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Inhibition of Protease Produced from Clinical Isolate of *pseudomonas aeruginosa* using Vasicine Alkaloid

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Abstract: ***This study was carried out using leaves of Adhatodavasicaplant, which is belongs to the family of Acanthaceae. This study includes extraction and partial purification of vacisinealkaloid and detection of active materials in the crude leaves extract using methanol80%.The partial purification for vasicine from crude leaves extracts was obtained by separation and precipitation with a percentage of 0.6%.The objective of this study is determination of effect of these plant extracts as an inhibitor of proteasesisolated from the pseudomonas aeruginosa bacteria, where 40 samples were collected from different hospitals in Baghdad,25 isolates were identified as Pseudomonas aeruginosa by routine biochemical tests, Api 20 E; Then the ability of these isolates for production of proteases was screened by using quantification and semi- quantitative methods, Pseudomonas aeruginosa (P1) was selected as the highest protease producer, which was identified as one of the strains ofP. aeruginosa. It was observed that The optimum culture medium and conditions for protease production by submerged culturesin thetryptic medium soya broth medium with pH 8 and incubated at a temperature of 37 ° C for 48 hours. The effect of vasicine on the effective protease enzyme showed that the activity of enzyme had decreased from 15.5U / ml to 1.2 U / ml when treated with vasicine at a concentration of 0.8ml. The inhibitory activity of the extract towards protease was 92.2%.***

Keywords: Protease, *pseudomonas aeruginosa*, vasicine, inhibitory activity.

1. Introduction

Microorganisms produced different types of products through metabolism of different types of substrates. Some of these products are Poisons that are produced naturally and considered as the final products of The metabolism which not intermediate These toxins have accumulated inside the cell known as endotoxins or release to the growth media is known as exotoxin, the toxins can be either small peptide molecules or proteins. *Pseudomonas aeruginosa* produces a large number of extracellular toxins which include phytotoxic factor, pigments, hydrocyanic acid, ,proteolytic enzyme phospholipase, interotoxin,exotoxin, and slime (Pollack et.al (2000). The most important factor in the pathogenesis of *P.aeruginosa*is develop a set of exotoxins (protein in nature), External toxins can cause leukopenia, acidosis, breakdown of blood circulation of the liver, pulmonary edema, hemorrhage and tubular necrosis of the kidneys. Passive administration of antitoxic sera against these exotoxins is capable of Protection against deadly infection with *P. aeruginosa* in absence antibodies against cellular antigens (Pollack 2000 and Stover *et al*.,2000) reported that *P aeruginosa*is an opportunistic pathogen and was able to causes intensive morbidity and mortality in individuals who are immunocompromised or have underlying medical conditions such as urinary tract, primarily causes of nosocomial infections, and it is frequently resistant to commonly used antibiotics and disinfectants. Microbial extracellular Protease is a cellular enzyme of *P. aeruginosa* that plays an "important" role in the pathogenesis of bacteria through invasion, concentration, and overcoming the immune defenses of the body (Leung and Stevenson, 1988), that hydrolyze peptide bonds of proteins and break down into polypeptides or free amino acids (Khan, *et al*., 2011).proteases which causes bleeding and tissue necrosis. One of the most important characteristics that determine the industrial suitability of proteases is their requirement of high pH for optimum enzyme activity (Khan, *et al.*, 2011). The occurance of several tissue damage related diseases assiociated with free radicals coupled with pathogen resistance to antibiotics has attracted finding alternative remedy against these deleterious molecules and pathogens .Alarge number of protease inhibitors have been isolated and identified from several plants that medicinal plant are of great importance to the health of individuals and communities in Nigeria and whole world. They contain phytoactive components that possess both curative and preventive properties (Hill and Lillicrap,2005).for example, *Adhatodavasica*(L.) belong to the family (Acanthaceae), commonly known as vasaka that Plants have evolved a wide variety of chemical compounds which are known as secondary metabolites were (Coumarines ,Flavones ,Phenolic compound ,Volatiles oil , Tannins , Saponines , Glycosides , Alkaloids , Resins and Terpenoids) (Rajni*et al*.,2014).*A. vasica* (,is widely used in Ayurvedic medicine in India,Sri Lanka and Pakistan due it is bronchodilator,expectorant , antiasthmatic,antiallergic activities against dermatitis and tuberculosis (Cupta*et al.*,1977). In china this plant has traditional use against skin disorders. Initial studies showed that the plant possesses anti-inflammatory properties(Cueller*et al.*,1998). The aims of this study are: Isolation and identification of *P. aeruginosa*from the clinical sample,Preparation of active compound vasicine , Determination of efficacy of isolates in Protease production. Producing the enzyme of the most virulent isolation Determination of active compounds concentrations for inhibition of protease .

