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Effect of aqueous red cabbage extract on uricase activity isolated from *Pseudomonas aeruginosa*

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Abstract

Separation of uricase from *Pseudomonas aeruginosa* was done using (70%) saturation ammonium sulphate, and purification of this enzyme was done by ion exchange chromatography on DEAE- cellulose column and eluted with linear NaCl (0-1M). Partial purified uricase gave an activity of (4.9 u/ml), protein concentration of (0.56 mg/ml), specific activity of (8.75 unit/mg) with purification folds (8.4) and a yield of (48%). The maximum purified uricase activity was detected at 35ºc and pH 8.5 with (0.12 mM.uric acid). The results shown that red cabbage extract (RCE) contain flavonoides which contain phenolic compounds and anthocyanines which glycoslated with mono or dimolecules of saccharides, while test for alkaloids, steroids, saponins and tannins were negative. Red cabbage extract (50mg/ml) have an inhibition activity against uricase compared with negative and positive control using allopurinol. So red cabbage considered as a novel inhibition for uricase whiel can used to treat gout disease in future.

Keywords: *Pseudomonas aeruginosa*, uricase, aqueous red cabbage, anthocyanins

تأثير مستخلص اللهانة الحمراء المائي على فعالية انزيم اليوريكيز المعزول من بكتريا *Pseudomonas aeruginosa*

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الخلاصة

فصل انزيم اليوريكيز من بكتريا *pseudomonas aeruginosa* باستخدام (70%) كبريتات الامونيوم المشبعة، ونقي هذا الانزيم نفذت بواسطة كروماتوكرافية التبادل الايوني على عمود DEAE−cellulose والمزال مع NaCl 0,1 مولر . يوريكيز المنقى جزئيا اعطى فعالية (4,9 وحده/مل) ، تركيز البروتين (0.56 ملغ/ مل)، الفغالية النوعية (8.75 وحدة/ ملغ) مع عدد مرات التنقية (8.4) والحصيله (48%) مع حامض اليوريك (0,12 ملي مولر.) اعلى فعاليه لليوريكيز المنقى قيست على 35م° والاس الهيدروجيني 8.5.،النتائج تعرض مستخلص اللهانة الحمراء فلافونويدات حيث تحتوي على مركبات فينولية وانثوسيانين كلايكوسايدات مع جزيئات احادية وثنائية من السكريات بينما اختبار القلويدات ،الستيرويدات ،السابونينات والتانينات كان سالبا.مستخلص اللهانة الحمراء (50 ملغ/مل) لديه فعالية تثبيطية ضد اليوريكيز مقارنة مع القياسي الموجب والسالب بأستخدام allopurinol. لذا تعتبر اللهانة الحمراء مثبط جديد لليوريكيز بينما وممكن استخدامها في علاج داء النقرص في المستقبل .

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Introduction

Cabbage is a popular cultivar of the species *Brassica oleraceaLinne* (Capitata Group) of the Family Brassicaceae and is vegetable of leafy green. It is a herbaceous, biennial, dicotyledonous flowering distinguished by stem upon which is crowded a mass of leaves green but in some varieties red or purplish, which while immature form a characteristic compact, globular cluster (*cabbagehead*) [1]. Cabbage is an excellent source of vitamin C. It also contains significant amounts of glutamine, an amino acid that has anti-inflammatory properties. Cabbage can also be included in dieting programs, as it is a low calorie food [2]. Cancer prevention all other of health research with best to cabbage and its late benefits. More than 475 studies have examined the role of this cruciferous vegetable in cancer prevention [3]. *Pseudomonas aeruginosa* is aerobic rod shaped a Gram-negative, bacterium with unipolar motility. An opportunistic human pathogen, *P. aeruginosa* is also an opportunistic pathogen of plants and animals [4]. It is found in soil, skin flora, water, and most man-made environments throughout the world. It development not only in normal atmospheres, but also with little oxygen, and has colonised many natural and artificial environments. *P. aeruginosa* secretes a variety of pigments, including pyocyanin (blue-green), fluorescein (yellow-green and fluorescent), and pyorubin (red-brown) [5]. Uricase is one of the oxidoreductases enzymes. This group of enzymes is the first group in the rules of Classification according to the Enzyme Commission (EC.) in the International Union of Biochemist (IUB). The systematic name of this enzyme is (Urate: Oxygen Oxidoreductase [EC 1.7.3.3] [6]. Uricase catalyzes the oxidation of uric acid with molecular oxygen to allantoin serving as an electron acceptor [7] **.**the aim of this study was detection of secondary metabolites of red cabbage extract and study the effect of red cabbage extract on uricase.

