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Original Research

Antimicrobial effect of polyphenolic extracts present in *ananas comosus*

Efecto antimicrobiano de los extractos polifenolicos presentes en *ananas comosus*

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Abstract

Introduction: The use of antimicrobials is a permanent challenge as it is constantly confronted with the ability of bacteria to develop resistance mechanisms. Objective: The objective of this research was to evaluate the antimicrobial effect of polyphenolic extracts present in Ananas comosus crown. Materials and Methods: 3.5 kg of Ananas comosus pineapple crown leaves were collected and taken to a tray dryer to reduce humidity, then, they were milled until a fine flour was obtained, for the extraction process of the oleaginous extract a Soxhlet equipment was used, using 70% ethanol as solvent, The identification of phenolic compounds was carried out by ultra-high resolution liquid chromatography with an Orbitrap mass detector. The microbiological analysis was evaluated by the standardized method of diffusion with discs using Mueller-Hinton agar, for which the Staphylococcus aureus strain (ATCC 25923) was used, using oxacillin as a positive control and DMSO as a negative control. Results: from the drying obtained, the humidity was reduced by 50%, with which the dry matter obtained was ground and used to carry out the extraction process of the oleaginous extract, obtaining 63 ml of which 27 phenolic compounds were identified. As for the microbiological analysis carried out, inhibition halos varying between 4.5 mm and 6.0 mm were observed. Conclusion: Finally, it was concluded that the polyphenols present in the extract of Ananas comosus showed antibacterial activity on Staphylococcus aureus, with a greater inhibition effect observed when a higher concentration of the extract was applied.

Resumen

Introducción: La utilización de antimicrobianos es un desafío permanente ya que se enfrenta en todo momento a la capacidad de las bacterias para desarrollar mecanismos de resistencia. Objetivo: el objetivo de esta investigación fue evaluar el efecto antimicrobiano de los extractos polifenolicos presentes en la corona Ananas comosus. Materiales y Métodos: se recolectaron 3,5 kg de hojas de coronas de piña Ananas comosus para ello se llevaron a un secador de charolas, para reducir humedad, posteriormente, se realizó la molienda hasta obtener una harina fina, para el proceso de extracción del extracto oleaginoso se utilizó un equipo Soxhlet utilizando etanol al 70 % como solvente, la identificación de compuestos fenólicos se realizó por cromatografía líquida de ultra-alta resolución con detector de masas Orbitrap,el análisis microbiológico se evaluó mediante el método estandarizado de difusión con discos utilizando agar Mueller-Hinton, para lo cual se utilizo la cepa Staphylococcus aureus (ATCC 25923), utilizando como control positivo oxacilina y como control negativo (DMSO). Resultados: del secado obtenido se disminuyó la humedad en 50 %, con el cual la materia seca obtenida fue molida y utilizada para realizar el proceso de extracción del extracto oleaginoso obteniendo 63ml del cual se identificaron de 27 compuestos fenólicos. En cuanto al análisis microbiológico realizado, se observaron halos de inhibición variables entre 4,5 mm y 6,0 mm. Conclusión: finalmente se concluyó que los polifenoles presentes en el extracto de Ananas comosus presentaron actividad antibacteriana en Staphylococcus aureus observándose mayor efecto de inhibición al aplicar de mayor concentración del extracto.

Keywords: antioxidant, bacteria, inhibition, pathogen, polyphenol, polyphenol.

Palabras clave: antioxidante, bacteria, inhibición, patógeno, polifenol.

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Why was it carried out?
This research was carried out in order to know the possible antimicrobial effect of polyphenolic compounds present in the oleaginous extract of Ananas comosus (pineapple), taking into account that this type of fruit is rich in polyphenolic compounds, which is why it was evaluated in the applicability of this in Staphylococcus aureus (ATCC 25923), microorganism that causes many respiratory diseases and infections, but currently some microorganisms have acquired resistance to common antibiotics, which is why the research seeks to be an antimicrobial alternative for this type of microorganism.

What were the most relevant results?
Among the most important results was the presence of 27 polyphenolic compounds in the oleaginous extract of Ananas comosus, being caffeine, vanillic acid and p-coumaric acid those of greater proportion, on the other hand, it was also found that inhibition effect with halos ranging between 4.5 and 6.0 mm results that occurred when increasing the concentration of the extract..

What do these results provide?
These results are important because they present polyphenolic compounds as a possible antimicrobial alternative due to the antibiotic resistance that microorganisms such as Staphylococcus aureus could present.

