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INVESTIGATE THE ANTIBACTERIAL ACTIVITY OF AMYGDALIN AGAINST SOME TYPES OF ANTIBIOTIC RESISTANT PATHOGENIC BACTERIA

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ABSTRACT

Extraction of Amygdalin from apple seeds by alcoholic extraction procedure were done and applied a high-performance liquid chromatographic (HPLC) procedure for quantification of it to investigate extraction efficiency and (16) bacterial isolates *(* 4 isolates of *Staphylococcus aureus,* 4 isolates of *Escherichia coli ,* 4 isolates of *Pseudomonas aeruginosa* and 4 isolates of *Streptococcus pyogenes* from skin infections samples are choose by accomplished of drug sensitivity tests as a multidrug resistant, the antimicrobial activity of methanolic extract of Amygdalin from crushed apple seeds was tested against each group of four pathogenic bacteria species of gram positive (*Staphylococcus aureus and Streptococcus pyogenes)* and gram negative (*Escherichia coli and Pseudomonas aeruginosa*) by well diffusion method, the results shown that plant extract gave antibacterial activity through the inhibition zone , but the concentration of the raw material was more inhibited than the dilute substance concentration, inhibition of the substance was obtained on all types of bacteria were in *Staphylococcus aureus*, inhibition zone of substances concentrations (100 mg/ml, 50 mg/ml) were (40, 28 mm) respectively , in *Pseudomonas aeruginosa* inhibition zone of substances concentrations (100 mg/ml, 50 mg/ml) were (50, 35 mm) respectively, in *Escherichia coli,* inhibition zone of substances concentrations (100 mg/ml, 50 mg/ml) were (40, 25 mm) respectively, also this results showed that three types of bacteria above were inhibited in synergistic effect, while inhibition zone of *Streptococcus pyogenes* in concentration 100 mg/ml was 30 mm and in concentration 50 mg/ml was 20 mm.

The present study is aimed to evaluate the antimicrobial effects of Amygdalin from crushed apple seeds plant extract against some pathogenic bacterial species *(Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa and Streptococcus pyogenes)*, and determination the synergistic effect of alcoholic extract of it.

INTRODUCTION

Amygdalin is classified as a cyanogenic glycoside because each amygdalin molecule includes a nitrile group, which can be released as the toxic cyanide anion by the action of a beta-glycosidase: eating amygdalin will cause it to release cyanide in the human body and may lead to cyanide poisoning. Cyanogenic glycosides are plant natural toxicant that has the ability to produce toxic hydrogen cyanide. Concentrations of cyanogenic glycosides vary widely in plants as a result of genetic and environmental factors, location, season and soil types. (1)

Cyanogenic glycoside is a large group of secondary metabolites that are widely, distributed in the plant kingdom, including many plants that are commonly consumed by humans. The diverse chemical nature of cyanogenic glycoside means that extraction and analysis of individual compounds can be difficult. In addition, degradation can be rapid under appropriate condition. (2).

Amygdalin is a cyanogenic glycoside derived from the aromatic amino acid phenylalanine. Amygdalin and prunasin are common among plants of the family Rosacea, particularly the genus Prunes, Phocaea (grasses), Fabaceae (legumes), and in other food plants, including flaxseed and manioc. Within these plants, amygdalin and the enzymes necessary to hydrolyze them are stored in separate locations so that they will mix in response to tissue damage. This provides a natural defense system (3).

Although apple contains compounds which may confer significant health benefits to humans (4)., apple seeds contain amygdalin. Amygdalin (from Ancient Greek: ἀμυγδαλή *amygdale* "almond") is a naturally occurring chemical compound, famous for falsely being promoted as a cancer cure. It is found in many plants, but most notably in the seeds (kernels) of apricot, bitter almonds, apple, peach, and plum. (5) .

MATERIALS AND METHODS

Reagents and standards

Amygdalin, ethanol, diethyl ether, and HPLC-grade methanol were all achieved from Sigma-Aldrich commercially. Water was prepared using a Millipore Milli-Q purification system. All other reagents were of analytical grade.

