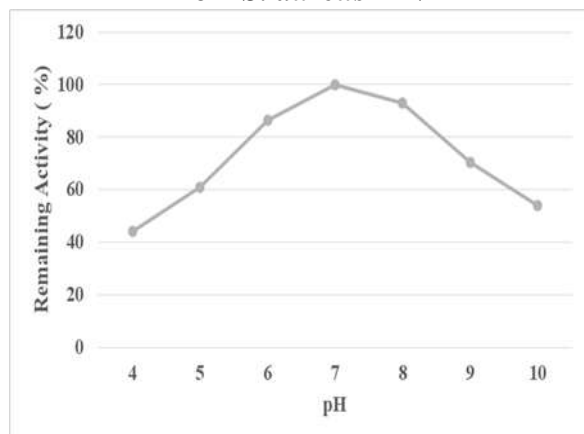
**Effect of pH on collagenase activity:** The effect of pH on partial purified collagenase from *S. aureus* HN77 was evaluated in a pH range of 4.0-10.0 as presented in (Fig. 9). The best activity was pH between (6.5-7.5) with maximum enzyme activity at pH 7.0. Collagenase activity at pH 7.0 with phosphate buffer 0.1 M reached to 13.5 U/ml, whereas in acidic pH (4 and 5) were 7.7 U/ml, 9 U/ml respectively, also the activity was decrease in alkaline pH (9 and 10) and reached to 12.9 U/ml and 12.4 U/ml respectively. The shift of optimum pH was attributed to the electrostatic interaction influenced by the carrier microenvironment. The results of this study was compatible with the results mentioned by



Gautam *et al* (15), they found that the optimum pH of collagenase activity purified from *S. aureus* was 7.0.



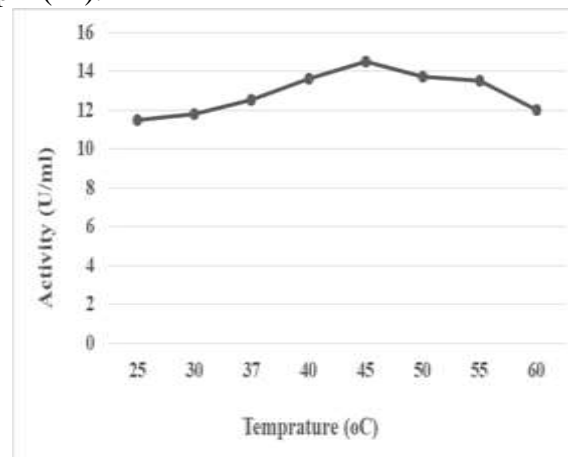
**Figure 9.** Effect of different pH values (4.0-10.0) on partial purified collagenase activity from *S. aureus* HN77



**Figure 10.** Effect of different pH values (4.0-10.0) on partial purified collagenase stability from *S. aureus* HN77

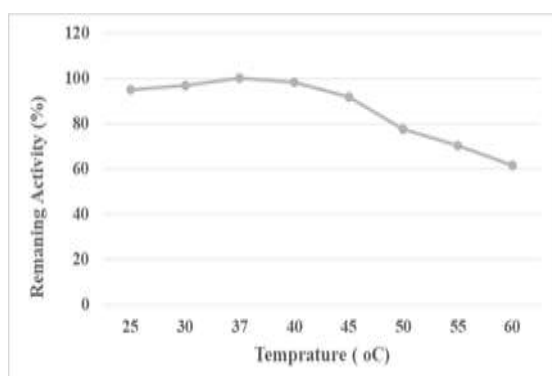
**Effect of pH values on stability of collagenase:** The results in Fig. (10) shows that pH ranged between 7.0-8.0 were the optimum pH for collagenase stability, the enzyme kept about 100% of its activity in pH 7.0, while kept about 93.13% in pH 8.0. The enzyme activity was decreased away either side of the optimum pH values. The residual activities were 86.47% at pH 6.0, The enzyme activity was very low at nearly acidic pH and it was reached to 61.08% at pH 5.0 almost half of activity was lost at pH 4.0 about 44.19%. Also enzyme activity decreased toward alkaline pH like 70.58% for pH 9.0 and littler about 53.89% for pH 10.0. Several types of research pointed to the optimum pH of enzyme stability, Baehaki *et al* (3) showed that the optimal pH between 7.0-7.5 for collagenase productivity.

**Effect of temperature on collagenase activity:** The activity of partial pure collagenase was measured at temperatures ranging from 25°C to 60°C. (Fig. 11), shows an increase in activity to 14.5 U/ml at 45°C, then a drop in activity with rising temperature up to 45°C, with a minimum activity of 12 U/ml at 60°C. At 25°C, the minimum activity of 11.48 U/ml was also recorded. Temperature, on the other hand, has a variety of effects on the enzymatic reaction, including pH (12).