2. Materials and Methods

Collection of bacterialsamples:

Fourty clinical samples were collected from burns and infected wounds of patients attending AL-Yarmouk Hospital

and Medical City Hospital during the period from December 2016 to February 2017.

Identification of *Pseudomonas aeruginosa*:
All collected swabs were cultured on Cetrimide agar, MacConkey agar, and blood agar, and incubated at 37C° for 24hrs under aerobic condition. Bacterial characterization determined depending on cultural characterization, biochemical tests [8], and for more conformation API 20E kit was used.

Determination of protease production from *pseudomonas aeroginosa*
Semi-quantitative method
Skim milk-peptone agar was inoculated with 24hbacterial culture and incubated for 24h. at 40°C. clear zone around the spots and underneath the growth indicate protease production. The diameter of colonies and clear zones were measured. The ratio of clear zone diameter to colony diameter which represents a semi quantitative assay of protease.

Quantitative method
10 ml. of casein - peptone broth was inoculated with 0.1 ml of 24h Activated bacterial suspension and incubated at 40˚C for 24h. The crude enzyme was extracted by centrifugation at 3500 rpm for 20 min . Then the enzyme activity was measured in the supernatant.

Assay of protease activity in samples.
Protease activity was determined according to by Sharma et al. (2006) and by three replicates for each bacterial isolation. 0.2 mL of the crude enzyme for each bacterial isolation was adde to 1.8 ml of reaction solution at 37 c for 30 min the blank consist of 1.8 of reacton solution and 3ml of 5%TCA solution (trichloroacetic acid)without addition of crude enzyme the reaction was stoped by addition of 3ml of 5%and the mixture was centrifuged for 30 min at 3500rpm and then supernantant was separated the absorbance (OD)at 280nm was measured enzyme activity was measured according to following equation

$$\text{Activity of enzyme}(U/ml) = \frac{\text{absorbance at 280 nm}}{0.2\text{ ml} * 0.001 * 30\text{min}}$$

The enzyme activity unit is defined as the amount of enzyme that gives an increase of 0.001 in adsorption (280) nanometers per minute, under standard conditions.

Determination of optimum conditions for protease production.

Production of protease in different media
The bacterial isolate was activated by culturing in nutrient broth and incubated at 37˚C for 24h. Each 100ml. of different media was inoculated with 2ml. of bacterial suspension and incubated at 37˚C for 24 h. The cells were precipitated by centrifugation at 3500rpm for 20min The supernatants (crude enzyme) were assayed for enzyme activity for select the best production medium.

Temperature
100ml. of tryptic soya broth medium was inoculated with 2ml. of activated bacterial suspension and incubated at different temperature s (37, 40, and 50˚C) for 24h. The supernatant was assayed for enzyme activity

PH value
100ml. of tryptic soya broth medium was prepared at different pH values (7.0, 8.0, 9.0 and 10.0) adjusted with 1N HCl and 1N NaOH. The medium was inoculated with 2ml. of activated bacterial suspension and incubated at 37˚C. for 24h. The supernatant was assayed for enzyme activity.

Incubation period
100ml. of tryptic soya broth medium at pH 8 was inoculated with 2ml. of activated bacterial suspension and incubated at 37˚C for different times (24, 48 and 72)h. The supernatant was assayed for enzyme activity .

Extraction of Protease
The production medium was inculcated with bacterial suspension and incubated at 37˚C. for 48h. The enzyme was extracted by centrifuge at 3500 rpm. for 20min. Activity of enzyme was assay.

Collection and drying of *Adhatodavasica*leaves
The leaves of A. vasica (Acanthaceae) were collected from different places of the gardens of the University of Baghdad. The leaves were washed with clean tap water and then left at room temperature at (22-25) °C for (2-3) weeks for drying; plant leaves were grinded by electric miller to be powder.