Microorganisms Isolates collection:

The pathogenic microorganisms (*Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa and Streptococcus pyogenes)* which choose in this study were form different samples of skin infections from different hospitals in Iraq/ Baghdad and tested by bacteriological and biochemical tests in Microbiology laboratory / College of biotechnology / AL-Nahrain University.

Fruit Sample Collection:

Firstly, the red apples purchase from local market were cutting to separate the seeds from it and collect a suitable quantity of seeds and wash them to get rid of the dust particles or any other triad, then leave it to dry and grind it by using the blender to become small molecules, then prepare this powder for their where been extraction process.

Alcoholic Extraction Method:

Weigh (2g) of dry powder (crushed apple seeds) and place it in flask (500ml) and add 100ml of methanol alcohol with a concentration of 80% and mixing it , then close the flask tightly to avoid evaporation and left it to heating on the hot plate in 50 C^0 for 100 min, after completing the boil cool the mixture ,then filter it by Filter paper for the disposal of impurities and residues non-solvent and after finishing the extract put in the rotary evaporator for 15 min to separate the alcohol from the powder residue ,and remain for (24-48) hour in room temperature to dry and become a powder and then take the dry extract and put it in 20 ml of Diethyl ether and mixing for 1 min for the purpose of dissolving the extract and then purify the extract by Millipore filter to avoid any pollution during the extraction process, by Millipore filter unite was used to sterilize crushed apple seeds extract. Size of Millipore filter was 0,4 mm. (6).

Preparation of dilution of materials:

The dilution of the extracted substance is 100% and 50% concentration. The dilution of 100% is the substance extracted only, the dilution of 50% is taking 1ml of the extract and adding it to 1ml of double distilled water thought to has been 50%.

Microorganisms Preservation:

The isolates have been used in this work were maintained by weekly activation in nutrient broth and were incubated at 37 ^{0}C for 24 hours .(7).

Minimum Inhibitory Concentration and Minimum Bactericidal Concentration Estimation:

Culture: Overnight Mueller Hinton broth cultures of *Staphylococcus aureus and Streptococcus pyogenes, Escherichia coli* and *Pseudomonas aeruginosa* at 370C were prepared. The culture was adjusted to obtain turbidity comparable to that of the turbidity of MacFarland 0.5 standard3. and then further diluted 1: 200 in Mueller Hinton broth. The inoculums thus prepared expected to obtain 105 to 106 C.F.U/ml. This procedure done according to Hossain MD., 2000 method. (8).

Testing the effect of alcoholic apple seeds extracts on bacterial isolates:

To detect the susceptibility of the bacteria and their effect on the plant extract, Muller Hinton agar medium, prepared, then inoculated by using cotton swab with a culture of four bacteria species after comparing it with standard suspension containing 1.5x10^{12} g (McFarland Turbidity Standard), then two well work on agar and fill one well with appropriate amount from plant extract only and fill other well with appropriate amount from diluted extract , after complete all this, should take these petri dishes and put them in the incubator at 37C^0 for 24 hours, After this period , taken this dishes and determine the inhibitory activity (mm) of the bacteria by measuring diameter of the inhibition zone around the bacteria .measure the inhibitory effect of the substance and the effect the bacteria on it by using the rulers. then two well used on agar, one well filled with suitable amount from plant extract handiest and other filled properly with suitable quantity from diluted extract , after entire all this, have to take those petri dishes and positioned them within the incubator at 37C0 for 24 hours, After this period , taken this dishes and determine the inhibitory interest (mm) of the bacteria by measuring diameter of the inhibition area across the microorganism .degree the inhibitory impact of the

substance and the effect the bacteria on it by using the rulers.

Preparation of extracts for HPLC analysis:

Aliquots of the extract was dispensed into Eppendorf tubes (1.5 ml), centrifuged (10 min, 22 ^{o}C, 14000 rpm, using a micro centrifuge) and filtered with 0.45 µm PTFE filters.