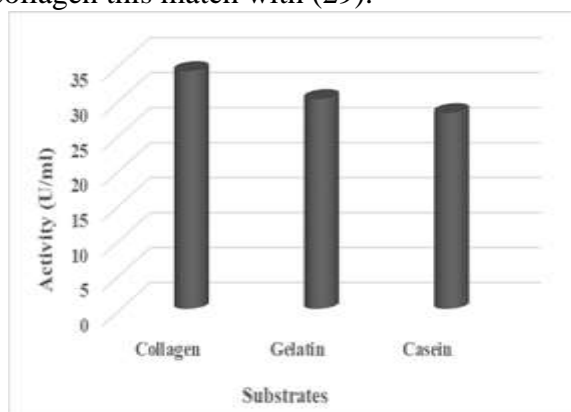
**Figure 11.** Effect of different temperature on partial purified collagenase activity from *S. aureus* HN77

**Effect of temperature on stability of collagenase:** The stability of collagenase at different temperatures was determined by incubating the purified enzyme at temperatures ranging from 25°C to 60°C for 30 minutes and then determining the remaining activity percent. Collagenase activity was maintained in the current investigation at temperatures ranging from 25 to 45°C Fig. (12), but activity began to decline with increasing temperature at 50°C to 60°C. The decreased activity of collagenase at temperatures above 45°C is due to its susceptibility to high temperatures; however, the increased thermal stability of collagenase resulting from immobilization should be beneficial for treating effluents at high temperatures. The thermal stability of *S. aureus* HN77 collagenase was close to others (14), when they reported that the enzyme produced from *S. aureus* was stable when incubated for 30 min at 30-40°C.



**Figure 12. Effect of different ranges of temperature on stability of partial purified collagenase from *S. aureus* HN77**

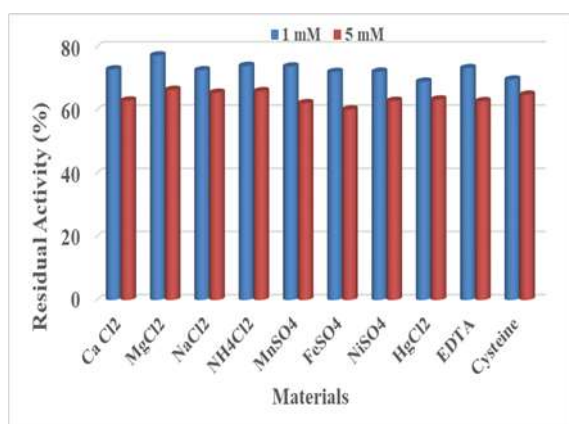
**Substrates specificity of collagenase** Best substrate required for collagenase activity was determined by incubating the purified enzyme with different substrate such as collagen, gelatin, and casein in pH 7.0 at 45°C. The results in Fig. (13) shows an increase in enzyme activity by using collagen as a substrate, it was reached to 34 U/ml, followed by using gelatin 30 U/ml whereas the activity by using casein reached to 28 U/ml. The result agreed with other studies that observed a specific substrate for collagenase enzyme that produced from microorganism in general its collagen this match with (29).



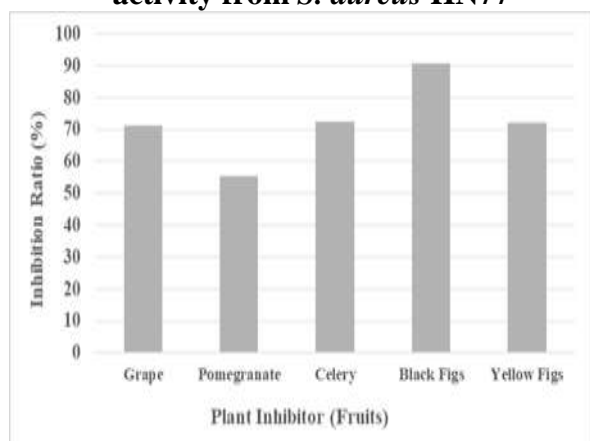
**Figure 13. Effect of different substrates on purified collagenase activity produced by *S. aureus* HN77 at pH 7**

**Effect some chemical compounds on collagenase activity:** Chemical materials were used to treat purified collagenase from *S. aureus* HN77. The results in (Fig. 14) reveal that all of these materials inhibited the collagenase enzyme, while there were some variances. Metal ions' influence on collagenase activity varies depending on the enzyme's origin, but at 1 and 5 mM,  $\text{CaCl}_2$ ,  $\text{MgCl}_2$ ,  $\text{NaCl}_2$ ,  $\text{NH}_4\text{Cl}_2$ ,  $\text{MnSO}_4$ ,  $\text{FeSO}_4$ ,  $\text{NiSO}_4$ , and  $\text{HgCl}_2$  were shown to suppress enzyme activity