Preparation of methanol extracts of plant leaves
Crude leaves extract were prepared by using methanol 80%, dried leaves powder (200gm) was kept in a thimble and extracted by soxhlet apparatus which contained 600ml of the solvent, each separately at 50°C for 36 h with an extraction ratio of (1:3) (Indian Herbal Pharmacopoeia, 1998).Extracted was filtered through a filter paper (whatman No.4)and the filtrate was concentrated by using rotary evaporator. The extract was kept in a glass container at -4°C in a refrigerator.

Estimation of crude extraction ratio for plant leaves
The extraction ratio was determined according to Al Balany (2003) using the following equation.

$$\text{ER\%} = \frac{\text{Concentration extract (yield)}}{\text{Initial weight of fresh leaves}} \times 100$$

Extraction and purification of vasicine
The vasicine alkaloid was isolated from the leaves of *A.vasica* by extracted the leaves with 80% methanol in soxhlet apparatus. After that, methanolic extract was treated with aqueous 2% H2SO4 at room temperature for 15 min, and defatted using Chloroform via separator funnel for three times. The aqueous layer was basified with liquid ammonium hydroxide and extracted with chloroform resulted crude vasicine which was purified by crystallization and recrystallization using a mixture of ethanol-Diethyl ether (1:1) (Indian herbal pharmacopoeia, 1998).

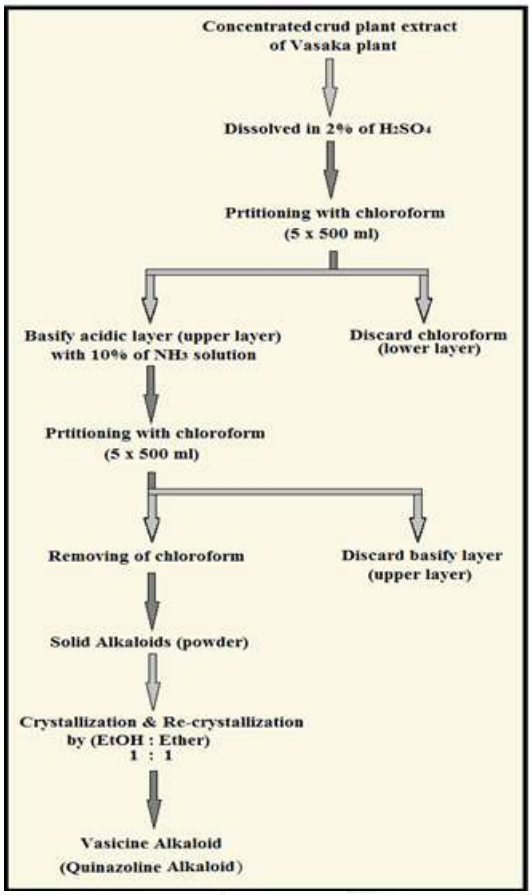


Figure 1-2: Steps of purification procedure of vasicine alkaloid from crude extract of *A. vasica*

Physical characteristics of vasicine alkaloid

Melting point

The melting point of the partial purified sample was measured according to *Harbone*,(1984) in order to view the vasicine alkaloid purity.

Chemical characteristics

The method was done according to *Al-Shah at*,(1986). A few drops of modified Dragendroff's reagent were added to 5ml of leaves extract by methanol. The appearance of orange –red color indicated a positive result for alkaloid presence.

Inhibitory Assay

The method adopted was described by Adu et al,(2013)with slight dfference . briefly 0.2ml of the crude enzyme extract and different concentration of vasicineseveraly (0.2,0.4,0.6,0.8,1ml)of 10 % w/v of plant crude in 1.8 of 5casein solution was added this reaction mixture was mixed and incubated at 37c for 10 minutes this reaction was stopped by adding 2ml of 5% trichloroacetic acid Protease assays was carried out and procedure was repeated without inhibitor.

3. Results and Discussion

Isolation and Identification of *Pseudomonas aeruginosa*

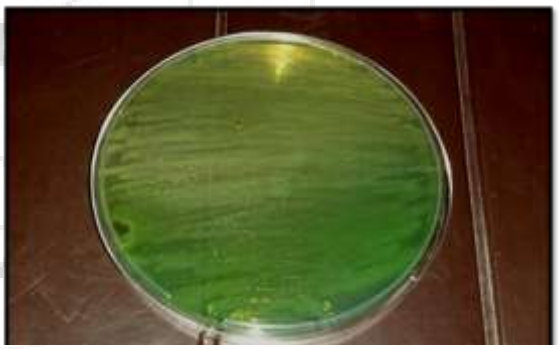
Isolation:

For isolation of Pseudomonas spp., 40 samples were collected from burning infections during the period From December to February in two hospitals (Baghdad Yarmouk Teaching Hospital and Medical City Hospital). The bacteria were obtained, and oxidase and catalase tests were used to isolate genus of pseudomonas.

