

# Antibacterial activity of the *Agaricus pampeanus* (Agaricaceae) ethanol extract against *Enterococcus faecalis*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*

Actividad antibacteriana del extracto etanólico de *Agaricus pampeanus* (Agaricaceae) frente a *Enterococcus faecalis*, *Staphylococcus aureus*, *Escherichia coli* y *Pseudomonas aeruginosa*

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## Abstract

The macro fungi of the genus *Agaricus* have so far recorded many bioactive compounds with antibacterial properties as a product of their metabolism. However, in Ecuador, *A. pampeanus* or “Kallamba de finados” is a species studied for its ethnomycological uses and as part of the fungal biodiversity of the country. In this study, the antibacterial activity of the ethanolic extract of *A. pampeanus* was evaluated on four ATCC strains and the bioactive compounds present in the extract were identified. Initially, an ethanol extract of *A. pampeanus* was performed with the use of a Soxhlet extractor. To identify the extracted compounds, a gas chromatograph coupled to a mass spectrophotometer was used. With the mother extract, four solutions of the *A. pampeanus* ethanol extract (APE) were made at different concentrations, classified in APE1 (50.32 mg/mL), APE2 (40.24 mg/mL), APE3 (30.16 mg/mL), APE4 (20.12 mg/mL), which were subsequently used in the analysis of antibacterial activity, using the Kirby-Bauer technique. Six chemical compounds were identified; among them Eugenol was found as one of the main compounds. Out of the four concentrations, the *A. pampeanus* ethanol extract (APE1) was the most effective against *Enterococcus faecalis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* and categorized as sensitive, while *Escherichia coli* did not present any activity and was cataloged as resistant. The APE4 was not effective in any case. The antibacterial effect of APE was verified. Furthermore, *A. pampeanus* is more effective against Gram positive bacteria.

**Keywords:** Active principles, antimicrobial, basidiomycetes, biocomposites, Kallamba de finados

## Resumen

Los macrohongos del género *Agaricus* registran, hasta el momento, gran cantidad de compuestos bioactivos con propiedades antibacterianas como producto de su metabolismo. Sin embargo, en Ecuador, *A. pampeanus* o “Kallamba de finados” es una especie estudiada por sus usos etnomicológicos y como parte de la biodiversidad fúngica del país. En este estudio se evaluó la actividad antibacteriana del extracto etanólico de *A. pampeanus* sobre cuatro cepas ATCC y se identificaron los compuestos bioactivos presentes en el extracto. Inicialmente, se realizó un extracto etanólico de *A. pampeanus* con la utilización de un equipo Soxhlet. Para la identificación de los compuestos extraídos se empleó un cromatógrafo de gases acoplado a un espectrofotómetro de masas. A partir del extracto madre se realizaron cuatro soluciones a diferentes concentraciones del extracto etanólico de *A. pampeanus* (EAP), clasificados en EAP1 (50,32 mg/mL), EAP2 (40,24 mg/mL), EAP3 (30,16 mg/mL), EAP4 (20,12 mg/mL), que posteriormente fueron empleadas en el análisis de actividad antibacteriana, mediante la técnica de Kirby-Bauer. Se identificó un total de seis compuestos químicos; entre estos

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se encontró al Eugenol como uno de los compuestos principales. De las cuatro concentraciones, el extracto etanólico de *A. pampeanus* (EAP1) fue el más efectivo frente a *Enterococcus faecalis*, *Staphylococcus aureus* y *Pseudomonas aeruginosa*, siendo categorizadas como sensibles, mientras que *Escherichia coli* no presentó ninguna actividad y se catalogó como resistente. Por su parte, el EAP4 no fue efectivo en ningún caso. Se comprobó el efecto antibacteriano del EAP. Además, *A. pampeanus* presentó mayor efectividad frente a bacterias Gram positivas.