HPLC determination of amygdalin

Amygdalin contents of desiccated apple seeds was determined by RP-HPLC, using a Shimadzu HPLC consisting of a 20ADXR pump, SIL-20ACXR autosampler and degasser. (4.60 mm, 3 µm) placed in a column oven set at 40 ^{o} C. The mobile phase was an isocratic elution that consisted of methanol and water (25:75, v:v) and the flow rate was 1 ml/min. The mobile phase was sonicated (20 min, 22o ± 2 ^{o}C) to remove gas bubbles before use. The sample injection volume was 5 µl. Amygdalin was detected using a photodiode array detector at 214 nm. Results were expressed as the amount of amygdalin in mg per gram or mg per milliliter of extracted samples (9).

Statistical analysis

The data obtained in this study were assessed statistically by using of The IBM SPSS statistics version 20 software was used for the analysis.

RESULTS AND DISCUSSION

HPLC analysis of amygdalin

Amygdalin detection was achieved by UV detection in an isocratic elution with an excellent peak area and the concentration of amygdalin. The amygdalin peak was completely separated from other materials without any pre-treatment. The recovery of amygdalin was greater than 98%.

Amygdalin content of apple seeds

The amygdalin contents of seeds from different varieties of apple are given ranged from 0.5-1 mg g-1. Which was not accepted with Haque and Bradbury (10). who reported the amygdalin

contents of Fuji apple seeds to be 5.4 mg g-1. This value is lower than the amygdalin content of Fuji apple seeds (1.89 mg g-1) reported in this study. The variation in the amygdalin content of apple seeds could be due to cultivation practices (e.g. different levels of fertilization, irrigation and use of pesticides) or environmental factors such as drought or infection by pathogens during fruit formation. Application of fertilizer to a field before planting has been reported to decrease cyanogenic glycoside levels in cassava tubers (11).

MIC and MBC Of Amygdalin Evaluation

The results of MIC were 12.5 mg/mL for all strains used in this study and MBC were 200 mg/mL for all strains used in this study.

Antibacterial Activity:

After preparation stock solution from the dried powder of crushed apple seeds extract (alcohol) ,and diluted it to one concentration (50%) by take 1ml from it and add to 1ml from distilled water ,for testing antibacterial activity on gram positive bacteria of (*Staphylococcus aureus and Streptococcus pyogenes)* and gram negative bacteria of (*Escherichia coli and Pseudomonas aeruginosa*) both, diluted extract and raw extract (without dilution) were positive ,but the concentration of the raw material was more inhibited than the dilute substance concentration, inhibition of the substance was obtained on all types of bacteria, both well are filled as control positive (trioxane antibiotic) and control negative (sterile water)

Table (1): Antibacterial activity of Amygdalin from alcoholic apple seeds extractions against *Staphylococcus aureus*.

Type of extraction	**Concentration mg/ml**	**Type of microorganism**	**Inhibition zone**
Alcoholic extraction	100mg/ml	*Staphylococcus aureus*	40
	50mg/ml		28

This table shows that inhibition zone of *Staphylococcus aureus* in concentration 100 mg/ml was 70 mm and in concentration 50 mg/ml was 70 mm, this type was inhibited in synergistic effect with other concentrations were tested as shown in Figure (1).

Figure (1): Inhibition zone of Amygdalin from the apple seeds extract on *Staphylococcus aureus* culture.

Table (2): Antibacterial activity of Amygdalin from alcoholic apple seeds extractions against *Streptococcus pyogenes*.

Type of extraction	**Concentration mg/ml**	**Type of microorganism**	**Inhibition zone (mm)**
Alcoholic extraction	100mg/ml	*Streptococcus pyogenes*	30
	50mg/ml		20

This table shows that inhibition zone of *Streptococcus pyogenes* at concentration 100 mg/ml was 30 mm and at concentration 50 mg/ml was 20 mm.