Graphical Abstract

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Introduction

The use of various antimicrobial substances that retard microbial growth (1), and also fight various infections has become a challenge (2), which is why antioxidant substances play a key role in the health of people, as they act in the protection against pathogens, oxidation reactions and even delay the growth of microorganisms (3), It has been proven that antioxidants are substances that are present in fruits and vegetables (4), that is why to reduce the impact generated by free radicals, industries such as cosmetic products have included in their components the use of these substances as mentioned (5) in his research. In the case of fruits, they are potential producers of a large amount of antioxidants that delay oxidative processes (6);(7), the presence of vitamins and other bioactive substances are also main antioxidants (8), which is why authors such as (9); (10), mention in their research that the hydroxyl groups of phenolic compounds are responsible for the deterioration of free radicals during the oxidative process, that is why *Ananas comosus* (pineapple) is one of the fruits of great importance for its high content of bioactive compounds (vitamin C, β-carotene and phenolic compounds), In addition, the residues generated from pineapple, such as the peel and crown, are an important source of dietary fiber and phenols (11). The prevention of respiratory diseases and others are derived from the trapping of free radicals and reactive oxygen species (ROS), as is the case of polyphenols (12);(13), which is why they are important in the prevention of diseases in the case of S. aureus is one of the microorganisms frequently isolated in hospitals and is also one of the most important nosocomial pathogens, responsible for infections and diseases (14), however this type of microorganisms have acquired importance for having developed resistance to antibiotics (15), which in the 60's induced resistance to methicillin (MRSA) as stated (16), which is why the objective of this research was to evaluate the antimicrobial effect of polyphenolic extracts present in *Ananas comosus.*

Materials and methods

Collection and adequacy

Pineapple (*Ananas comosus*) crowns were collected from the waste found in the city's central supply center. 3.5 kg of pineapple crown leaves were taken and cleaned using a 0.9% saline solution and then weighed.

Drying and grinding

For the drying process, a tray dryer, model PS-ECE-001/PE and series GEN-0412-237 was used for 72 hours at 55 °C. to reduce as much humidity as possible, and then they were milled to obtain a fine flour, for which a grain mill was used.

Extraction and identification of phenolic compounds

In the process of obtaining the oleaginous extract, a Soxhlet equipment was used in a solid-liquid extraction for seven hours using 96% ethanol as solvent, then a simple distillation was performed for 60 minutes to obtain the purest extract and evaporate the solvent, then the yield obtained from the extract was calculated using equation 1:

(Equation 1)

$$\%R = \frac{PI-PF}{PI} * 100$$

The identification of phenolic compounds was carried out by ultra-high performance liquid chromatography with Orbitrap mass detector (UHPLC-ESI+-Orbitrap-HRMS), the analyzed samples

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were dissolved in a mixture of methanol: water (1:1 v/v) with formic acid 0.2 % v/v, vortexed (5 min) and sonicated (5 min) and then injected into the chromatographic equipment. As reference standards were used the xanthines: caffeine (Part N° C8960-250G, Sigma-Aldrich), theobromine (Part N° T4500-25G, Sigma-Aldrich) and theophylline (Part N° T1633-25G, Sigma-Aldrich); the catechins: (±)-catechin (C) (Part N° C1788-500MG, Sigma-Aldrich), (-)-epigallocatechin gallate (EGCG) (Part N° E4143-50MG, Sigma-Aldrich), (-)-epicatechin (EC) (Part N° E1753-1G, Sigma-Aldrich), (-)-epicateqin gallate (ECG) (Part N° E3893-10MG, SigmaAldrich), (-)-epigallocateqin (EGC) (Part N° E3768-5MG, Sigma-Aldrich); flavonoids: caffeic acid (Part N° C0625, Sigma-Aldrich), p-coumaric acid (Part N° C9008, Sigma-Aldrich), rosmarinic acid (Part N° 536954-5G, Sigma-Aldrich), quercetin (Part N° Q4951-10G, Sigma-Aldrich), naringenin (Part N° N5893-1G, Sigma-Aldrich), luteolin (Part N° L9283-10MG, Sigma-Aldrich), kaempferol (Part N° K0133-50MG, Sigma-Aldrich), pinocembrin (Part N° P5239, Sigma-Aldrich), apigenin (Part N° A3145-25MG, Sigma-Aldrich); anthocyanins: cyanidin 3-rutinoside (Part No. G36428, Sigma-Aldrich), pelargonidin 3-glucoside (Part No. 53489, Sigma-Aldrich). Ultra-high efficiency liquid chromatograph (UHPLC), Dionex Ultimate 3000 (Thermo Scientific, Sunnyvale, CA, USA), equipped with a binary gradient pump (HP G3400RS), an automatic sample injector (WPS 300TRS) and a thermostatted unit for the column (TCC 3000). The LC-MS interface was electronebulization (ESI) and the mass spectrometer was high resolution with an Obitrap ion current detection system. Operated in positive mode with a capillary voltage of 3.5 kV. A Hypersil GOLD Aq Column (Thermo Scientific, Sunnyvale, CA, USA; 100 x 2.1 mm, 1.9 µm particle size) was used at 30 °C. The mobile phase was A: a solution of 0.2 % v/v formic acid in water, and B: 0.2 % v/v formic acid in acetonitrile. The initial gradient condition was 100 % A, changing linearly up to 100 % B (8 min); it was maintained for 4 min; the return to initial conditions in 1 min; the total run time was 13 min, with three min for post-run. Compound identification was performed using full scan acquisition mode and extraction of ionic currents (EIC) corresponding to the [M+H]+ of compounds of interest, mass measurement with accuracy and precision of Δppm < 1 and using a standard solution-mix of the compounds (certified reference material), for quantification of the analytes of interest the external standardization method was used.