**Palabras clave:** principios activos, antimicrobiano, Basidiomicetos, biocompuestos, Kallamba de finados

## INTRODUCTION

The bacterial resistance to antibiotics is a public health problem because of the high rates of mutations, selection of multi-resistant strains, and various mechanisms of adaptation that these organisms have as a defense against antimicrobial compounds (Benavides-Plascencia, Aldama-Ojeda, and Vázquez, 2005; Cavalieri et al, 2005; Fernández, López, Ponce and Machado, 2003). In recent years, large quantities of drugs have been produced, and the indiscriminate use of these contributes to antibacterial resistance (Cohen, 1992; World Health Organization, 2015, 2020). For this reason, the World Health Organization developed an action plan to combat antibiotic resistance by supporting new alternative treatments such as natural medicine (World Health Organization, 2015, 2020). Thus, institutions and researchers are oriented to find new sources of active compounds (Fernández et al, 2003). The antibacterial property is attributed to synthetic or natural compounds, products of the metabolism of plants and fungi (Nascimento, Locatelli, Freitas, and Silva, 2000; Singdevsachan, Patra, Tayung, and Thatoi, 2017).

Among the diversity of living organisms, fungi are an essential source of bioactive compounds with antibacterial characteristics (Singdevsachan et al, 2017). In fact, the extraction and analysis of compounds present in these organisms is currently essential (Castillo-Machalskis, D'Armas, Malaver, and Núñez, 2007). Macro fungi extracts, obtained through grinding, maceration, infusion, etc., possess a wide range of uses in traditional medicine (Milenge-Kamalebo et al, 2018; Ruan-Soto, 2017); they are described as having anti-tumor, anti-cancer, anti-fungal, anti-viral, anti-bacterial, immunomodulating, and cytostatic properties (Brizuela, García,

Pérez, and Mansur, 1998; Sorimachi and Koge, 2008; Liébana-Ureña, 2002; Wang, Wei, and Chou, 2008).

Some of the studied macro fungi with active ingredients are of the genus *Agaricus*. Natural extracts of species belonging to this genus are used as alternative medications and nutritional supplements (Wang et al, 2008). Among its extracts metabolites such as diazonium salt (Agaritin) and eugenol, which have inhibitory and bacteriostatic properties in the growth of fungi and bacteria (Gram-positive and gram-negative), were identified and analyzed through susceptibility tests (Dornberger, Lich, and Zureck, 1989; Schulzová et al, 2009; Soković and Van Griensven, 2006; Sun et al, 2014). In addition, it was determined that *A. placomyces* Peck 1878, *A. bitorquis* (Qué.) Sacc 1887, *A. bisporus* (J.E. Lange) Imbach 1946, *A. brasiliensis* Fr 1830, and *A. blazei* Murrill 1945 have antibacterial effects on Gram-negative and Gram-positive strains (Bernardshaw, Johnson, and Hetland, 2005; Jagadish, Venkata, Shenbhagaraman, and Kaviyaran, 2009; Mazzutti et al, 2012). Meanwhile, *A. essettei* Bon 1983 and *A. silvicola*, updated in *indexfungorum* as *A. silvicolae-similis* Bohus and Locsmáncsi 1994, only have activity on Gram-positive strains (Barros, Cruz, Baptista, Estevinho, and Ferreira, 2008; Öztürk et al, 2011).

In Ecuador, the use of fungi in traditional medicine has been recorded, and this ethnomycological use is only surpassed by its employment in food (Gamboa-Trujillo et al, 2014; Singdevsachan et al, 2017). *Agaricus pampeanus* Speg 1880 known as "Kallamba de finados" is used in local communities; however, there are no recorded studies about its medicinal properties (Gamboa-Trujillo et al, 2014). In this context, the present study seeks to evaluate the antibacterial activity of *A. pampeanus* on ATCC strains of *Enterococcus faecalis*, *Staphylococcus aureus*, *Escherichia*

*coli* and *Pseudomonas aeruginosa*.

## MATERIALS AND METHODS

### Study design

The present study is framed in the lines of a cross-sectional observational-descriptive research according to the study time.

### Collection of fungal material

Approximately 200 g of fresh fruiting bodies of *A. pampeanus* were obtained at the César Chiriboga market located in Sangolquí, Cantón Rumiñahui. The samples were identified in the Laboratory of Applied Mycology of the Faculty of Chemical Engineering of the Central University of Ecuador.

