Evaluation of antimicrobial activity of Ethanol Extracts of potato Glycoalkaloids against some pathogenic bacteria

Dheyaa khaleel Ismael

Directerate of Materials Research, Ministry of Sciences &Technology – Baghdad -Iraq

Abstract :-

This study included extraction and partial purification of glycoalkaloids compounds in potato tuber, Preliminary chemical screening of the glycoalkaloids extract detected the presence of alkaloids by addition of some specific reagents such as Mayer's reagent, Dragendroff's reagent and Wagner's reagent.

In the extraction process, two methods were used: alcoholic extract and Soxhlet extraction, The extraction of glycoalkaloid was screened in vitro for antibacterial activity using Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus cereus*) and Gram-negative bacteria (*Pseudomonas aeruginosa* and *Escherichia coli*).. Antimicrobial activity of glycoalkaloids was evaluated by well diffusion method and agar dilution assay. The zone of inhibition against the tested bacteria was found ranging from (6 to 28)mm. The results were displayed antibacterial activity of the two extracts on the pathogenic bacteria. The highest effect for soxhlet extract as a concentrated solution was found on *P aregonosa*, *S. aureus*, *B cereus* and *E.coli* was (28, 22,15 and 12) mm respectively, The Minimum Inhibitory Concentration (MIC) value was determined against all the tested bacteria. The MIC values of Soxhlet Extract were found to be 50mg/mL, whereas the Minimum Bactericidal Concentration (MBC) values were found 100mg/mL. Extracted glycoalkaloids (Stock solution) showed a degree of inhibition ranging from medium to good against the growth of all tested bacteria, whereas the results were demonstrated a slightly decrease or no alteration in viable colony count reduction in dilute solution (1/4 and 1/8).

Key words Antimicrobial activity, Glycoalkaloid, Potato tuber

تقيم الفعالية البايلوجية للمركبات القلويدية السكرية المستخلصة بالايثانول من درنة البطاطا ضد بعض أنواع البكتريا المرضية

ضياء خليل إسماعيل

دائرة بحوث المواد – وزارة العلوم والتكنولوجيا-بغداد –العراق

المستخلص :-

تضمنت الدراسة استخلاص والتنقية الجزئية والكشف الكيميائي لبعض المركبات القلويدية السكرية المستخلصة من درنة البطاطا، . استخدمت الكواشف التمهيدية (الاستدلالية)عن وجود القلويات في المستخلص وذلك بإضافة بعض العوامل الكاشفة عن القلويات مثل كاشف ماير Mayer's reagent وكاشف كاشف دراغن دروف Dragendroff وكاشف فاجنر Wagner.

استخدمت طريقتين في عملية الاستخلاص: الاستخلاص بالكحول والاستخلاص بواسطة السوكسليت، تم التحقق عن الفعالية البايلوجية للمركبات السكر القلوية خارج الجسم باتجاه البكتيريا إيجابية الجرام (المكورات العنقودية

الذهبية *Staphylococcus aureus* ، العصوية الشمعية *Bacillus cereus*) والبكتيريا سالبة الجرام (الزائفة الزنجارية *P seudomonas aeruginosa* والإشريكية القولونية *Escherichia coli*). تم تقييم هذه الفعالية بطريقة الانتشار بواسطة الحفر وفحص التخفيف على الاكار. وجد إن منطقة تثبيط البكتيريا المختبرة تتراوح ما بين 6 إلى 28مليمتر. و أظهرت النتائج الفعالية البابولوجية لاثنين من المستخلصات على البكتيريا المرضية، حيث أظهرا تأثيرات مختلفة على نمو البكتيريا، ووجد أن أعلى تأثير كان للمستخلص بواسطة السوكسليت عند المحلول المركز على *P aregonosa* ، *S. aureus* ، *B cereus* و *E.coli* كانت (15,22,28و12) ملم وعلى التوالي .كذلك تم تحديد قيمة الحد الأدنى للتركيز المثبط (MIC) ضد كل أنواع البكتيريا المختبرة. حيث وجدت إن قيمة الحد الأدنى للتركيز المثبط (MIC) للمستخلص بواسطة السوكسليت كانت 50 مغم / مل، في حين أن الحد الأدنى لتركيز القاتل للبكتريا (MBC) كان 100مغم / مل، مركبات السكر القلوية المستخلصة أظهرت درجة تثبيط تتراوح من متوسطة إلى جيدة ضد نمو جميع سلالات البكترية التي اختبرت عند المحلول المركز ، في حين أظهرت النتائج انخفاضا طفيفا أو بدون تغيير في أعداد المستعمرات البكتيرية النامية في المحلول المخفف (4/1 و 8/1).

