International Journal of Scientific Research and Engineering Development-– Volume 5 Issue 1, Jan-Feb 2022

Available at www.ijsred.com

RESEARCH ARTICLE OPEN ACCESS

Isolation of the Substances Responsible for the Antibacterial Activity of Peanut Shells

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Abstract:

Peanut shells are agricultural waste. They are generally used in animal feed. Our study focused on the isolation of the substances responsible for the antibacterial activity in these by-products. To do this, methods of extraction, fractionation, isolation, antibacterial tests and nuclear magnetic resonance method were used. The results obtained showed that two fractions have antibacterial activities. These activities are due to the presence of the two compounds coded CAA2 and CM01. These compounds were respectively extracted from ethyl acetate and methanol. These two compounds are, respectively, called Luteolin and Eriodictyol. They are therefore responsible for the antibacterial activity of peanut shells.

Key words: **peanut shell, by-product, extract, antibacterial activity, luteolin, eriodictyol**.

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I. INTRODUCTION

Groundnut is native to tropical America and has been introduced to tropical countries [1]. Among the various known species, *Arachis hypogeae* is the most economically important. Peanut shells are the main waste obtained after peanut shelling. Some concerns have been raised about the dangerous nature of peanut shells and their environmental and health impacts. This waste has harmful effects on the soil, flora and fauna, degrading landscapes and affecting human health and the environment [2]. In Madagascar, especially in the District of Mandritsara, Fokontany Ambohimahavelona, peanut shells are the most important agricultural by-products. Peanut shells represent 20 to 32% of the pod weight [3]. During the 2005-2006 growing season in this District, production was 750 tons, 30% of which consisted of groundnut shells, which gave 225 tons of empty shells and 70% were seeds [4].

Peanut shells are famous worldwide for their medicinal properties and their use by the local population to cure diseases. Many legume species have therapeutic properties and are used in traditional medicine [5], [6]. Currently, the multiplication of microbes, viruses and the growing installation of chemoresistance in humans, vis-à-vis the drugs produced by the pharmaceutical industries are at the center of problems for scientists. At the same time, these drugs have exorbitant prices, and they are not within everyone's reach, especially for the majority of Malagasy people. Our country has many plants endowed with therapeutic virtues [7], [8] and which are just waiting to be studied and exploited. Plants are extremely complex in terms of their chemical composition. In light of the information provided by other researchers and by the richness of its chemical composition, peanut shells appear to be very interesting materials for analyzing and identifying bioactive molecules. This prompted the present study, in order to optimize the valorization of these agricultural by-products.

The objective of this work was to implement the isolation of antibacterial substances contained in peanut shells by chemical and bacteriological studies of the various extracts prepared from these agricultural by-products.

II. MATERIALS AND METHODS

A. *Plant material*

Our study focused on peanut shells of the 'Voanjo mena' variety called Valencia. It is the most common variety, belonging to the Leguminosae family and the Papilionaceae subfamily.

B. *Culture medium*

The culture media used during this study are Mueller Hinton Agar, BCO102M. They are respectively used to test the sensitivity of extracts to bacteria and to determine the minimum inhibitory concentration.

C. *Microbial strain*

The microbial strain used during the realization of this study is *Streptococcus pneumoniae*.

D. *Sample Collection*

Peanut shells were harvested in May 2015 at Fokontany Ambohimahavelona, urban commune of Mandritsara, Region of Sofia, Madagascar.

E. *Preparation of crude extract*

Peanut shells (600 g) were ground using an electric machine for 10 minutes. In this case, 500 g of ground material were suspended in 90 % ethanol for 7 days. The suspension thus obtained was subjected to magnetic stirring at 130 revolutions per minute for 7 days. The suspension was then filtered using filter paper. The filtrate thus obtained was centrifuged at 5000 revolutions per minute for 5 min. The pellet was discarded, while the supernatant was evaporated to dryness using a rotary evaporator. The evaporation residue was taken up in distilled water and the suspension thus obtained constitutes the crude extract.

F. *Splitting*

The method of Markham (1982) [9] was used for the fractionation of the crude extract. It consists of the successive exhaustion of crude extract using four solvents of increasing polarity: hexane, dichloromethane, ethyl acetate and methanol, from the apolar solvent to the more polar solvent. The extraction was carried out with continuous stirring. After filtration of each extract, the filtrate obtained was concentrated by evaporation. The raw extract and the different extracts have been subjected to antibacterial tests.