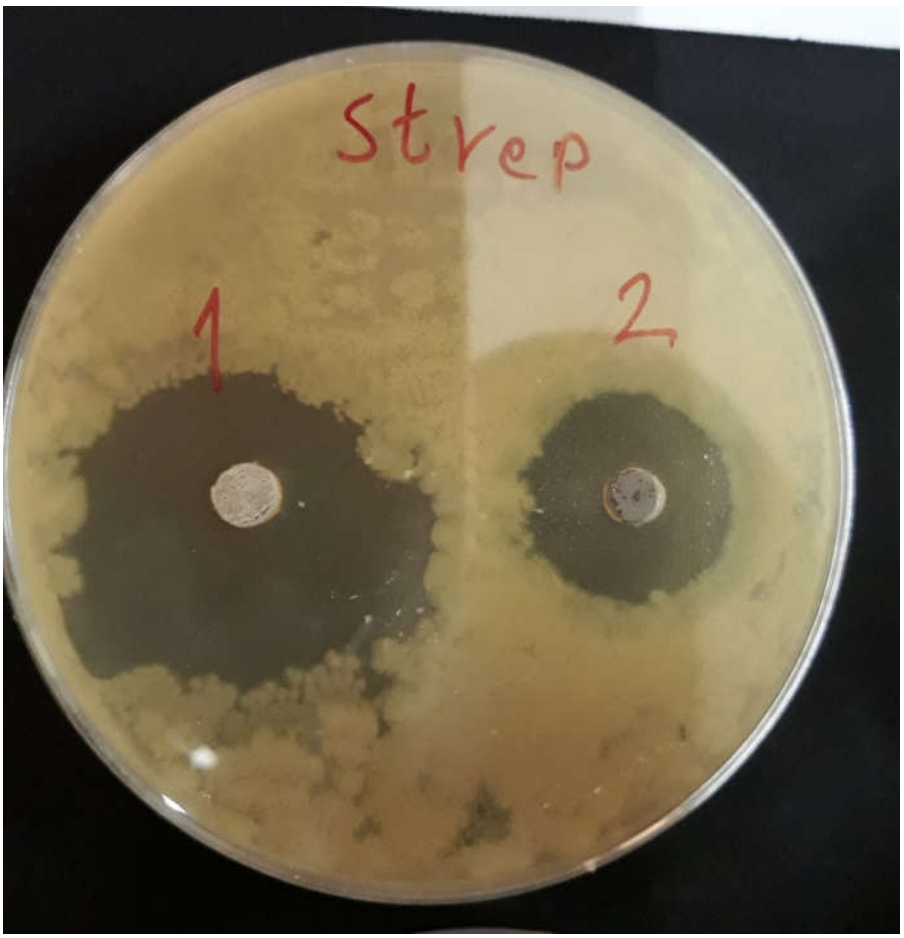

Figure (2): Inhibition zone of the Amygdalin from apple seeds extract on *Streptococcus pyogenes* culture.

Table (3): Antibacterial activity of Amygdalin from alcoholic apple seeds extractions against *Pseudomonas aeruginosa*.

Type of extraction	**Concentration mg/ml**	**Type of microorganism**	**Inhibition zone**
Alcoholic extraction	100mg/ml	*Pseudomonas aeruginosa*	50
	50mg/ml		35

This table shows that inhibition zone of *Pseudomonas* in concentration 100 mg/ml was 40 mm and in concentration 50mg/ml was 35 mm ,this type was inhibited in synergistic effect as shown in Figure (3).

Figure (3): Inhibition zone of the Amygdalin from apple seeds extract on *Pseudomonas aeruginosa* culture.

Table (4): Antibacterial activity of Amygdalin from alcoholic apple seeds extractions against *Escherichia coli*.

Type of extraction	**Concentration mg /ml**	**Type of microorganism**	**Inhibition zone (mm)**
Alcoholic extraction	100mg/ml	*Escherichia coli*	40
	50mg/ml		25

This table shows that inhibition zone of *Escherichia coli* in concentration 100 mg/ml was 40 mm and in concentration 50 mg/ml was 25 mm, this type was inhibited in synergistic effect with other concentrations were tested as shown in figure (4).