مفتاح الكلمات: الفعالية البايلوجية، المركبات القلويدية السكرية، درنة البطاطا

INTRODUCTION

Plant based antimicrobials remain a vast untapped source for medicine with enormous therapeutic potentials. They are effective in the treatment of infectious diseases. Herbal products are promisingly important source for therapeutics and may be a viable solution for disease control (Murray, 1995).

Numerous plants produce toxins, they are regarded as defensive chemicals against a number of pathogens and predators including fungi, viruses, bacteria, insects, and worms (Friedman 2005).A large group of such natural toxins are the alkaloids counting very toxic compounds such as strychnine, morphine, cytisine, and atropine. (Raffauf,1996).

Glycoalkaloids a class of nitrogen-containing steroidal glycosides are in nature occurring secondary metabolites, The term "secondary metabolites" indicates compounds that are not required for plant growth and development but presumed to function in communication or defense (Luckner,1990). There are different types of steroid alkaloids, some of which are conjugated to carbohydrates to form glycoalkaloids that are commonly found in the Solanaceae family which includes many significant agricultural plants, such as tomato, potato, pepper and nightshade (Dinan *et al*., 2001).

Glycoalkaloids are naturally occurring compounds present in potato. the Potatoes that become sunburned during growth and start to turn green, due to lack of soil cover, tend to taste very bitter as a result of their high glycoalkaloid content. Consequently, higher concentrations formed within aerial tuber tissue and sprouted tubers may confer increased host resistance during growth and development (Mader *et al.,*2009).also the highest concentrations of glycoalkaloids are usually associated with mechanical damage (Machado et al., 2007). There are many factor that may affect the concentration of glycoalkaloids in potato plants such as harvest and postharvest treatments, drought (Bejarano *et al.* 2000), high temperature (Lafta and Lorenzen, 2000), and wounding (Choi *et al*., 1994).

The most important of these compounds is solasodine, a steroidal alkaloid that is widespread in Solanum species, More than 95% of the glycoalkaloids are made up of α solanine and α chaconine, they are known to be highly toxic to humans and animals. It has been reported that low doses of Glycoalkaloids in take can cause gastrointestinal disturbances (Hellenäs *et al.,* 1992); while at higher doses, the toxicity of Glycoalkaloids leads to acute intoxication and more severe symptoms, including rapid pulse, low blood pressure, neurological disorders and, in severe cases, coma and death(Langk-ilde *et al.,* 2009).

An official safety or acceptable limit of total glycoalkaloid content for human consumption in tubers is 1 mg/kg body weight . Glycoalkaloid contents below this guideline are not thought to represent health risks for humans(Vorne & Hallikainen, 2003).

The major toxic properties of glycoalkaloids are due to the ability of glycoalkaloids to bind with membrane and to disrupt membrane function (Roddick, 1996).

Although in high doses they are toxic, glycoalkaloids may also have beneficial effects. These include lowering of blood cholesterol (*Friedman et al.*,2000a, b), protection against infection by *Salmonella typhimurium* (Gubarev *et al.*, 1998), and chemoprevention of cancer (Friedman *et al.*, 2005. 2007).

The solubility of α- solanine in water is low, and high affinity of the compound for sorption to natural organic matter. therefore both the cationic and uncharged form will be present in most agricultural soils having pH in the range 5–7. Both glycoalkaloids are quite resistant to acidic conditions and hydrolyses only slowly.