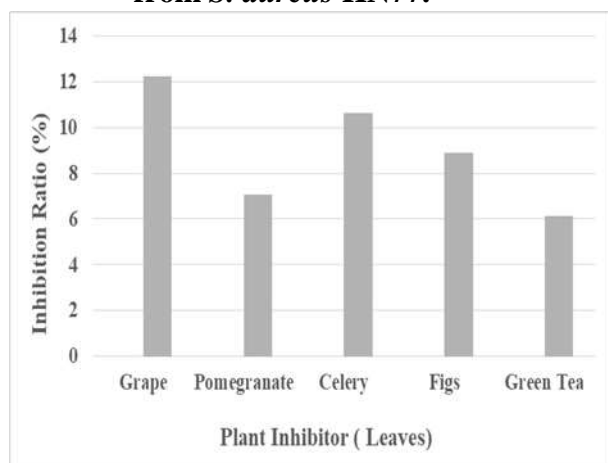
to levels below their original activity (control value). At 1mM and 5mM,  $\text{CgCl}_2$  inhibited the enzyme, with residual activity of 72.9% and 60%, respectively.  $\text{MgCl}_2$  inhibits enzyme activity, with remaining activity equal to 77.3% and 66.4% at concentrations of 1 and 5 mM, respectively, Fig. (14). Also, the results show that enzymatic activity was decreased by using 1 and 5 Mm of  $\text{NaCl}_2$ ,  $\text{NH}_4\text{Cl}_2$ ,  $\text{MnSO}_4$ ,  $\text{FeSO}_4$ ,  $\text{NiSO}_4$ , with (72.6, 74, 73.8, 72 and 72)% at 1mM and (65.5, 66, 62.2 and 62.9)% at 5mM respectively, This could indicate that the enzyme activity changed when the concentration was increased. The recent study's findings revealed that ions have varying impacts on enzyme activity depending on their type and concentration, and that these ions' effects often increase as concentration rises. The decrease in activity is due to the development of complexes with the enzyme, which prevents the enzyme from attaching to the substrate and converting it to product (22).  $\text{HgCl}_2$  inhibited the enzyme at 1mM and 5mM, indicating the presence of SH groups in the enzyme active site, which  $\text{HgCl}_2$  oxidized. Furthermore, the presence of  $\text{HgCl}_2$  in the substrate processing solution may form a complex with the enzyme, preventing it from binding to the substrate and forming the product (20). EDTA was used to test the effect of inhibitors on collagenase activity. Based on the residual enzyme activities, it was discovered that the enzyme displayed a decrease in enzyme activity as well as an increase in inhibitor concentration. When purified collagenase was incubated with EDTA at 1 and 5 mM, the residual activity was 73.3% and 72.8%, respectively (Fig. 14). The findings revealed that the enzyme was a metallo-enzyme and that divalent ions played a key role in the generated activity. When the enzyme was treated with cysteine at 1mM and 5mM, it lost 69.7% and 64.6% of its activity, respectively. When an enzyme is incubated with reducing chemicals such as cysteine, the disulfide bonds in the protein structure and conformation are reduced, resulting in fragmentation of the protein into its constituent units, which has a negative impact on its activity (3). These findings suggested that the enzyme under investigation included disulfide bonds.



**Figure 14. Effect of some chemical compounds and ions on purified collagenase activity from *S. aureus* HN77**



**Figure 15. Effect of some plants fruits extracts on purified collagenase activity from *S. aureus* HN77.**



**Figure 16. Effect of some plants leaves extracts on purified collagenase activity from *S. aureus* HN77**

**Effect some plants sources on collagenase activity :** This study was used some local plants (fruit and leaves) as a source of inhibition material for purified collagenase from *S. aureus* HN77. The results in Fig. (15) show that the black fig fruit was inhibited 90.7% of collagenase followed with celery

seed 72.5%, then the inhibition ratio of collagenase was decreased by treated with other fruits, the lowest inhibition activity was observed with pomegranate 55.4%. While the results in Fig. (16) observe that the leaf of grape had a higher value of inhibition it was reached to 12.2% followed with celery leaf 10.6%. The inhibition ratio of collagenase by using fig, pomegranate and green tea leaves have a lowest value of inhibition, it was reached to (7, 8.9 and 6.1) % respectively. Plant is one of the most important group for collagenase inhibition, plant considered as an important for healthy human (8). Secondary metabolites and whole extracts from plants have been widely investigated and found to have anti-collagenase activities. Plants contain a wide variety of compounds including polyphenols such as flavonoids, tocopherols, phenolic acids and tannins which have been found to provide ChC inhibitory compounds or a platform on which to synthesize active molecules (5).

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