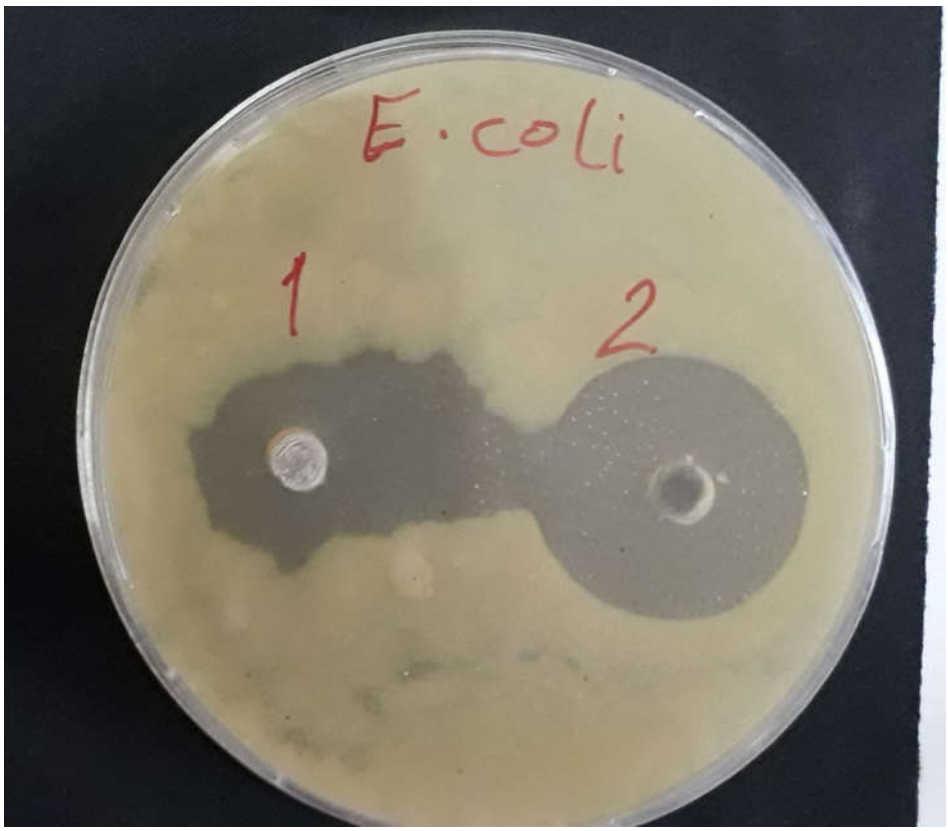

Figure (4): Inhibition zone of the Amygdalin from apple seeds extract on *Escherichia coli* culture.

In these test all types of bacteria affected with effective substance and the result was positive due to high concentration of substance and according to the size of each zone measured the inhibitory effect of the substance and its effect, through the measure of inhibition zones of activity of bacteria proved that three types of bacteria were inhibited in synergistic effect with other concentrations were tested due to increasing of the size of inhibition zone that could be attributed to presence of active compounds of apple seed alcoholic extract against these types of isolates.

It seems that amygdalin has antibacterial activity. The antibacterial activity may brought by amygdalin decomposition by bacterial enzymes to give hydrogen cyanide and benzaldeyde,

the toxic compounds which killed the bacteria. The results of this report are in agreement with the study of (12). They reported the antibacterial activity of bitter apricot extract against several bacterial strains. They supposed that the antibacterial activities might be brought by amygdalin, alkaloids, flavanoids, tannins and phenolic compounds.

CONCLUSION

Through this study the active substance of apples seeds plant alcoholic extract prove the ability to inhibit some types of pathogenic bacteria growth , were methanolic extract of crushed apple seeds has antibacterial activity against (*Staphylococcus aureus ,Escherichia coli ,Pseudomonas aeruginosa and Streptococcus pyogenes)*, the activity inhibition zone increased when the raw extracted substance (without dilution) increase in concentration. Finally, it is necessary to determinetoxicity and their side effects.

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