The toxin is heat – stable, but it will eventually break down at high temperatures, Deep frying temperatures of over about 760 ° C.

The growing interest in secondary metabolites of plants has directed attention to methods for their extraction. Natural products are extracted by conventional methods such as Soxhlet and room temperature solvent extraction.

Many of the medicinal properties of solanaceous species are now known to be attributable to the glycoalkaloids they contain, some of these compounds have become increasingly important in the pharmaceutical industry, as they can be chemically converted to steroid drugs (Jadhav *et al.*, 1973).

The study was conducted to investigate and evaluate the antibacterial activity of glycoalkaloids compounds in potato tuber extracts against some pathogenic bacteria.

Materials and Methods

Instrumental :

- Soxhlet unit
- rotary evaporation

Chemicals:-

All agars and chemicals used in this study were of highest grade of purity and were purchased from Oxoid (UK), Fluka Chemika, and Difco Laboratories, USA.

Detection of alkaloids

a) **Mayer's test:** (with most alkaloids in slightly acid solutions)A fraction of the extract was treated with Mayer's reagent (1.36g of mercuric chloride and 5g of potassium iodide in 100mL of distilled water).

b) **Dragendroff's test:** two stock solutions are prepared:

i.0.5g bismuth sub-nitrate was dissolved in 2mL concentrated hydrochloric acid. and 10mL water,

ii.4g potassium iodide was dissolved in 10mL distilled water.

These stock solutions are mixed together and three drops of this reagent were added to detect presence of alkaloids in each extracts, the formation of a precipitate was taken as an indication for the presence of alkaloid (Saldanha et al.,1984) .

c) **Wagner's test:** A fraction of the extract was treated with Wagner's reagent (1.27g of iodine and 2g of potassium iodide in 100mL distilled water) (Sousek *et al.*,1999).

Plant Collection

The first step is the sample preparation. The fresh sample of the potato (6kg) were collected and purchased from a local market in Baghdad city. Prepared potatoes tuber with high and low levels of glycoalkaloid (according to turn green color) were obtained from the surface layers of the potatoes. Surface samples were prepared by scraping with (2 to 3) mm thick surface from the entire tuber of the hand-peeled potatoes and then was air-dried in the laboratory for 2 weeks to get dry Peels of potato. The dry prepared sample of potato (140g) was reduced to a moderately coarse powder by using Blender and sieves. to facilitate maximum effective contact of the solvent with the ruptured alkaloid bearing tissues and cells. In the case of potato tuber that are containing oils and fats, these non-alkaloidal chemical components need to be eliminated completely by extraction with a suitable polar solvent like ethanol, in a soxhlet apparatus, which would used for extraction the alkaloids.

Preparation of crude extract.

A) Alcoholic extract.

The Adebayo and Ishola (2009) method of extraction was used for the preparation of the extract by taking 80 grams of dry prepared sample (air-dried) of potato and added to ethanol (98%) at room temperature until it became with a final volume of (200) mL , left for half an hour in magnetic stirrer then sedimentation of sample for 48 hrs. at room temperature (25°C) using Whatman Grade No.1 filter paper. Under reduced pressure (vacuum),the extract was dried to give the crude extract which stored at 4°C until further use.

B) Soxhlet extraction

Procedure (Harborne1984) with some modifications was followed, fifty grams of powdered potato tuber were kept in Soxhlet extraction unit for 18 hours and exhaustively extracted with 510 mL of C_2H_6O / H_2O / CH_3COOH (400/100/10,V/V/V) then concentrated to 100 mL by a rotary evaporator. After that , few drops of ammonium hydroxide solution NH_4OH were added to extract solution to obtain pH = 9, The solution was transferred into a separation funnel and extracted separately with 100 mL of a mixture of ethanol-chloroform (2:1). The mixture was separated into two layers. The lower layer which containing chloroform with alkaline compounds was concentrated to 10 mL , then filtered to remove the powder samples. The chloroform solvent was evaporated using a rotary evaporator for 30 min. The extract solution became more concentrated with a semi solid crude extract (4.65g). The chloroform was evaporated to dryness in a water-bath. The dried powder (2.64g) and fractions were weighed and their percentage in terms of the dry weight of potato tuber material was estimated by the following formula and given in:

$$\text{Percent extractive} = \frac{\text{weight of dried powder}}{\text{weight of dried plant materials}} \times 100$$ (Zhang *et al.*, 2007)