Phenotypic test of *P. aeruginosa*

This study included thirty *P. aeruginosa* isolates identified using a variety of techniques, which are morphological and biochemical characterization. The result showed that *P. aeruginosa* produces green pigment and characteristic dour on cetrimide agar. *P. aeruginosa* reacted positively to catalase and oxidase tests and Simmon Citrate test, while it was negative for methyl red, VogesProskauer and indole. These characteristics of the isolates were consistent with the description of typical *P. aeruginosa* according to (8; 16). Further biochemical tests were carried out to confirm characterization by using the API 20E system as in figure (1), which revealed that all 30 tested isolates belonged to the *P.aeruginosa*.

Figure 1-3: identification of p.aeruginosa by Epi20E



A

B

Figure 2-3A: *P. aeruginosa* on skim milk agar incubated at 37C° for 24 hours.

Figure 2-3B:*P. aeruginosa*on Cetrimide agar incubated at 37C° for 24 hours.

Screening of *pseudomonas aeruginosa* that produces proteaseenzyme:

Semi-quantitative screening

The bacterial susceptibility to the production of the protease on the skim milk agar was investigated (table 3-3). It was determined that isolated bacterial isolates with high ability to produce the enzyme through the size of lysis

Table 3-1: Hydrolysis ratio of Protein in Skim milk- agar by *pseudomonas aeruginosa* that incubated at pH7.0 for 24 h. and at 37°C

number of isolates		Hydrolysis ratio (Diameter of clear zone)(Cm)
P1		4
P2		2
P3		3
P4		2.4
P5		1.1
P6		2
P7		3
P8		1
P9		1.9
P10		2
P11		1.5
P12		1.3
P13		2.7
P14		2.3
P15		2.9
P16		1.1
P17		1.5
P18		1
P19		1.4
P20		3.5

Quantitative screening

The amount of enzyme that produced by bacterial isolates was measured using method of Sharma et al., (2006) and calculate the efficacy of the enzyme (unit / milliliter) was conducted depending on the previous results.

Table 3-2: Activities of protease produced by pseudomonas aeroginosa after 24h. with incubation at 40 C°, pH 8 on tryptic soya broth

Number of isolates	Enzymatic activity(U/ml)
p1	12.2
p20	10.1
p7	9.6
p3	7.2
P13	6.4
p1	8.3
p15	11.8

Determination of optimal conditions for Proteins production:

Effect of medium compositions:

P. aeruginosa (p1) were grown in different media and enzyme production was measured. The results indicated that tryptic soya broth was the best production media for protease with a specific activity of 14.4U /mL, while Cassin-yeast extract medium was 13.1U/ mL and casein peptone medium showed less specific activity of protease 11.9U/ ml. The differences among the productivity of the enzymes in the cultivated media can be attributed to the variation in the quality of organic matter and its concentration in the food medium, such as protein and carbohydrates, as well as inorganic substances and their concentration as salts of calcium, phosphates, etc. and their role in stimulating the productivity of the bacterial enzyme. Complexity stimulates the production of protease from the microorganism better and more efficiently than the "simple nitrogen" source of the microorganism (Chen *et al.*, 1997).

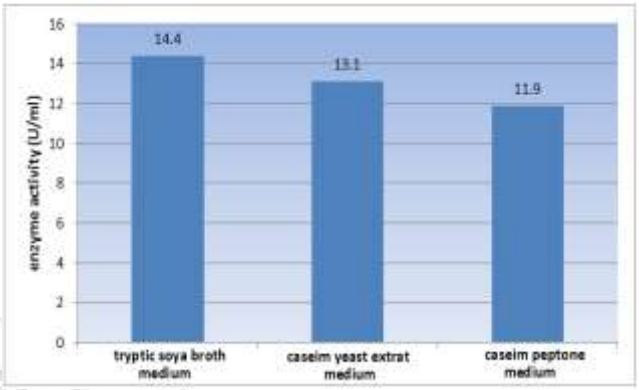


Figure 3-1: Protease production by *P. aeruginosa P1* cultured on different media with incubation at 37˚C and PH 8 for 24h.