Materials and Methods

Sepharose-6B, DEAE-cellulose, Column were purchased from Pharmacia Fine Chemicals. Coomassie Brilliant Blue, Uric acid, Boric acid, Glucose, Ethanol absolute, other chemicals were supplied by BDH Chemicals.

Plant materials

Fresh Samples of red cabbage plant were purchased from Iraqi local market in Baghdad. The samples were washed with clean tap water to remove dirt on the leaves. The dried plant material was manually powdered and the powder kept in polyethylene bags until used. The plant material was authenticated at the Department of Biology College of science, University of Baghdad, Iraq.

Preparation of Red Cabbage extracts

Leaves were sliced into small pieces and oven-dried at 50 C. Dried plants (100 gm) were extracted. After overnight maceration, the extract was filtered through gauze and water was evaporated under reduced pressure at 50C by using a rotary evaporator. After evaporation, dried samples were placed in a desiccator over calcium sulfate to remove any remaining water. The resulting dried pigments were then used for further studies.

Preliminary phytochemical screening

Standard screening test of the extract was carried out for various plant constituents. The crude extract was screened for the presence or absence of secondary metabolites such as alkaloids, steroidal compounds, phenolic compounds, flavonoids, saponins, tannins and anthraquinones using standard procedures [8].

Uricase production and separation

The bacterial isolated used in this study was previously isolated from poultry waste and was identified as *Pseudomonas aeruginosa* by specific biochemical tests [9]. The *P. aeruginosa* growth was optimized for mass level uricase production by using basal media containing several components such as 10.0g dextrose; 2.0g yeast extract; 3.0g uric acid and 5.0g NaCl. All ingredients were dissolved in 1000ml distilled water with 7.0 pH. The dextrose and yeast extract were used as a carbon and nitrogen source. The organism was allowed to grown at 35°C temperature for 36 hours incubation to obtain uricase for further analysis [10]. The broth culture was centrifuged at 8000 xg for 30 minutes, at 4^0C. The pH of supernatant was adjusted to 8.5 with NaOH. Uricase activity and protein concentration were estimated [10].

Precipitation step was by addition different concentrations of solid ammonium sulfate to the crude extract. Solid ammonium sulfate was slowly added at 4 C° with continue mixing for 30 minute, then centrifuged at 8000 xg for 30 minutes at 4 °C. Precipitates were dissolved in a small volume of 0.01M

borate buffer (pH 8.5), then dialyzed against 1000 ml of 0.01M borate buffer (pH 8.5) for 24 hours, and concentrated by using sugar. Uricase activity and protein concentration were determinate [11].

Enzyme assay and protein concentration

In practical analysis, 0.1ml enzyme solution was incubated with a mixture of 0.6 ml sodium borate buffer (pH 8.5, 0.1M) containing 2mM uric acid, 0.15 ml 4-aminoantipyrine (30 mM), 0.1ml phenol (1.5%), 0.05ml peroxidase (15 unit/ml) at 37 °C for 20 min. The reaction was stopped by addition of 1 ml ethanol, and the absorbance at 540 nm was read against the blank by a spectrophotometer. One unit of enzyme was defined as the amount of enzyme that produces 1ml of H_2O_2 per minute under the standard assay conditions [10].protein concentration was determination by Bradford assay [12].

