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APPLICATION OF IMMUNOENZYMATIC TECHNIQUE IN DETERMINATION OF GALANTHAMINE IN TWO *PANCRATIUM* SPECIES

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ABSTRACT

Enzyme-Linked Immunosorbent Assay (ELISA) was established to qualitatively and quantitatively determine galanthamine in the alcoholic extract of *Pancratium foetidum* Pomel bulbs and in the callus culture of *P. arabicum* Sick. The amount of galanthamine was 168 ng/g dry weight of bulbs and 314 ng/g dry weight of the callus. The measuring range for galanthamine extended from 0.1-100 ng per assay. The least amount to be detected was 0.01 ng and the recovery was 98 ±1.8 % .

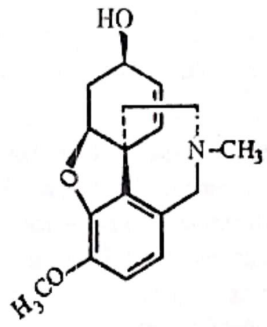
INTRODUCTION

Recently, immunoassays has been widely used to detect and quantitatively determine a wide range of plant constituents as well as important pharmaceutical products[1-3]. This is because enzyme immunoassays have been successfully used in the determination of a wide range of high as well as low molecular weight compounds[4]. The principle advantage of enzyme immunoassay is that, it can be carried out easily even under field conditions and it requires neither specialized nor costly equipment[2].

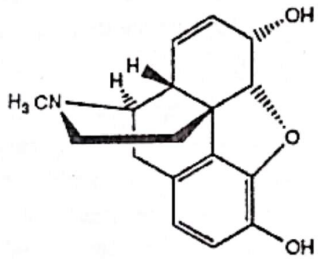
Galanthamine is an alkaloid derived biosynthetically from dibenzofuran . It is the only alkaloid of the family Amaryllidaceae alkaloids which shows certain therapeutic applications[5]. These include its extensive use as a curare-reversal agent as it reverses the neuromuscular blockade induced by tubocurarine by increasing the sensibility of the skeletal muscles to acetylcholine and reactivates the neuromuscular conduction. Recently, it has been approved in Austria for treatment of mild-to-moderate Alzheimer's disease due to its central anticholinesterase effect[6,7]. Furthermore, galanthamine-at doses of 0.5 to 1.5 mg /kg-produced 1.6 to 13-fold increase in the analgesic effect of morphine[8]. Also, galanthamine has a remarkable prophylactic action against irreversible anticholinesterase substances like organophosphorous compounds. Moreover, pretreatment with galanthamine increases the enzyme sensitivity for enzyme reactivator[9].

Thus, the development of precise and sensitive method for detection and quantitation of galanthamine in its natural sources or pharmaceuticals would be a valuable advancement for phytochemical, medicinal and biosynthetic studies.

In this study, ELISA procedure is presented for qualitative and quantitative determination of galanthamine in crude extracts of *Pancratium foetidum* Pomel bulbs and callus culture of *P. arabicum* Sick.



Galanthamine



Morphine

EXPERIMENTAL

Plant material:

The bulbs of *Pancratium foetidum* Pomel-during the fruiting stage-were collected from plants growing at Tajura, near Tripoli, Libya in October 1992. Identity of the plant was verified by Prof. Dr. A. El-Gadi, Department of Botany, Faculty of Science, Al-Faateh University, Tripoli, Libya. The seeds of *Pancratium arabicuum* Sick. were collected from plants growing near Alexandria, Egypt in October 1992 and the identity of the plant was verified by Prof. Dr. N. El-Hadidi, Department of Botany, Faculty of Science, Cairo University, Cairo, Egypt. Voucher specimens are deposited at the Department of Pharmacognosy, Faculty of Pharmacy, University of Zagazig, Egypt.