Estimation of Alkaloids: 200mg of the crude extract and 20 mL of 10% acetic acid in ethanol were mixed. The mixture was covered and allowed to stand for four hours, filtered, and the extract was concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide solution was added dropwise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitate was then filtered, washed with dilute ammonium hydroxide solution, dried, and weighed. The content of alkaloids was determined with the following formula (Drzewiecki *et al.*, 2003).

$$\text{Alkaloids (\%)} = \frac{\text{weight of precipitate}}{\text{weight of sample}} \text{X100}$$

Selection of microorganisms used in the study

The selection of the microorganisms for antibacterial evaluation in this study was based on their known pathogenic effects in human .The following strains of bacteria were used in this study *Escherichia coli*, *Bacillus cereus*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* were obtained

as"gratis" from genetic engineering and biotechnology for post graduate studies, University of Baghdad and college of Science in University of Al-Mustansiriya. Bacteria were used in this study were clinical isolate obtained from previous researches and maintained brain heart infusion (B.H.I agar) slants at (4± 2)ºC.

All The bacterial cultures were revived every 2 weeks in Nutrient Broth medium and incubated at 37°C for 48 hours. Each bacterial culture was further maintained at 37°C on nutrient agar slants. Before testing, the bacterial inoculums were prepared and cultivated in nutrient broth for 24 hrs at incubation temperature of 37°C. The antimicrobial activity screening was carried out using agar well diffusion and agar dilution method technique (Tijjani *et al.*,2005).

Medium Preparation and Antibacterial Activity

Nutrient Agar was enrichment medium for the growth of microorganisms. Medium was prepared according to the manufacturing company by adding 28g of dehydrated powder using electrical balance into liter of distilled water, boiling to completely dissolving, and sterilizing by autoclaving at 15 pounds per square inch (p.s.i.) for 20 minutes. After that sterilized nutrient agar medium was prepared and poured into the Petri dish (25mL),then The plates were stored at 4°C in refrigerator for future use.

Preparation of test solution:

The dry isolated extract (100 mg) were dissolved in acetone to give a 100mg/mL of these stock solution and used for testing. and volumetrically diluted (1:2,1:4 and 1:8).

Screening of antimicrobial activity:

Test pathogenic microorganisms: Two strains of Gram-positive bacteria (*Staphylococcus aureus*, and *Bacillus cereus*) and two strains of Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) were used for antibacterial assay: The microbial cultures were serially diluted (10 fold increments) in sterile broth to obtain the cell suspension of 1.0×10^8 CFU/mL. by compared with McFarland standards tubes, The effect of potato tuber extract on the pathogenic bacterial strains were assayed by Agar well diffusion method and further confirmed by Agar dilution method.

Agar well diffusion method was performed for screening on Mueller Hinton agar plates were inoculated with bacteria (inoculum size 1×10^8 CFU/mL) by streaking in different directions with the aid of glass spreader, The plates were cut with help of cork borer (5mm) In each plates disc shaped pores were produced, each filled with a (50µL) of each dilute extract solution. The plates were kept at 4°C for 1 hr. for diffusion of extract. Finally, it was placed in incubator at 37 °C. for 48 hrs (Baek *et al.*,2008).

Agar dilution method: The potato tuber extracts were volumetrically diluted (1:2,1:4 and 1:8) where each plate contained 50 percent of the concentration of the antimicrobial in the previous dilution. The extract agent, thus diluted were incorporated into the Muller–Hinton agar medium, when the temperature reached around 50°C (Washington and Wood, 1995; Pottumarthy *et al.*, 2006). Mixed by gentle rotation and poured into Petri plates. thereafter were inoculated by bacterial suspension by glass spreader and incubated at 37 °C for 48 hours. After incubation, the inhibition zones of each bacteria were measured in millimeter by the ruler.