### Extract preparation

The impurities in the fruiting bodies were removed by washing them with distilled water. The fruiting bodies were dried with the help of a stove and pulverized in an electric mill. A total of 20 g of sample were obtained. The sample was placed in a paper filter cartridge and then in the body of the Soxhlet extractor. Then it was hydrated with 50 mL of 96 % ethanol and 100 mL of ethanol were placed in a round flask, which was boiled at a constant temperature of 78°C for approximately one hour, by refluxing the extraction system and accomplishing three siphons (Saldaña-Sunci6n and Garc3a-Medina, 2016). Once the mother extract was obtained, it was stored in an amber bottle at -4°C for its preservation.

### Extract efficiency

To calculate the extract yield, the initial weight of the sample was taken versus the final weight (Martinez, Valencia, Jimenez, Mesa, and Galeano, 2003):

$$\% \text{ efficiency} = \frac{\text{final sample weight (g)}}{\text{initial sample weight (g)}} \times 100$$

To determine the grams dissolved in the final liquid extract, the initial and final volume of the alcohol used, as well as the volume of the sample under extraction were considered.

### Preparation of concentrations

From the mother extract, named ethanolic extract of *A. pampeanus* (APE), dilutions with distilled water were prepared. The initial extract, which was called APE1, was kept at a concentration of 100% with 50.32 mg/mL. The following extract, designated APE2, was diluted 80% with 40.24 mg/mL. The third extract, known as APE3, was prepared at a concentration of 60% with 30.16 mg/mL. Finally, the APE4 extract was at 40% of concentration with 20.12 mg/mL.

### Activation of ATCC strains

The ATCC bacterial strains and culture medium were purchased from authorized commercial stores. The strains in Kwik-Stik presentation were activated in Trypticase Soja Agar (TSA). Subsequently, the young strains were inoculated into Brain Heart Infusion (BHI) liquid medium from which the bacterial suspension of 0.5 McFarland (1.5 X 10<sup>-8</sup> CFU/mL) was done for the antibacterial analysis.

### Analysis of antibacterial activity by Kirby-Bauer technique

Mueller Hinton Agar (MHA) solid culture medium was used for *S. aureus* ATCC<sup>®</sup> 25923<sup>TM</sup>, *E. coli* ATCC<sup>®</sup> 25922<sup>TM</sup> and *P. aeruginosa* ATCC<sup>®</sup> 9027<sup>TM</sup>, and Mueller Hinton blood agar was used for *E. faecalis* ATCC<sup>®</sup> 29212<sup>TM</sup>. The four bacterial strains were inoculated in 15 cm diameter Petri dishes, four repetitions of each inoculation were performed and each test was triplicated. Then, disks loaded with 15 μL of each concentration of the ethanol extract were properly distributed in each Petri dish. In addition, as a positive control, 120 μg gentamicin disks were applied for *E. faecalis* ATCC<sup>®</sup> 29212<sup>TM</sup> and 10 μg gentamicin for *S. aureus* ATCC<sup>®</sup> 25923<sup>TM</sup>, *E. coli* ATCC<sup>®</sup> 25922<sup>TM</sup> and *P. aeruginosa* ATCC<sup>®</sup> 9027<sup>TM</sup>, while as a negative control disks with ethyl alcohol were applied. The incubation process was applied at 37°C in a range of 18 to 24 hours (Cavalieri et al, 2005).

## Inhibition zone measurement

Once the incubation time elapsed, the inhibition halos were measured in millimeters with the help of a calibrator and dark cardboard (Cavalieri et al, 2005).

## Interpretation of inhibition zones

Reference points for each antibiotic used as a control were established taking into account the criteria established by the National Committee for Clinical Laboratory Standards (NCCLS), with this it was determined whether the bacteria in the study were sensitive, sensitive intermediate or resistant (Cavalieri et al, 2005; Clinical and Laboratory Standards Institute-CLSI, 2014). For the analysis of results, the arithmetic mean, standard deviation and variance of the set of repetitions applied for each of the extracts against each bacterium were calculated. With this data, tables and graphs were made.