Media:

Nitsch medium[10]; **Gamborg** medium (B5)[11]; **DAX** medium: Gamborg medium (1L) plus 2,4-dichlorophenoxy acetic acid (0.2 mg); **PAX** medium :

Gamborg medium (1L) plus 2,4- dichlorophenoxy acetic acid (1.0 mg), 4X medium : Gamborg[11] medium (1L), 2,4-dichlorophenoxy acetic acid (2 mg), 1-naphthyl acetic acid (0.5 mg), IAA (0.5 mg) and Kinetin (0.2 mg); LSK medium:LS medium[12] (1L) plus KNO_3 (4 g) and Th medium : LS medium (1L) plus tyrosin (1 g).

Authentic sample of galanthamine was obtained from Prof. Dr. M.H. Zenk, Institute of Pharmaceutical Biology, University of Munich, Munich, Germany.

Bovine serum albumin (BSA), complete and incomplete forms of Freund's adjuvant, rivanol (6,9-diaminoethoxyacridine lactate), sodium periodate and horseradish peroxidase (HRP, type VI-A) were obtained from Sigma (Munich, Germany). ABTS (2,'2- azino-di (3 ethyl-benzthiazoline sulfonic acid-6) ammonium salt was obtained from Boehringer (Mannheim, Germany). Precision microplat reader : Molecular device E max (USA).

Methods:

I- Formation of specific antibody against galanthamine :

Galanthamine has to be coupled with a large molecule (bovine serum albumin, BSA) to be immunogenic[13] but galanthamine lacks functional group to allow covalent conjugation with BSA, so it has to be functionalized by being imparted a carboxyl group[14] . The derivatized galanthamine can be linked through its carboxylic group to the amino group of protein (BSA) using carboimide as a linkage reagent[15,16] as shown in Scheme 1.

a- Synthesis of galanthamine- 2-0-hemisuccinate and coupling to BSA:

Galanthamine hydrobromide (90.1 mg) and succinic anhydride (150 mg) were dissolved in pyridine (1.5 ml). The mixture was left in oil bath at 100°C with continuous stirring for 22 hr. The reaction mixture was concentrated under reduced pressure at 55°C and the residue was dissolved in chloroform- methanol mixture (1 : 1) , This was followed by purification using PTLC on silica gel precoated plates and $CHCl_3$-MeOH-NH_4OH (90:9:1) as a solvent system to afford galanthamine -2-O- hemisuccinate (78.1 mg). Galanthamine 2-O- hemisuccinate (46. 5 mg) was dissolved in pyridine- water mixture (1:1, 6 ml) to afford solution [1]. 1- Ethyl-3- (3-dimethylaminopropyl) carboimide (52 mg) was dissolved in pyridine-water mixture (1:1, 3 ml) to give solution [2]. Solution [2] was added to solution [1] dropwise followed by continuous stirring for 20 min. at room temp. to afford solution [3]. BSA solution (72 mg in 6 ml deionized H_2O) was added dropwise to this mixture and stirred for 20 hrs. at room temp. This reaction mixture was dialysed (1L deionized H_2O/ 12 hr, 6 days) to afford galanthamine-2-O- hemisuccinate-BSA conjugate (62 mg) which was kept lyophilized.

b- Immunization and antiserum production:

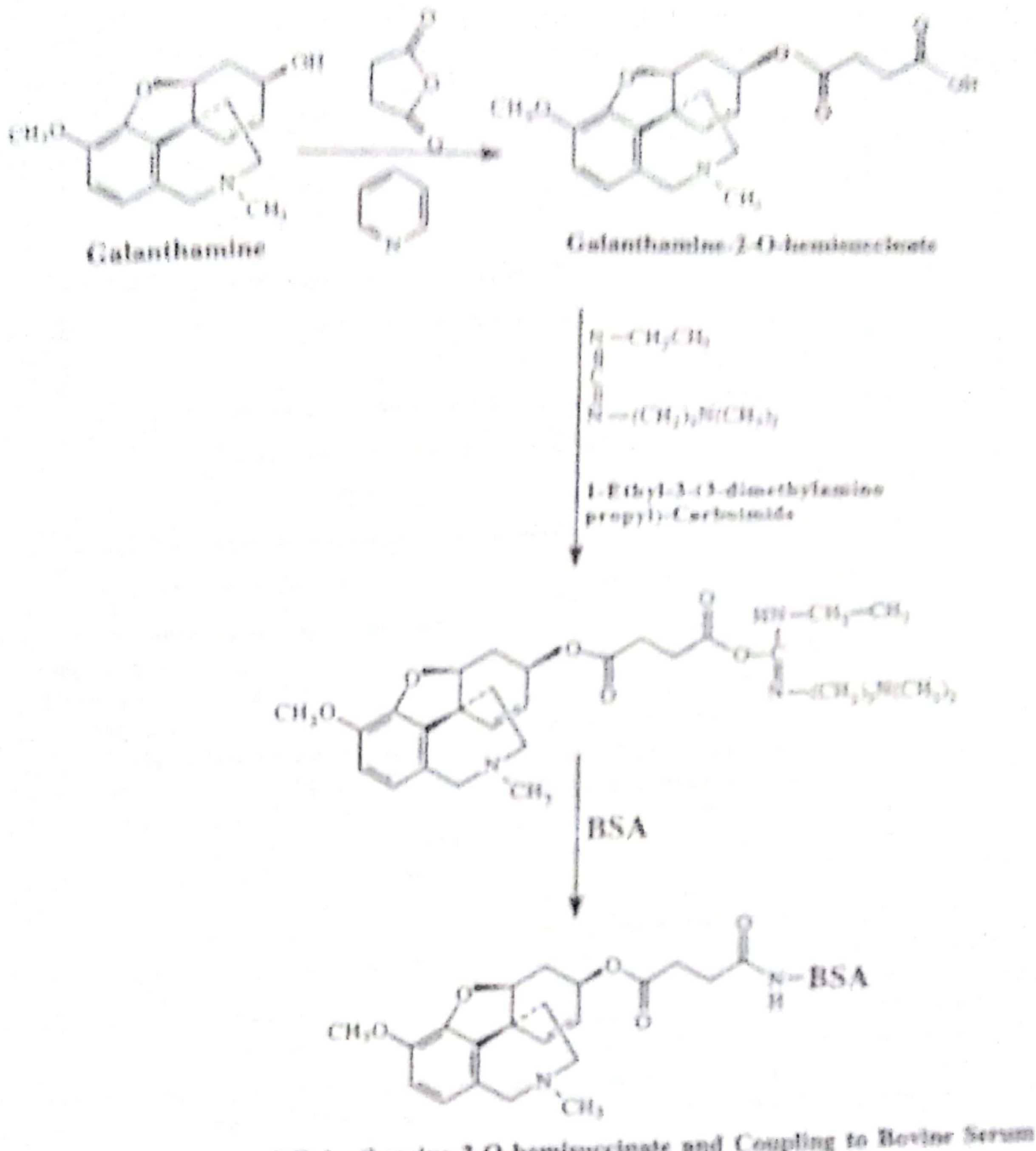
The freez - dried hapten - BSA conjugate (0.5 mg) was dissolved in 250 µl normal saline, emulsified in an equal volume of Freund's adjuvant and injected intradermally (once a week for 4 consecutive weeks) into 4 albino rabbits (12-week old, each weighing about 2 kg). Complete Freund's adjuvant was used during the first two weeks of initial immunization while the incomplete form is used during the following two consecutive weeks. The following immunization steps that were taken are shown in Table 1. Starting from the ninth week the blood was collected, allowed to clot at room temp. for 2 hr, centrifuged at 4000 rpm for 10 min. The antiserum was decanted and stored in aliquots in air-tight vials (10 ml) and kept at -20°C.

c- Isolation and purification of Immunoglobulin G (IgG) from antiserum[17] :

The pH of the antiserum was adjusted to 8.5 (NaOH, 0.1 N). Rivanol (25 ml, 0.4 %) was added to the antiserum (10 ml) dropwise with slow mixing, centrifuged at 4000 rpm for 10 min. and the supernatant was decanted. Charcoal (100 mg) was added to 10 ml of supernatant and stirred for 10 min. The solution was filtered and the filtrate was centrifuged at 4000 rpm for 10 min. Equal volumes of saturated solution of ammonium sulphate was added dropwise to the supernatant . The mixture was kept overnight at 4°C for sedimentation and centrifuged at 4000 rpm for 10 min. The sediment was dissolved in Tris buffer (5 ml, 0.2 M, pH 7.8) and dialysed (1 L deionized H_2O/12 hr, 3 days). The sulphate free product left after dialysis (2 L deionized H_2O/ 12 hr, 3 days) was frozen with liquid nitrogen and lyophilized. The lyophilized IgG-fraction was dissolved in $NaHCO_3$ solution (0.01 M, pH 9) and stored in 1 ml portions at -20°C.