G. *Antibacterial activity tests*

1) *Disc method*: The method used is that of Pyun et al (2006) and Ngameni et al (2009) [10], [11]. This involves determining the antibacterial activity of each extract by the inhibition diameter. Five extracts, crude extract, hexane extract, dichloromethane extract, ethyl acetate extract and methanol extract, were subjected to the antibiogram test. The product obtained after evaporation of the crude extract (200 mg) was dissolved in 1 ml of methanol and served as a stock solution. The other extracts were taken up in distilled water at a rate of 200 mg/ml. The activity of each extract was assessed by the diameter of the zone of inhibition around each disc [12].

2) Method by dilution (Liquid medium): This is the determination of the antibacterial activity of each extract by the value of the Minimum Inhibitory Concentration (MIC) which is the lowest concentration of the series of tubes where there is no visible concentration of germ. The

bacteria were inoculated into the enrichment medium and adjusted to 0.5 Mac Farland. The bacterial inoculum was distributed over the tubes, and then the extract to be tested was added to a precise volume [13]. Bacterial growth in each tube was examined by its turbidity [14].

H. *Chromatographic Analysis (Isolation and Purification)*

The ethyl acetate extract (7 g) was fractionated by chromatography on a silica gel glass column at 112 µm in diameter. The eluent used is a binary solvent system composed of hexane and ethyl acetate (AcOEt). The elution was done by solvent gradient Hexane/AcOEt (10/0 to 0/10) V/V [15], [16]. The fractions collected were analyzed on Thin Layer Chromatography (TLC) [17] which made it possible to bring together the fractions presenting the same chromatograms. Six fractions were obtained noted A1 to A 6. Fraction A 2 was subjected to washing with AcOEt, because its physical appearance shows that it is formed from a mixture of 2 or 3 products maximum. We proceeded to wash to get rid of the other products. In this case, the undissolved AcOEt product was recovered as the pure product named CA-A1. We chose the A4 fraction, because it has a high mass and the molecules that constitute it are less numerous. The refraction by column chromatography of fraction A 4 by elution with a Hexane/AcOEt solvent gradient made it possible to obtain five fractions ranging from TS1 to TS5, including TS3, depending on its physical aspect (formed from the mixture of two products), was subjected to washing with ethanol to obtain a pure product named CA-A 2.

The methanolic extract (MeOH) of mass 4 g was fractionated by chromatography on a silica gel glass column at 112 µm in diameter. The eluent used is a binary solvent system composed of AcOEt and MeOH. The elution is done by solvent gradient AcOEt/MeOH (10/0 to 0/10) V/V. The fractions collected were analyzed on TLC, which made it possible to combine the fractions showing the same chromatograms. Eight fractions were obtained ranging from M1 to M8. Afterwards, we chose the M1, M3 and M5 fractions to purify them, because their physical appearance shows that they are made up of 2 or 3 products maximum. Washing the M1, M3 and M5 fractions with AcOEt favors their separation from the other products. In this case, the products not dissolved in AcOEt were recovered as the pure products named respectively CM-01, CM-03 and CM-02.

I. *Nuclear Magnetic Resonance Spectrometry (NMR)*

NMR is the method to elucidate the structure of a molecule. NMR data are recorded as one-dimensional 1D spectra and two-dimensional 2D spectra [18], [19].

III. RESULTS

A. *Fractions*

Depending on the type of solvent used, 4 molecular fractions extracted according to their polarity were obtained. These are the hexane extract, dichloromethane extract, ethyl acetate extract and methanol extract. Only two extracts ethyl acetate and methanol show antibacterial activity. This is why only two extracts were subjected to chromatographic analysis.

B. *Antibacterial activity*

1) *Results obtained by the disc method*

The antibacterial activity of the extracts in solid medium is presented in Table I.

Table I: Value of the diameters of the growth inhibition zone of *Streptococcus pneumoniae* in a solid medium

Parameters	Types of extracts						
	Crude extract			Hexanique Extract	DCM Extract	AcOEt Extract	MeOH Extract
Masses (in mg/disque)	2	1	0,5	2	2	2	2
Diameters (in mm)	10	0	0	8	0	12	11

The antibacterial activity of the extracts is shown in the figure below.