Purification of uricase by ion exchange chromatography

A glass column (1.5×60 cm) was packed with DEAE-cellulose. The concentrated and dialyzed cell free supernatant was applied to a column, which previously was equilibrated with 0.01M borate buffer (pH 8.5). The column was washed with 3 times volumes of 0.01 M borate buffer, pH 8.5 at a flow rate of 40 ml/hour and the bound proteins were eluted with a linear NaCl gradient (0-1 M) in the same buffer by using fraction collector (LKB Radi-Rac) and analyzed by UV spectrophotometer at 280nm . Fractions containing uricase enzyme were pooled and concentrated by using sugar [13].

Effect of different concentration of red cabbage extract on uricase activity.

The experiment was performed to investigate the effect of different concentration or RCF on activity of uricase (20, 50, 100, 200, and 300 mg/ml) on uricase activity .as follow:Mix well and leave for 10 min. Then stopping the reaction, calculation the uricase activity [10].

0.5 ml	**uricase + 1 ml uric acid + 0.5 ml D.W.**
0.5ml	**uricase + 1 ml uric acid + 0.5 ml RCE(20 mg/ml)**
0.5ml	**uricase + 1 ml uric acid + 0.5 ml RCE(50 mg/ml)**
0.5ml	**uricase + 1 ml uric acid + 0.5 ml RCE(100 mg/ml)**
	uricase + 1 ml uric acid + 0.5 ml RCE(200 mg/ml)
	uricase + 1 ml uric acid + 0.5 ml RCE(300 mg/ml)

$$\textbf{Uricase activity} = \frac{volume\ of\ reaction}{12.2x1xvE} \textbf{ X } \Delta \frac{A290}{\Delta\ min}$$

Results and Discussion

Using aqueous solvent, yield of recovering red cabbage extract was observed. High yield of crude aqueous extract was produced from 100 g of dried whole plant was 11.0 gm (11.0 %) with the violet dry material similar to the optimum condition reported in the literature to extract high yield of active compounds without degradation were 70% ethanol as solvent for extraction, 50C for temperature at overnight swirling. The variations yields as seen between alcoholic and aqueous extract depend on several factors, such as the method of extraction and solvent polarity that was used in the extraction process**.** Phenolic compound can be extracted from plant samples. Usually before extraction plant samples are treated by grinding and homogenization, which may be preceded by freeze-drying. Generally, freeze-drying retains higher levels of Phenolic content in plant samples than air-drying. However, drying processes, including freeze-drying, can cause undesirable effects on the constituent profiles of plant samples, therefore, caution when prepare planning and analyzing research studies on the medicinal properties of plants [3,8].

The aqueous extract of RC powder was carried out and result was 6.0 gm (6.0 %) while alcoholic extraction of red cabbage was 5.4 gm (5.4 %).[14**]**. The solubility of phenolic compounds is governed by the chemical nature of the sample of plant, as well as the polarity of the solvents used. Plant materials may contain phenolics varying from simple e.g., phenolic acids, anthocyanin). Moreover, phenolic may also be associated with other plant components such as proteins and carbohydrates. Therefore, there is no union extraction procedure suitable for extraction of all plant phenolic. Depending on the solvent system used during exaction, a mixture of phenolic soluble in the solvent will be extracted from plant materials. It may also contain some non-phenolic material such as sugar, organic acids and fats. As a result, additional steps may be required to remove those undesirable components [8,15].

Anthocyanin are polar molecules with hydroxyl, carboxyl, methoxyl and glycolyl groups bound to aromatic rings. They are more soluble in water than in non-polar solvents and this characteristic helps extraction and separation processes, as described by Corrales [16].