II- Preparation of enzyme-labelled galanthamine[18]:

Galanthamine-2-O-hemisuccinate (4.2 mg) was dissolved in dimethylformamide (125 µl) and tri-n-butylamine (5 µl) was added. The mixture was cooled (-15°C) using cooling mixture (NaCl- ice, 1:3) for 5 min. Isobutylchloroformate (5 µl) was added to the mixture and left at -15°C for 20 min. to form the anhydride mixture. Horseradish peroxidase (10.6 mg) was dissolved in $NaHCO_3$ solution (360 µl, 5%) and



Scheme (1) : Synthesis of Galanthamine-2-O-hemisuccinate and Coupling to Bovine Serum Albumin .

dioxan (360 µl) and added to anhydride mixture (65 µl). The mixture was left for 20 min.

Then, another 70 µl of the anhydride mixture was added, stirred at 0°C for 1 hr and at 10°C for 30 min. phosphate buffer solution (1.8 ml, 0.1 M, pH 7) was added to the solution and dialysed (1L, 0.1 M phosphate buffer / 8 hr, 3 days). Sodium azide (21 µl, 0.02% was added to the dialysed product (4.7 ml) and kept at 4 °C.

III - ELISA procedure[19] **:**

Microtiter plate (96 wells, Nunc) was coated with antibody solution (0.75 µg/ well). Wells intended for determination of unspecific binding were coated with $NaHCO_3$ (200 µl, 0.01 M, pH 9). The microtiter plate was incubated overnight at 4°C. The antibody solution was discarded and the wells were washed with phosphate-buffered saline (PBS), (400 µl /well, pH 7.4). All the wells received 200 µl of BSA (0.5% in 0.1M $NaHCO_3$, pH 9), incubated at 4°C for 1 hr and washed with PBS (400 µl / well, pH 7.4). All wells received 150 µl of PBS (pH 7.4), 50 µl of either test sample or standard and enzyme tracer (dilutions range 1 : 10^4) and

Table 1: Immunization Schedule for Induction of Antibody Against Galanthamine-2-O- hemisuccinate conjugate.

Week No.	Procedure
1 and 2	Initial immunization; intradermally with the conjugate and complete Freund's adjuvants; (1:1)
3 and 4	Initial immunization; intradermally with the conjugate and incomplete Freund's adjuvant; (1:1)
5	Pause.
6	Boosting injection, intramuscular in the right thigh (1 mg conjugate / 0.5 ml normal saline)
7	Boosting injection; intramuscular in the left thigh (1 mg conjugate / 0.5 ml normal saline)
8	Pause
9	Collection of blood from the marginal vein of the right inner ear.
10	Collection of blood from the marginal vein of the left inner ear.
11	Pause

mixed using a vibrating shaker for 30 sec. The plate was incubated at 37°C for 1hr, the wells were washed twice with PBS (400 µl, pH 7.4). ABTS / H_2O_2 solution (8 mg ABTS in 20 ml phosphate-citrate buffer, pH 4.5 + 50 µl of 10 - fold diluted H_2O_2 solution) 200 µl was added to each well, mixed using vibrating shaker and incubated at 37°C for 90 min. The enzyme reaction was measured photometrically at λ_{max} 405 nm using a microplate reader.