## Extract identification

For the analysis 1.5 mL of extract were placed in a vial, filtered at 0.45 micrometers at a temperature of 4°C to 230°C being analyzed in a gas chromatograph coupled to an Agilent Technologies™ mass spectrophotometer 5977 EMSD and 7820 AGC System.

## RESULTS

After doing the quantitative and qualitative analysis of the ethanolic extract of *A. pampeanus* by gas chromatography coupled to mass spectrophotome-

try, six compounds were obtained, characterized and described (table 1). Based on this, it was determined that the yield of the ethanolic extract of the fruiting bodies of *A. pampeanus* was 25.67%, equivalent to 5.536 g diluted in 110 mL of the ethanolic extract.

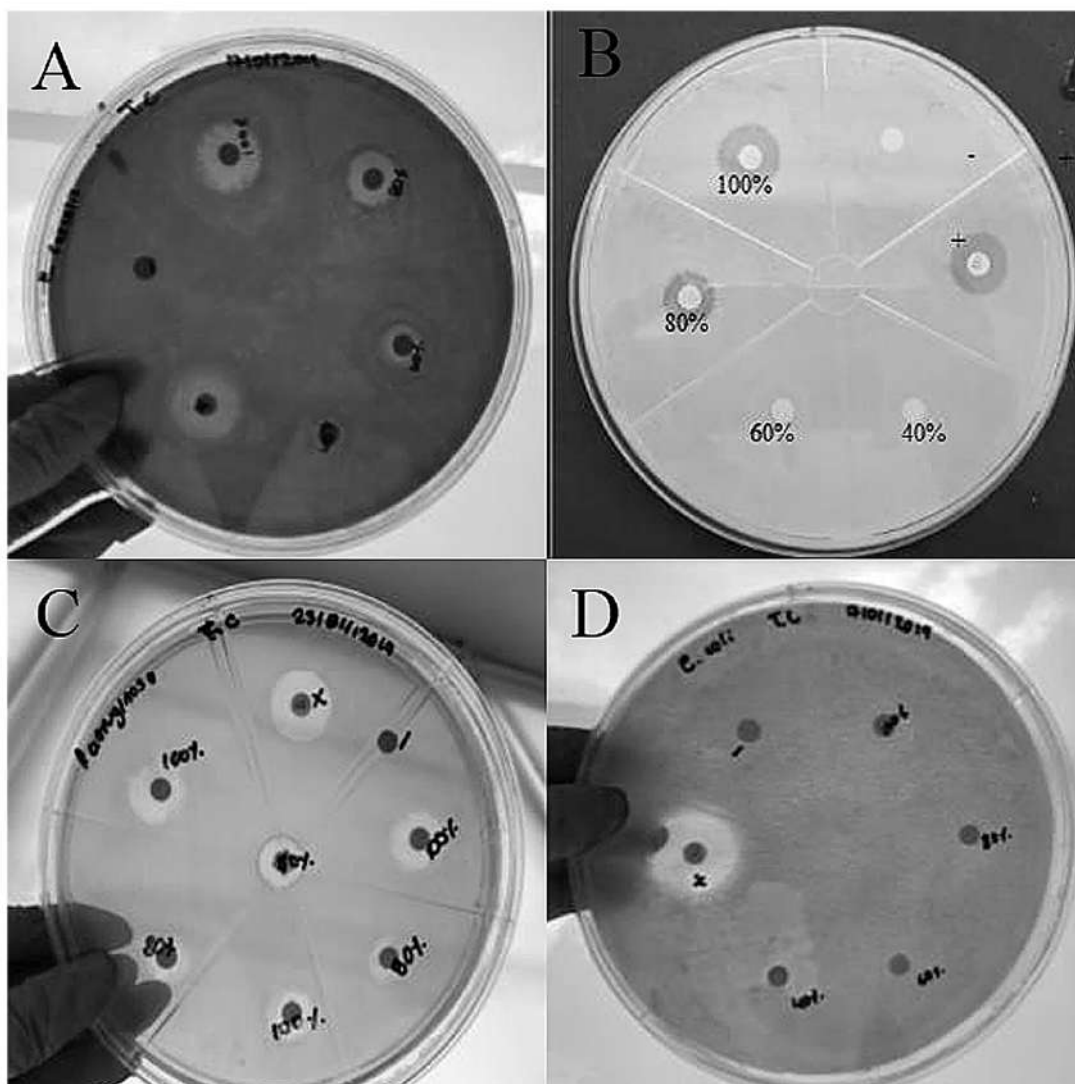
## Antibacterial activity of ethanolic extract of *A. pampeanus* by Kirby-Bauer technique