The total viable count was calculated for the inoculated plate, and the controls without extract to observe the bactericidal effect of the Isolated extract,

Total viable count was carried out for the plates that contain bacterial colonies in the range of (30-300) CFU. The number of bacteria in one milliliter is calculated as follows (CLSI. 2012); (NCCLS.1998):

Forming Unit (CFU)/1ml = No. of Colonies * Dilution Factor

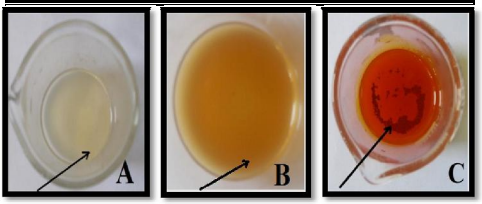
Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC):

Minimum inhibitory concentration (MIC) was determined for potato tuber extract activity against test pathogens. agar dilution method was followed for determination of MIC values. Potato tuber extracts were re suspended in acetone (which has no activity against test microorganisms) to make 100 mg/ml final concentration then five fold serially diluted; added to agar media before poured into the Petri dish. Thereafter 100 μl inoculum (for bacteria 1×10^8 CFU/mL) was culturing to each plate. Bacterial growth were used as negative control, while agar containing diluted extract was used as positive control. Plates were incubated at 37°C for 48 hrs. The MIC values were taken as the lowest concentration of the extracts in the agar plate that showed growth inhibition and reduce in number of bacterial Colonies. The Minimum Bactericidal Concentration (MBC) was no visible growth of microorganisms after incubation. In other words, least concentration of extract showing no visible growth on sub culturing was taken as MBC (Sharififar *et al*., 2007).

Results and Discussion

Extraction solution. Using two solutions of Alcoholic extract and Soxhlet extraction which had previously been mentioned for extraction of glycoalkaloids from potato tubers, The yield of Alcoholic extract and Soxhlet extracts were 2.64g and 1.54g respectively. The extraction yield obtained from the Soxhlet method was about (5.28%) and for Alcoholic extraction method (1.925%). Estimate of Percent of Alkaloids was about (75%). The chemical identifications of alkaloids were as following :-

Reagent/ test	Composition of the reagent	Result
Meyer's reagent	Potassium-Mercuric iodide solution	formation of cream colored precipitate. fig (**A**)
Wagner's reagent	Iodine in potassium iodide	formation of reddish brown colored precipitate. fig (**B**)
Dragendorff's reagent	Solution of potassium bismuth iodide	Formation of Orange or reddish-brown precipitate fig (**C**)



Figs(A,B,C) Reaction of reagents with isolated alkaloids

For comparative activity of alcoholic extract and Soxhlet extraction, the antibacterial activities of the extracts was investigated against four microorganisms. Soxhlet extracts showed high range of antimicrobial activities against (two Gram positive and two Gram negative bacteria).After incubation antibacterial activities were determined by measuring the diameters of zone of growth inhibition in mm in agar well diffusion assay technique fig(1). The diameters zones of growth inhibitions for the dilution was tabulated as shown in (table1).Furthermore, the extracts of glycoalkaloids exhibited medium to good degree of inhibition against the growth of all the tested bacteria strains with in (stock extracted solution) whereas the results was demonstrated a slightly decrease or no alteration in viable colony counts reduction in dilute solution (1/4 and 1/8).(fig 3 and fig 4)..