Microbiological analysis

For the microbiological analysis, the methodology proposed by (17) was followed, with some modifications, in which antimicrobial susceptibility was evaluated by the standardized method of diffusion with discs (Bauer-Kirby Method), using Mueller-Hinton agar according to the standards established by the Clinical and Laboratory Standards Institute Clinical and Laboratory Standards Institute. CLSI (2017). For this, the Staphylococcus aureus strain (ATCC 25923) was used, using as positive control oxacillin (10 µL), and as negative control (DMSO), in the elaboration of the treatments of Ananas comosus was implemented the methodology used by. (18) and (19), where the extract was diluted in dimethyl sulfoxide (DMSO), with three replicates for each treatment applied, with the inoculum applied, the plates were prepared at concentrations of 25, 50, 75 and 100 mg/ml respectively see (Table 1)

Table 1. Preparation of treatments.

Treatments	Concentration mg/ml	Extract (µl)	DMSO (µl)
1	25	250	750
2	50	500	500
3	75	750	250
4	100	1000	0

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Likewise the turbidity of the medium was fixed in 0.5 units (1.5x108 UFC/ml) according to the Mc Farland scale pattern, which correlates with the number of bacteria present (20), likewise its absorbance was verified at a wavelength of 625nm, then we proceeded to the sowing in agar, pouring 25 µL of the extract and the controls in filter paper discs (Whatman No. 42), these were placed in the agar plate with a sterile clamp at 15 mm of the edge of the plate, pressing on the agar for its adherence with three repetitions for each applied treatment. 42), these were placed on the agar plate with sterile forceps 15 mm from the edge of the plate, pressing on the agar for adherence with three replicates for each treatment applied, the plates were incubated at 37 °C for 24 h in an inverted manner, then the inhibition halos were measured including the diameter of the discs.

Statistical analysis

A 1-factor Anova statistical analysis was performed on the data obtained in each treatment, determining the significant differences between them, for which the inhibition halos present in each treatment were used as a parameter, with a confidence base of 95 % and an error percentage of 5 %, showing the treatment with the best results in the process.

Results and discussions

After finishing the drying process of the pineapple crowns, 1766 g of pineapple crown were obtained.

$$\% R = \frac{3500 - 1766}{3500} * 100 = 49.5\%$$

With which a yield close to 50 % was obtained, later the obtained material was milled to obtain 900 g of fine flour, observing a decrease in the humidity present in the pineapple crown, which according to (21), the humidity depends on the irrigation before the harvest and by the state of conservation of the fruit.

Extraction and identification of phenolic compounds

According to (22),(23) solvent extraction is the most common method for the extraction of phenolic compounds, for which ethanol was used as solvent, using the Soxhlet extraction method, which, according to (24), can be attributed several factors that influence the extraction yield, Among these are the extraction temperature, extraction time and the type of solvent used, in this process it was possible to obtain a volume of 63 ml of extract of dried leaves of pineapple crown, in this case 96% ethanol was used as solvent. An HPLC/DAD type chromatographic analysis was performed using a 25 ml sample of the extract obtained, where a qualitative analysis was performed to identify the presence of phenolic compounds present in the extract, which are reported below (Table 2).

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Table 2. Phenolic compounds identified in pineapple (Ananás comosus).