Effect of pH media

For determination the effect of pH values on protease production, *P. aeruginosa (P1)* were cultured in tryptic soya broth with different pH values. The results showed that the enzyme was produced at pH ranged from 7.0 to 10.0, the maximum value of enzymatic activity was 15.5 that observed at pH 8.0The most important characteristic of microorganisms are their dependence on thextracellular pH for cell growth and enzyme production (Kumar et al., 1999)

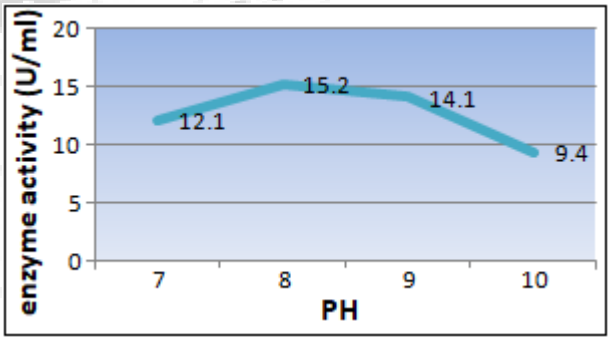


Figure 3-2: Protease production by *P. aeruginosa*P1 cultured in tryptic soya broth medium prepared at different pHs and incubated at 37˚C. for 24h.

Effect of incubation temperature

Protease activity was evaluated at different incubation temperatures (30, 37, 40 and 50 ˚C). The results showed that the best temperature for Protease production by p. aeruginosa(P1) at 37 ˚C with a specific activity of 14.953U / ml (Fig. 3-3) and decreased productivity to 12.184 U / ml and 8.861U /ml at 30 ° C and 50 ° C respectively((Chi and Zhao et al., 2003, Uyar et al., 2011) reported that Temperature is one of the most important factors affecting the enzyme production and the temperature could regulate the synthesis and secretion of extracellular protease by microorganisms (Ray et al. 1992).

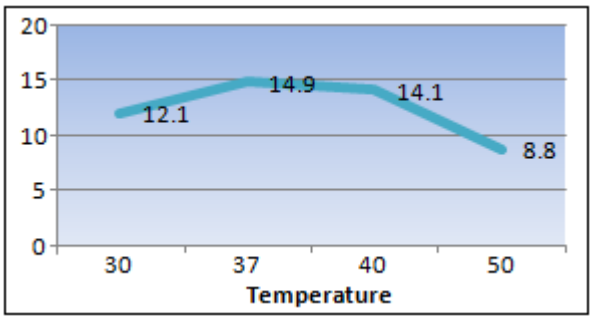


Figure 3-3: Protease production by *P. aeruginosa* P1 cultured in tryptic soya broth medium at pH 8.0 and incubated at different temperatures for 24 h.

Determination of optimum incubation time

Enzyme production was observed by p. aeruginosa (P1) after 24, 48 and 72 hrs. of incubation period that showed a maximum activity (15.276U / ml) was obtained after 48hrs. Then, the activity decreased with increasing incubation period (Figure 3-6). (Mona, and Goud et al., 2006) showed that protease is produced during logarithmic phase and reaches its maximum value at stationary phase. (Nguyen, and Quyen et al., 2011) attended that Enzyme production in culture medium did notchange in the stationary phase.

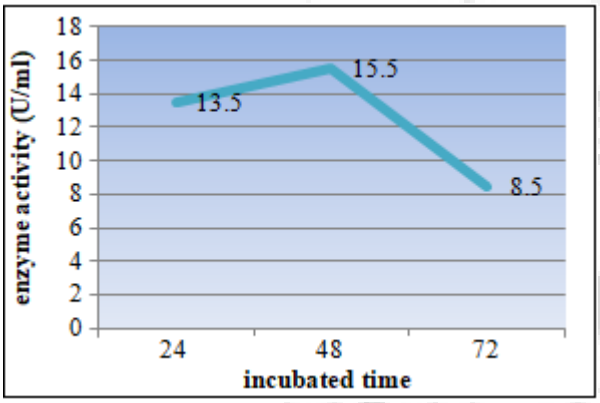


Figure 3-4: Protease production by *P. aeruginosa P*1 cultured on tryptic soya broth medium prepared at pH 8.0 and incubated at 37˚C for different times.