For calibration curve, each concentration of a serial dilution of either the standard or the test substance was applied in duplicate or triplicate. Wells intended for determination of unspecific binding (UB) and maximal binding (Bo) received no sample but they received PBS (50 µl, pH 7.4). Calculations[20,21] were made using immunoassay computation software :

$$\% \text{ Unspecific binding (UB)} = \frac{UB}{Bo - UB} x100$$

$$\% \text{ relative binding of the maximal binding of the enzyme tracer(*B/Bo)} = \frac{*B - UB}{Bo - UB} x100$$

Linear dose interpolation was obtained through the logit-log transformation :

$$\text{Logit [*B/Bo (\%)]} = \text{In } \frac{\% \text{ B/ Bo}}{100 - \% \text{ B/ Bo}}$$

(*B is the sample reading)

Estimation of galanthamine content in the extract of *Pancratium foetidum* Pomel :

The powdered dry bulbs (2.5 g) of *Pancratium foetidum* Pomel was extracted with EtOH (95%, 20 ml) by heating with stirring on a water bath at 45°C for 60 min, protected from light. The extract was filtered, the marc was washed with EtOH (95%). The filtrate and washing were combined and the volume was adjusted to 10 ml with EtOH (95%). Serial dilutions with PBS were made (1:1, 1:10, 1:100 and 1:1000) and subjected to ELISA procedure. Preparations for the standard curve (Fig. 1; slope - 0.68) were made by making serial dilutions of galanthamine from 1 mg /1 ml stock solution. The dilutions for the test solution were applied along with the standard in the same microtiter plate.

Estimation of galanthamine in callus culture of *Pancratium arabicum* Sick. :

Seeds (deprived of testae) were sterilized by transferring into a conical flask (500 ml) containing EtOH (300 ml, 70%) and shaken for 3 min. to affect surface sterilization. Alcohol was discarded and the seeds were wrapped in a gauze and submerged in a beaker filled with sodium hypochlorite solution (5%) containing 0.01% Tween 20 for 15 min. The seeds were washed with sterile deionized water and placed over a double-layer, sterilized filter paper in a Petri dish to remove remains of water. The sterilized seeds were transferred into Petri dishes containing Nitsch media, then sealed with parafilm and incubated at 23°C for 60 days.

Explants were transferred into different media; Nitsch, B5, DAX, PAX, 4X, LSK and Th., after six monthes, radicles and plumules were developed. The primary seedling radicles and plumules that emerged were excised (5 mm in length) and subcultured into