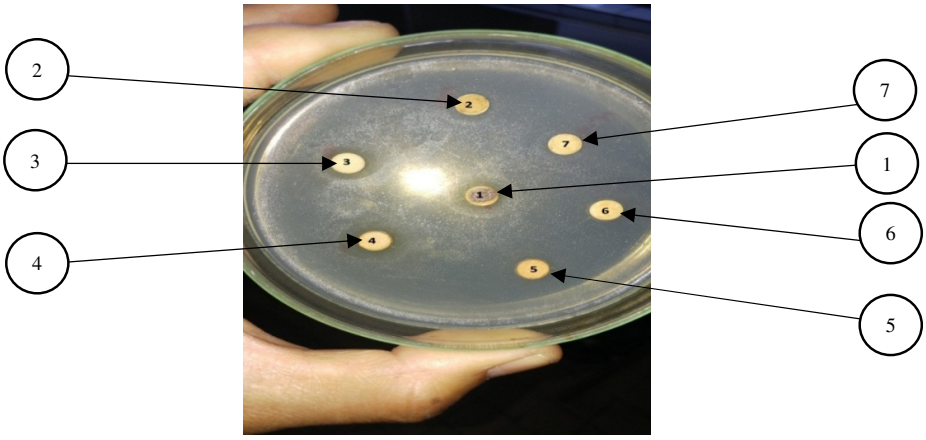


Fig. 1- Antimicrobial activity test result of the different extracts on *Streptococcus pneumoniae*
1: Pure crude extract, 2: Crude extract ½, 3: Crude extract ¼, 4: Hexane extract
5: DCM extract, 6: Ethyl acetate extract, 7: Methanolic extract

The crude extract (1), the AcOEt extract (5), the MetOH extract (6) show anti-bacterial activity. The Hexane extract shows low activity. The Dicloromethane extract is in contact and shows no activity.

2) *Results obtained by the method in liquid medium*

The MIC value for the crude extract is 6.25mg/ml, 3.125mg/ml for the ethyl acetate extract and 3.125mg/ml for the methanolic extract.

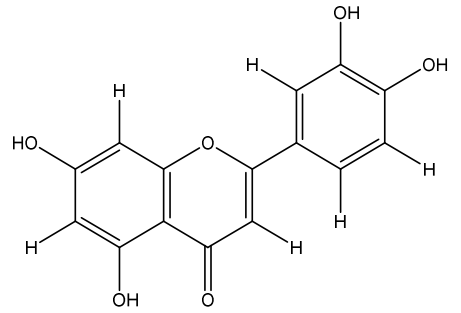
C. *Products obtained*

The ethyl acetate extract was fractionated on silica gel column chromatography. Finally, two products named CA-A1 and CA-A2 were isolated and purified from the AcOEt extract. Among the two products, CA-A1 precipitates during NMR.

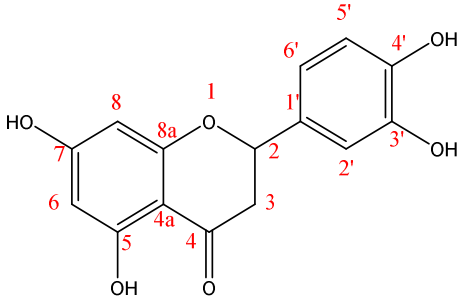
Fractionation and purification of the MeOH extract on silica gel column chromatography yields three products named CM-01, CM-02 and CM-03. Among these three products, CM-02 shows contamination during bacterial analysis and the amount of CM-03 is insufficient for NMR. Thus, during this study, only two products, such as CAA2 and CM-01 were considered.

D. *Identified Products*

Thanks to the different methods of spectral analysis, the structure of the two compounds isolated from peanut shells, such as CA-A2 and CM-01, were identified. These are, respectively, Luteolin and Eriodictyol. Luteolin is a flavonoid belonging to the group of flavones, while Eriodictyol is a flavonoid belonging to the group of flavanones.



5, 7,3',4'-tetrahydroxyflavone or Luteolin



5, 7,3',4'-tetrahydroxyflavanone ou Eriodictyol

Fig. 2- Structure of Luteolin and Eriodictyol

IV. DISCUSSION

The alcoholic maceration favors that the active substances are soluble in water. In order to target the maximum number of molecules, ground peanut shells were subjected to extraction by successive exhaustion using solvents of increasing polarity. Thus, hexane was used to degrease and depigment the organs, dichloromethane for apolar compounds, ethyl acetate to obtain moderately polar compounds and MeOH to extract polar compounds. The use of solvents with different polarities makes it possible to separate the compounds according to their degree of solubility in the extraction solvent.