Extraction of crude leave extracts of *Adhatodavasica*

The solvent that used for extraction of A. vasica was methanol 80%, because this concentration had a high extraction capacity with good polarity. This result was agreed with (Koresh et al., 2001) and (Raul et al. 2003). Also, the water polarity is high, but not all plant material can be dissolved and extracted with it. Therefore, methanol 80% consists of methanol and water at some time.

Estimation of extraction ratio for crude leaves extracts:

The result of extraction ratio showed that was determined according to the percentage of concentrated crude extracts was reached 26 % (Koresh et *al*., 2001).

Detection of phytochemicals in the crude leaves extracts of *A. Vasica*

The results of the search for secondary metabolic compounds of the methanolic extract of the leaves of AdatodaVasica showed the presence of flavonoids, volatile oils, tannins, saponine, glycosides, alkaloids and resins, while coumarin compounds were not found (Table 3-9) these results are agreement with many research .

Determination of the separated vasicine

According to the result, the percentage of separated vasicine about 1.6% and partial purified of vasicine alkaloids were about 0.6%. These results correspond to what the researchers obtained in (Al-balany, 2003). It is worth mentioning that the amount of vasicine may change and decrease during un-flowering period of plant, while, increase during flowering period because this period is usually considered a vegetative defense period that requires an increase in quantity of vasicine alkaloid. In addition to all the above and when making simple economic calculations of the amount of total alkaloids obtained from the dry leaves that were extracted using methanol 80%, the percentage of isolated total alkaloids were (1.5%) and this means to get (15) grams of alkaloid from (1) Kg of dry leaves.These quantities of alkaloids obtained are good quantities compared to the quantities obtained by other researchers Isolation of alkaloids of A. vasica by H. Topenshaw and (2.1%) with pure alkaloids (0.8%) Mansak and Holmes, 1953). Also, the alkaloids were separated as pure alkaloid that was (1.3%) (Indian herbal pharmacopeia, 1998).

Determination of melting point for separated vasicine:

The measurement of melting point was used as one of the basic and important tests for diagnosing the compounds for the characterization of vasicine alkaloid. The melting point was 210°C, which is agreement with those of other studies (Pandita et al, 1983) and (Indian herbal pharmacopoeia et al., 1998), which referred to the range of vasicine melting points are 204-210°C. This range may due to the flowering period and the purity of vasicine.

Biological activity of vasicine and catechins against protease activity:

The activity of active compounds (vasicine and catechins) was tested against proteases activity. This was done by use different dilution of active compounds. This was done by identifying the range of response of enzyme to inhibition according to the type of active compounds (vasicine and catechins) and identify the range of enzyme inhibition depending on different dilutions of active compounds as shown in Table (3-11), that shows the response of the enzyme protease towards (vasicine and catechins) that extracted by using methanol 80% and hot D.W, at different concentration of these compounds. The mean response of protease inhibition was very high at the inhibitory dilution (4) is 1.2 U/ml, when the treated crude enzyme with vasicine while when treated crude enzyme with catechins that extracted by methanol, chins (hot water the activity of enzyme was 3 and 2U/ml respectively. At the same time, the mean response was lowest at inhibitory dilution (1) is 4.5 _5_6 U/ml for vasicine, catechins (water extract) and catechins (methanolic extract).

Enzyme inhibition

Different concentrations (0.1, 0.2, 0.3, 0.4 and 0.5 ml) of active compounds (vasicine) were used as natural inhibitor for Protease. Protease was measured before and after the addition of the compounds to know the effect of these compounds on the protease enzyme activity.

Table (3-11): The effect of active compounds on the inhibition of protease enzyme activity.

NO.	Enzyme : extract (Concentration)	Enzyme activity (U/ml)
1	1:1	4.5
2	1:2	4.0
3	1:3	3.5
4	1:4	1.2
5	1:5	1.2

These results show the best ratio of (enzyme:extract) for inhibition of enzymatic activity of protease was 1:4 (0.2ml of enzyme treated with 0.8 ml of compound). The enzymatic activity decreases from 15.5 to 1.2 U/ml .

Table (3-12): Determination of inhibition activity of active compounds depending on its concentration

Active compounds	Concentration of active compounds(ml)	Inhibition activity %
Vasicine	0.2	70.96
	0.4	74.1
	0.6	77.4
	0.8	92.2
	1	92.2

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