Compond	**$t_{R,}$ min**	**NMC*, mg Kg^{-1}**	**Concentration in samples , mg kg-1 993315-01-EB Sample of pineapple crown extract** Ananas comosus
Theobromine	2,3	0,1	28
Theophylline	2,5	0,1	51
Epigallocatechin (EGC)	2,6	0,1	0,5
Catechin (C)	2,7	0,1	<0,1
Epicatechin (EC)	2,9	0,1	1,7
p-Hydroxybenzoic acid	2,9	0,1	< 0,1
Caffeine	2,8	0,1	**4899**
Caffeic acid	3,7	0,1	< 0,1
Vanillic acid	2,9	0,1	**108**
Epigallocatechin gallate (EGCG)	3,0	0,1	<0,1
p-coumaric acid	3,3	0,1	**193**
Epicatechin gallate (ECG)	3,4	0,1	<0,1
Ferulic acid	3,4	0,1	9,5
Quercetin	3,2	0,1	52
Rosmarinic acid	3,7	2,0	< 2,0
Cianidina	3,5	0,1	1,0
Luteolin	4,2	0,1	0,4
Kaempferol	4,6	0,1	0,1
trans-cinnamic acid	4,4	0,4	19
Naringenin	4,6	0,1	0,3
Apigenin	4,5	0,1	0,6
Pinocembrina	5,5	0,1	0,1
ursolic acid	8,4	0,1	6,9
Cyanidin 3-rutinoside	2,7	0,1	< 0,1
Pelargonidinagg 3-glucoside	2,8	0,1	< 0,1
Kaempferol 3-glucoside	3,5	0,1	0,8
Routine	3,2	0,1	21
Gallic acid	1,9	0,1	< 0,1

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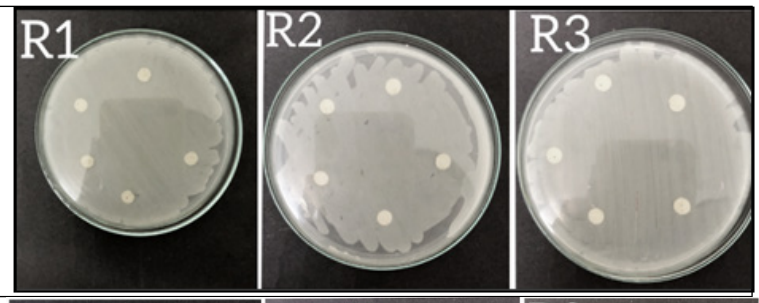
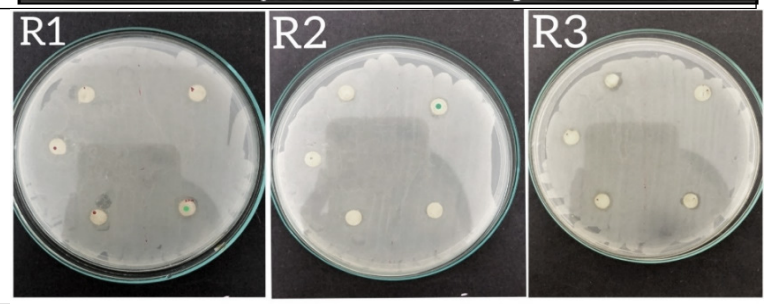
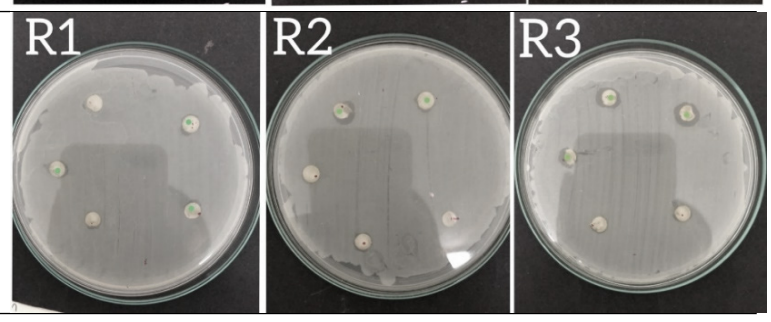
Twenty-seven phenolic compounds were identified, among them caffeine, vanillic acid and p-coumaric acid, these being the ones with the highest concentration. In addition, other structures related to polyphenols, such as gallic acid and quercetin, were detected, These compounds have a high potential to be used as antioxidants and antibacterials, as pointed out in previous studies by (25), and when these results were compared with the extraction process carried out by (26) in which a total of 27 polyphenolic compounds were identified, gallic acid, vanillic acid, protocatechuic acid, 2,5-dihydroxybenzoic acid and 4-hydroxybenzoic acid predominated. In particular, rosmarinic acid was reported as the compound with the highest concentration present in the extract, which is a polyphenol known for its antioxidant properties and has been associated with various health benefits, which according to Ramirez et al. (27), the concentration of rosmarinic acid (RA) increases with increasing K, which can have a significant impact on the phenolic composition, contributing to the antioxidant capacity (28), likewise it can be mentioned that one of the possible mechanisms proposed for the antimicrobial effect of phenolic compounds is that they can break the integrity of the membrane and cause loss of cell integrity and eventual cell death (29), (30).