Extraction of glycoalkaloids from potato tuber was found sensitive to Gram negative *P aeruginosa and* gram positive *S aureus* more than other bacteria. Stock extract produced zone of inhibition 28 mm and 22 mm against *P aeruginosa* ,and *S aureus* respectively. In addition stock extract produced zone of inhibition of 12 mm and 15 mm against *E coli*, and *B cereus* respectively.However, it exhibited highest zone of inhibition (28 mm)against *P aeruginosa*. The MIC value was also determined against all the tested bacteria. The MIC values of Soxhlet Extract were found to be 50mg/ml while the MIC values were found 100mg/mL. Negative control well containing only acetone showed no zone against any bacteria.

Table 1 : Antibacterial activity of two alkaloids extracts of potato tuber

S. No.	alkaloids (Soxhlet Extract)	Zone of inhibition in mm			
		S aureus	*B cereus*	*E coli*	*P aeruginosa*
1	Stock Sol-	22	15	12	28
2	1/2 Dilute	14	8	6	18
3	1/4 Dilute	8	6	-	12
4	1/8 Dilute	6	-	-	8
	alkaloids (Alcoholic Extract)	Zone of inhibition in mm			
		S aureus	*B cereus*	*E coli*	*P aeruginosa*
1	Stock Sol-	14	10	8	18
2	1/2 Dilute	8	6	-	10
3	1/4 Dilute	-	-	-	7
4	1/8 Dilute	-	-	-	-

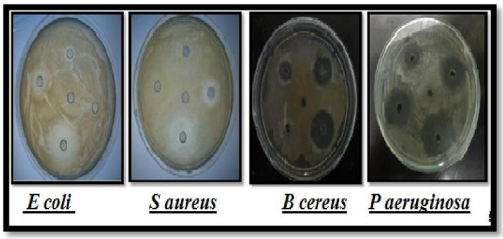


Fig. (1). Zone of inhibition of glycoalkaloids from potato tuber extract on Gram-negative and Gram-positive bacterial strains.

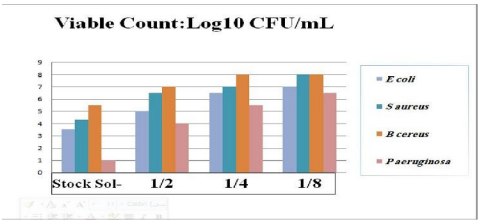


Fig. 3. Viable count of pathogenic bacteria after added various dilution of alkaloids extracts (Soxhlet extraction method)

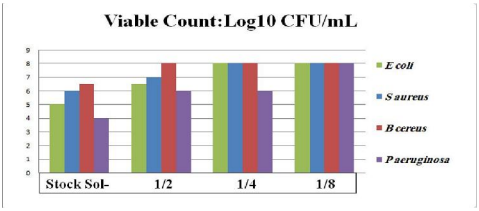


Fig. 4. Viable count of pathogenic bacteria after added various dilution of alkaloids extracts (Alcoholic extraction method)

In general, the Gram-negative bacteria have shown less sensitivity to glycoalkaloids extracts maybe as a result of their extra lipo-polysaccharide and protein cell wall that provides a permeability wall to the antibacterial agent (Adwan and Abu-Hassan, 1998). The Gram negative bacterial cell wall outer membrane appears to act as a barrier to several substances including synthetic and natural antibiotics (Tortora *et al.*, 2001).

The polarity in the membrane is determined by access of water to the polar-lipid inter phase (Parasassi et al., 1998)Since the mechanism of membrane disruption by glycoalkaloids implies first the insertion of the aglycone in the bilayer and then the complex formation between the sugar moieties. The two toxic glycoalkaloids (α-solanine and α-chaconine) and their aglycones were able to insert in the phospholipid bilayer by association with the sterols. In absence of the sugar moiety, hydration of the bilayer occurs. In contrast, if the hydrophilic sugar moieties are present as for α-solanine, regardless of other changes occurring in the membrane, the water concentration of the membranes would remain unchanged, likely due to hydrophilic interaction between the sugar moieties with water molecules present in the surroundings (Keukens et al. 1995).

The extracts of glycoalkaloids from potato tuber were found to be effective antibacterial agents against human pathogens. This study paves the way for further attention and research to identify the active compounds responsible for the plant biological activity.

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