Hydrochloric acid diluted in methanol is generally used to extract anthocyanins. Since methanol has toxic characteristics, food scientists prefer other extraction systems. Water: ethanol mixture of 80: 20 (v/v) is commonly used as a solvent in the food industry, and it is as good as methanol .In aqueous extractions, the most used and efficient acids are acetic, citric, tartaric and hydrochloric. Studies on anthocyanin extraction using these solvents are found in literature although, most of the literature concerns to the identification of anthocyanins in several vegetable sources, studies are carried out to develop the process and establish good operational conditions.

Generally, temperatures higher than 70°C cause rapid degradation and discoloration of anthocyanins .Opening of the pyrylium ring with a loss of the B-ring and chalcone formation which further decompose to coumarin glucoside derivatives was considered as the first anthocyanin degradation step hydrolysis of the glycosidic moiety and formation of aglycon even at acidic pH as the initial degradation reaction has been reported [17].

Phytochemical screening was done using color forming and precipitating chemical reagents on the dried whole leaves of red cabbage. Results obtained from the tests It was shown that red cabbage extract (RCE) contain flavonoides which contain phenolic compounds and anthocyanines which glycoslated with mono or dimolecules of saccharides.The principle constituents of red cabbage extract anthocyanins such as cyanidine 3-galactoside ,cyanidine 3-glucoside ,cyanidine 3-5 diglucoside and acetylated anthocynins with sinapic acid ,coumaric acid ,ferulic acid in addition to isothiocyanates (glucosinolate), vitamins A, B, C and β-carotenes . Anthocyanins, a group of phenolic natural pigments present in RCE, were found to have the strongest antioxidizing power of 150 flavonoids.

Anthocyanins are potent antioxidants *in vitro,* quench free radicals and terminate the chain reaction that is responsible for the oxidative damage. Demonstrated the antioxidant effects of anthocyanins *in vitro* using human colon cancer cell line (CaCO2). Cyanidin and Cyanidin-3-glucoside treatment reduced cell growth and cell proliferation in a dose-dependent way, decreased ROS level by any concentration of Cyanidin and only at the lowest concentration, by Cyanidin-3-glucoside and increased cell cycle/stress proteins expression [18,19]. Both the compounds affected DNA fragmentation, highlighting their antioxidant activity. The authors concluded that anthocyanins exhibit antioxidant properties and could therefore be useful in the treatment of pathologies where free radical Production plays a key role. The antioxidant activity of anthocyanins is largely because of the presence of hydroxyl groups in position 3 of ring C and also in the 3‘, 4‘ and 5‘ positions in ring B of the molecule. Anthocyanidins have superior antioxidant activity compared to their respective anthocyanins and the activity decreases as the number of sugar moieties increase. The antioxidant activity of many anthocyanins was comparable to the commercial antioxidants such as tert-butylhydroquinone (TBHQ), butylated hydroytoluene (BHT), butylated hydroxyanisole (BHA) and vitamin E..By analyzing TLC chromatogram of falvonoids as seen in figure 1- 1,we can note the presence of several spots corresponding to compounds with flavonid behavior(yellow, yellow –brown after spraying with diphenylboriloxiethilamine) or de hydroxycinnamic acid derivatives (blue or green-blue fluorescensce, after spraying with the mentioned reagent),.Among these, quercetin (Rf = 0.92) have been identified in all the analyzed plant extract. The consulted scientific data mentions flavonoids glycosides of quercetin with xylose , galactose as monosaccharides [16, 14, 19].

Separation of uricase

Separation of uricase from culture media of *Pseudomonas aeruginosa*. Activity of enzyme and protein concentration was determined. Results shown in table-1.

Ammonium sulfate precipitation

Ammonium sulfate was used at different concentration to precipitate the enzyme. Results indicate that precipitation of crude uricase was done with saturated ammonium sulfate 70%, and specific activity was estimated as 6.23 U/mg, purification fold of 6 and yield of 67% as show in table-1.