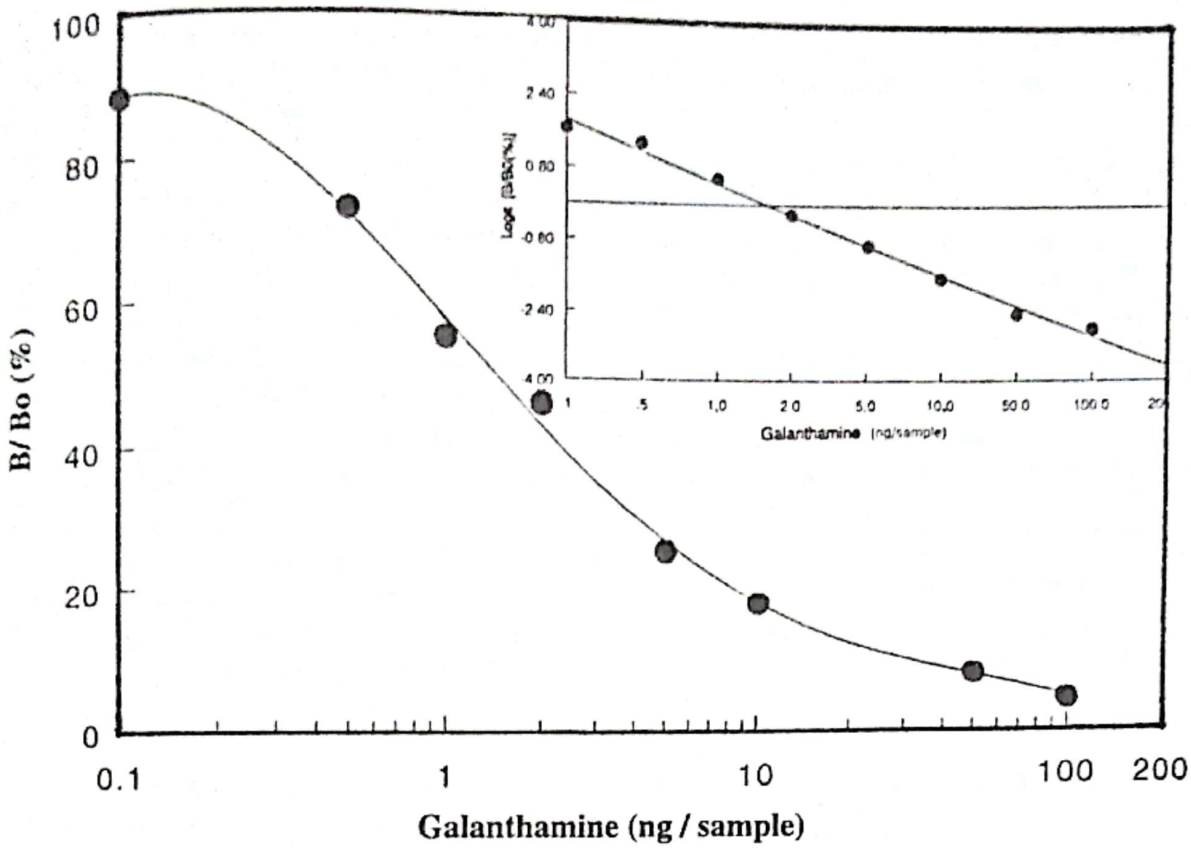


Fig. (1) : Standard ELISA Curve for Galanthamine (Inserted figure gives a linearized logit-log plot).

fresh media untill explants have been used up. About 3.0 g of fresh callus was extracted with MeOH at 45°C with continuous stirring away from light. The extract was filtered and the filtrate was dried under reduced pressure at 45°C to afford 65.0 mg of yellowish-brown residue. The residue was dissolved in MeOH and submitted for the previously mentioned ELISA procedure of galanthamine.

RESULTS AND DISCUSSION

As a result of a previous study[22] on *Pancratium foetidum* Pomel, trispheridine, crinamine, haemanthidine, lycorine and crinine were isolated and identified. Most of assay processes of alkaloids in plant material depend on primary purification techniques followed by sophisticated procedures which may be titrimetric, gravimetric, chromatographic and spectroscopic methods. These methods are efficient in high concentrations or in concentrations of mg /g of plant material. The present ELISA quantitates galanthamine (168 ng /g dry weight of powdered *Pancratium foetidum* Pomel) and this proves the efficiency of ELISA in detection of traces of galanthamine (detection limit 0.01 ng) and determine its percentage in the presence of other major constituents without interference.

Callus cultures of *Pancratium arabicum* Sick. were developed successfully from radicle on PAX medium but plumule cutlets failed to form culture callus. The development of the callus was slow as it reached 2 cm in diameter in twenty monthes. Galanthamine was previously isolated from *Pancratium arabicum* Sick. plant material[23]. As a result of application of ELISA to the determination of galanthamine content in the callus of this plant, galanthamine was found in a concentration of 314 ng /g dry weight of the callus tissue without interference with numerous components of the culture media. This work of ELISA for determination of galanthamine in *Pancratium foetidum* Pomel bulbs and *Pancratium arabicum* Sick. callus culture represents the first application of the assay in genus *Pancratium* [24].

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تطبيق تقنية التحاليل المناعية الانزيمية فى تعيين الثالاكتمين فى نوعين من جنس البانكريشيوم

تحية مصطفى سرج - ماينهارت زنك* - سميح ابراهيم الدهمى - عفاف السيد عبد الغنى - عايدة ابو هاشم

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فى هذا البحث تم تطبيق إحدى تقنيات التحاليل المناعية الانزيمية وهى الاليزا للتعيين الكيفى والكمى لقلويد الثالاكتمين فى الخلاصة الكحولية لأبصال نبات البنكريشيوم فوتيدم بومل كما تم تعيين هذا القلويد فى مستنبت نسيجى لنبات البنكريشيوم أرابيكم سك الذى استنبت للمرة الأولى وقد بلغت حساسية هذه التقنية ٦٠ر٠ نانوجرام ومدى التطبيق ٩ر٠ - ١٠٠ نانوجرام والاستعادة ٩٨ ± ٨ر١٪.