Microbiological analysis

The absorbance of the S. aureus culture was measured, obtaining an average value of 0.142 on the McFarland scale, which is within the range established by (31), in his research on the validation of the membrane filtration technique. The treatments applied and the inhibition halos generated by the Ananas comosus extract in its different concentrations and the replicates of each treatment against the S aureus strain, including the positive and negative controls corresponding to each test, were also observed

Treatment 1 concentration 25mg/ml	
Treatment 2 concentration 50mg/ml	
Treatment 3 concentration 75 mg/ml	

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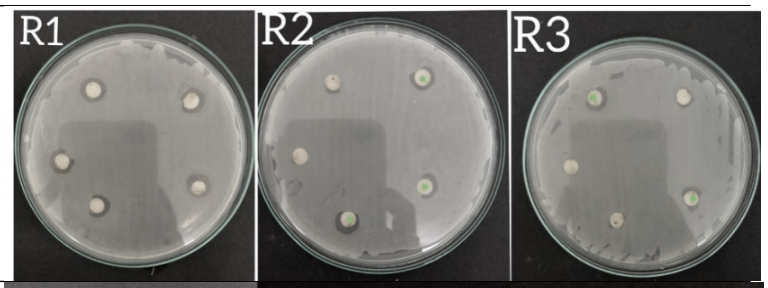
Treatment 4 concentration 100 mg/ml		
Positive control and negative.		

Figure 1. Inhibition halos present in each treatment applied per replicate.

The diameters of each halo generated were also measured and recorded (Table 3).

Table 3. Halo decade diameter obtained for each replicate.

Treatments	Diameter obtained in mm for each replica			Average of measurements
	R1	R2	R3	
T1 25 µl	0	0	0	0
T2 50 µl	5	4	4,5	4,5
T3 75 µl	5	6	6	5,6
T4 100 µl	6,2	6	6	6,0
Positive control	43,5	45	41	43,1

It should be noted that the evaluation process was only focused on observing the possible inhibition effect, so the dose-response analysis was not performed, then the average of the inhibition halos generated by the pineapple crown extract in S aureus was calculated, obtaining diameters ranging between 4.5 mm and 6, 0 mm, where the analysis of variance showed that with respect to the positive control and the applied treatments determined the difference, taking into account the p-value, which according to (32), this value indicates the probability of obtaining by chance, a difference greater than that observed in the process, this is how the obtained value of p = 5. 80E-13, which was less than 0.05, so there is a significant difference in the analysis of variance of inhibition halos (Table 4).

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Table 4. Analysis of variance of the inhibition halos.

Origin of variances	Sum of squares	Degrees of freedom	Mean squares	F	Probability	Critical value for F
Intergroup	3740,56	4	935,14	999,08	5,80E-13	3,47
Within the groups	9,36	10	0,93			
Total	3749,92	14				

On the other hand, the microbiological analysis carried out showed a variability in the inhibition halos which is significant because according to (33) previous results of inhibition halos of 28 and 18 mm have been reported, as reported by (34) and (35), as well as in the research carried out by (36), establishes that bacteria tend to produce resistance mechanisms against antimicrobials, which in the case of Staphylococcus aureus are related to the activation of cell wall synthesis, with the production of proteins, which once these resistance mechanisms are activated, create a greater firmness against the applied agent. It was also observed that treatment three corresponds to the highest concentration of pineapple crown extract, being the most representative in terms of inhibitory capacity, which indicates a gradual inhibition capacity, with a smaller inhibition halo being observed at lower concentrations of the extract. These results are consistent with the findings reported by (37), who indicated that treatments with higher concentrations usually generate larger inhibition halos, demonstrating a direct and effective action in the suppression of pathogenic microorganisms, so in this research it is established that the antimicrobial activity of the extract is proportional to its concentration.

Conclusions

The polyphenols present in the extract of pineapple crowns Ananas comosus presented an antibacterial activity on Staphylococcus aureus, observing a greater inhibition effect when applying a higher concentration of pineapple crown extract Ananas comosus on the sensidisc in Staphylococcus aureus, suggesting that the antimicrobial activity of the extract is proportional to its concentration.

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