Purification of uricase by ion exchange chromatography

Purification of uricase from *P. aeruginosa* was done use ion exchange chromatography. During the experiment, it was found that washing with borate buffer pH 8.5, three peak was observed,. as shown in figure -1. Addition of 300ml of borate buffer with NACL gradients (elution step) allows four peak to be obtained and represented by fraction 58-73 ,85-90 ,94-104,110-115 . Each fraction was tested for

uricase activity and protein concentration. The fraction in elution step – were showed uricase activity. Result also indicates that uricase produced from *P. aeruginosa* carried negative charges which attraction with DEAE-cellulose of positive charge therefore uricase was eluted and goes to elution step. As described in table 1 protein concentration of 0.56 mg/ml, activity of 4.9 U/ml, and specific activity of 8.75 U/mg, with purification fold of 8.4 and yield of 49% were obtained.

Table 1-purification step of uricase from *Pseudomonas aeruginosa*.

Steps	Volume ml	Activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg protein)	Total activity (units)	Fold of purification	Yield%
Crud extract	50	1.43	1.39	1.03	71.5	1	100
Pellet after 70% ammonium sulphate and Dialysis	8	5.99	0.96	6.23	47.9	6.0	67
Ion-exchange DEAE cellulose and sucrose	7	4.9	0.56	8.75	34.32	8.4	48

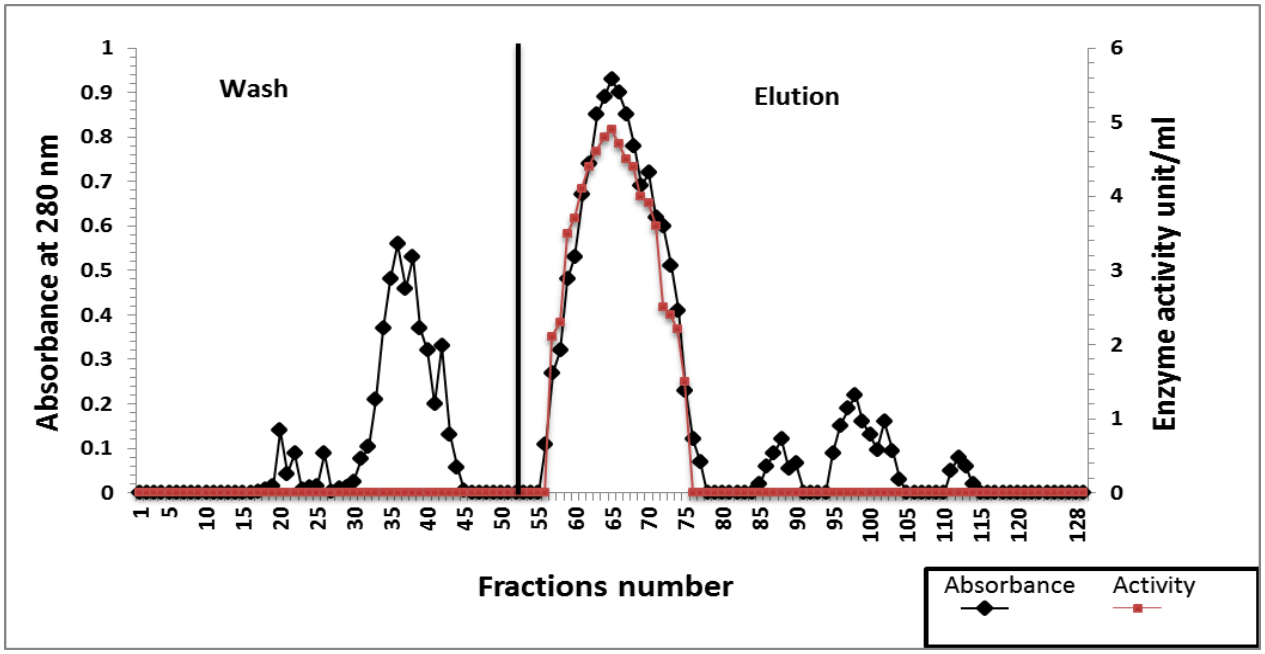

Figure 1-ion exchange chromatography for purification of uricase from *P. aeruginosa* by using DEAE-cellulose column, equilibrated with borate buffer (0.005 M, ph 8.0), eluted with borate buffer with NaCl gradient (0.1-1 M).