According to the results obtained, the crude extract, the AcOEt extract and the MeOH extract exhibit the antibacterial activity against the *Streptococcus pneumoniae* strain with the respective inhibition zone diameters of 10 mm, 12 mm and 11 mm. The AcOEt and MeOH extract have a strong inhibition on the growth of microorganisms (MIC: 3.125 mg/ml). According to the Kouitcheu team in (2013) [20], an extract was considered active against any microorganism tested if its MIC is less than or equal to 8 mg/ml. If the interpretation of the Kouitcheu team [20] has been taken into account, it can be said that the extracts used could be qualified as good antimicrobials.

The antibacterial activity of the extracts is due to the presence of biologically active constituents contained in both AcOEt and MeOH extracts. The active ingredient is concentrated on each of these products. It acts individually without combining the compounds and is linked to its chemical structure [21]. The AcOEt extract and MeOH contain more pure compound than the crude extract and they are more concentrated in active ingredients. This loss of inhibitory activity for the other extracts may be due to the separation of molecules that will act synergistically in the crude extract. The antibacterial activity is related to the polarity of the bioactive substances.

The main target of these natural compounds is the bacterial membrane. The antibacterial activity of natural substances is explained by the lysis of these membranes. The amount of polyphenols is abundant in the ethyl acetate extract. The presence of two free hydroxyl groups is essential for activity. They will cause the normal disruption of ion transport across the cytoplasmic membrane such as potassium ion leakage, inactivation of microbial enzymes and inhibition of bacterial motility [22]. Eriodictyol is one of the phenolic compounds resulting from chemical fractionation of the methanolic extract. The presence of two free hydroxyl groups is essential for activity. Indeed, these compounds have the ability to increase membrane permeability and inhibit bacterial mobility.

The fractionation and purification of the constituents of these extracts by different chromatographic methods allowed the isolation of 2 pure products coded CA-A1 in the form of a yellow powder with a mass of 0.013 g and CA-A2 in the form of a white powder of mass 0.015 g from the AcOEt extract. Three pure products coded CM – 01 in the form of yellow color powder with mass 0.013g, CM – 02 white color crystals with mass 0.015g and CM – 03 in the form of yellow color powder with mass 0.015 g were obtained from of the MeOH extract. The two pure products isolated from the AcOEt extract present an antibacterial activity. For the MeOH extract one out of three

pure products, denoted CM-01 presents an antibacterial activity. On the other hand, the CM-02 product was contaminated during the bacterial analysis. Therefore, during this study, two pure products, such as CA-A2 and CM-01, were considered, since CA-A1 precipitates upon introduction into the device.

Thanks to the different methods of spectral analysis, the structures of the two isolated compounds, such as Luteolin and Eriodictyol, were determined. Luteolin is a flavonoid belonging to the group of flavones and Eriodictyol is a flavonoid belonging to the group of flavanones [23]. These two products have already been isolated from Cordia globosa (Jacq.) Kunth (Boraginaceae) [23].

V. CONCLUSION

This research work contributes to the isolation of the substances responsible for the antibacterial activity of peanut shells. They therefore open up new avenues of research on the possibilities of exploiting their active ingredients. The ethyl acetate extract and the methanolic extract show antibacterial activity on *Streptococcus pneumoniae* strain. Two pure products, coded CA-A2 and CM-01, obtained after fractionation and isolation of the substances contained in the AcOEt, MeOH extract were subjected to nuclear magnetic resonance to determine their structure. The in-depth investigations carried out on peanut shells have therefore enabled us, from a chemical point of view, to isolate Luteolin and Eriodictyol. They are flavonoids. They are compounds of flavanone and flavone groups respectively. From a biological point of view, these investigations have shown the strong potential of extracts as a source of natural antimicrobial molecules.

Through this research work, we hope to have made our modest contribution to the recovery of this agricultural waste. In this case, they will be considered effective and accessible agricultural residue remedies for the local population.

ACKNOWLEDGEMENTS

We extend our sincere thanks to the researchers of the Laboratory of Chemistry Applied to Natural Substances, Ampasampito, Antananarivo (Madagascar) and the Bacteriology Laboratory of the Hospital Joseph Ravoahangy, Andrianavalona, Anosy, Antananarivo (Madagascar) who accepted our request for analysis of our samples.

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