Effect of different concentration of red cabbage extract on uricase activity.

The experiment was performed to investigate the effect of different concentration from RCF on uricase activity. The results in figure -2 , indicated that uricase activity was decreased with increased concentration ,red cabbage extract.

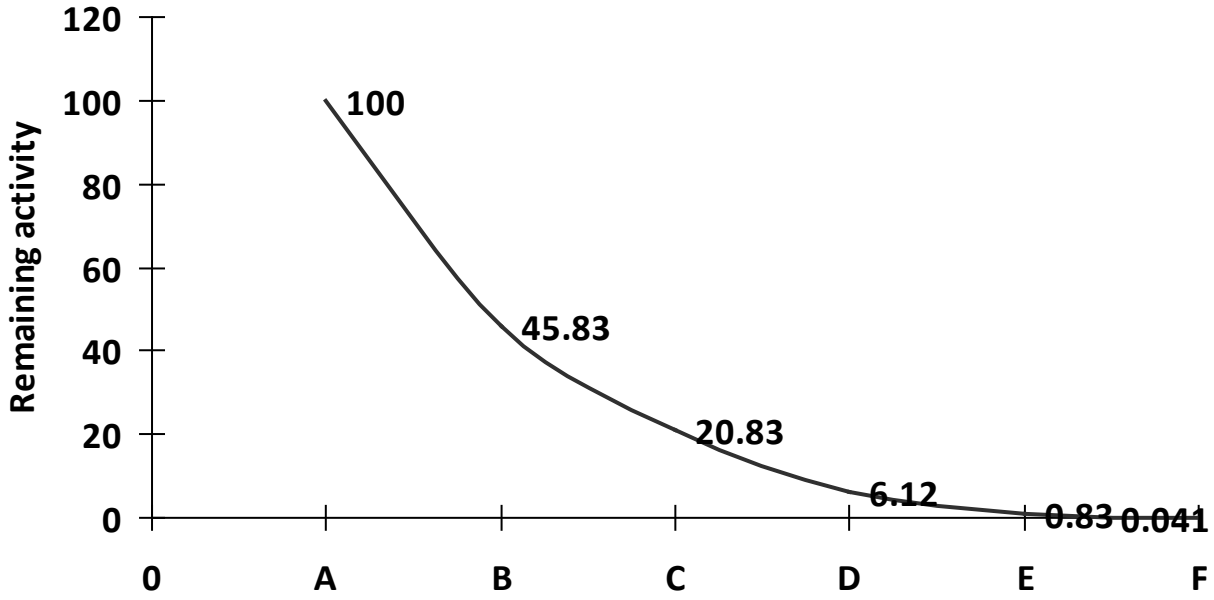

Figure 2- Effect of different concentration of red cabbage extract on uricase remaining activity.
A- 0.5 ml of uricase + 1 ml uric acid + 0.5 ml D.W. **B-** 0.5 ml of uricase + 1 ml uric acid + 0.5 ml RCE (20 mg/ml). **C-** 0.5 ml of uricase + 1 ml uric acid + 0.5 ml RCE (50 mg/ml). **D-** 0.5 ml of uricase + 1 ml uric acid + 0.5 ml RCE (100 mg/ml). **E-** 0.5 ml of uricase + 1 ml uric acid + 0.5 ml RCE (200 mg/ml). **F-** 0.5 ml of uricase + 1 ml uric acid + 0.5 ml RCE (300 mg/ml).

Aknowlgment: faithful thanks for professor Ali AL-Mousawi for classification of plant.

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