By exposing the APE against the target bacterial strains, it was possible to identify that it is more effective on Gram-positive strains of *S. aureus* and *E. faecalis*, on Gram-negative bacteria, the activity was verified only against *P. aeruginosa*, excluding *E. coli*. The latter was categorized as resistant since its growth was normal around the APE discs, however, the positive control showed sensitivity with an average halo of 22 mm in diameter, demonstrating the feasibility of the methodological application (figure 1). Furthermore, the activity observed against *P. aeruginosa* is markedly decreased compared to Gram-positive bacteria.

As a result, it was determined that APE1 was the most effective with a concentration of 50.32 mg/mL (100%) by showing greater diameter in the inhibition halos against *S. aureus* (18 mm), *E. faecalis* (17 mm), and *P. aeruginosa* (16 mm), followed by APE2 with a concentration of 40.24 mg/mL (80%) with halos of 15 mm, 14 mm and 11 mm respectively. Regarding APE3, with 30.16 mg/mL (60%), it was the only one that had an inhibitory effect against *E. faecalis* with an average halo of 8 mm. Finally, APE4 with 20.12 mg/mL (40%) does not have antibacterial activity in

**Table 1.** Description of the compounds present in the ethanol extract of *A. pampeanus*

Compound name	Formula	Synonyms	Total percentage (%)
Pyrimidine-2,4 (1H, 3H) - dione, 5-amino-6-nitroso	C <sub>4</sub> H <sub>4</sub> N <sub>4</sub> O <sub>3</sub>	Hydrazoic acid Azoimide Diazoimide	37.526
Acetic acid	C <sub>2</sub> H <sub>4</sub> O <sub>2</sub>	Ethanoic acid	13.448
p-Menth-8-en-3-ol	C <sub>10</sub> H <sub>18</sub> O <sub>2</sub>	Cyclohexanol	15.918
Eugenol	C <sub>10</sub> H <sub>12</sub> O <sub>2</sub>	Phenol, 2-methoxy-4-(2-propenyl) - Eugenic acid	15.244
Acid (1,1'-bicyclopropyl)-2-octanoic 2'-Hexyl, ester methyl	C <sub>21</sub> H <sub>38</sub> O <sub>2</sub>	Isopropyl Linoleate Linoleic acid	11.104
Ethyl (9Z, 11E)-octadeca-9,11-dienoate	C <sub>20</sub> H <sub>36</sub> O <sub>2</sub>	Ethyl linoleate	6.760



**Figure 1.** Antibacterial activity of the ethanolic extract of *A. pampeanus* in concentrations of APE1 (100%), APE2 (80%), APE3 (60%) and APE4 (40%) on the four bacterial strains. A) *E. faecalis*. B) *S. aureus*. C) *P. aeruginosa*. D) *E. coli*.

any case (figure 2). Furthermore, it should be noted that the inhibition halos exhibited by the different concentrations of APE in the strains under study did not exceed those generated by the positive controls.

For the classification of antibacterial activity by the Kirby-Bauer method, the previously mentioned halos' average was taken into account with the following criteria: R) resistant, I) intermediate, S) sensitive. The limits of the positive control Gentamicin (10  $\mu$ g) ranged from < 12 (resistant), 13-14 (intermediate),

> 15 (sensitive) and Gentamicin (120  $\mu$ g), < 6 (resistant), 7-9 (intermediate) and > 10 (sensitive) (table 2), in which APE4 was excluded due to not having any activity.

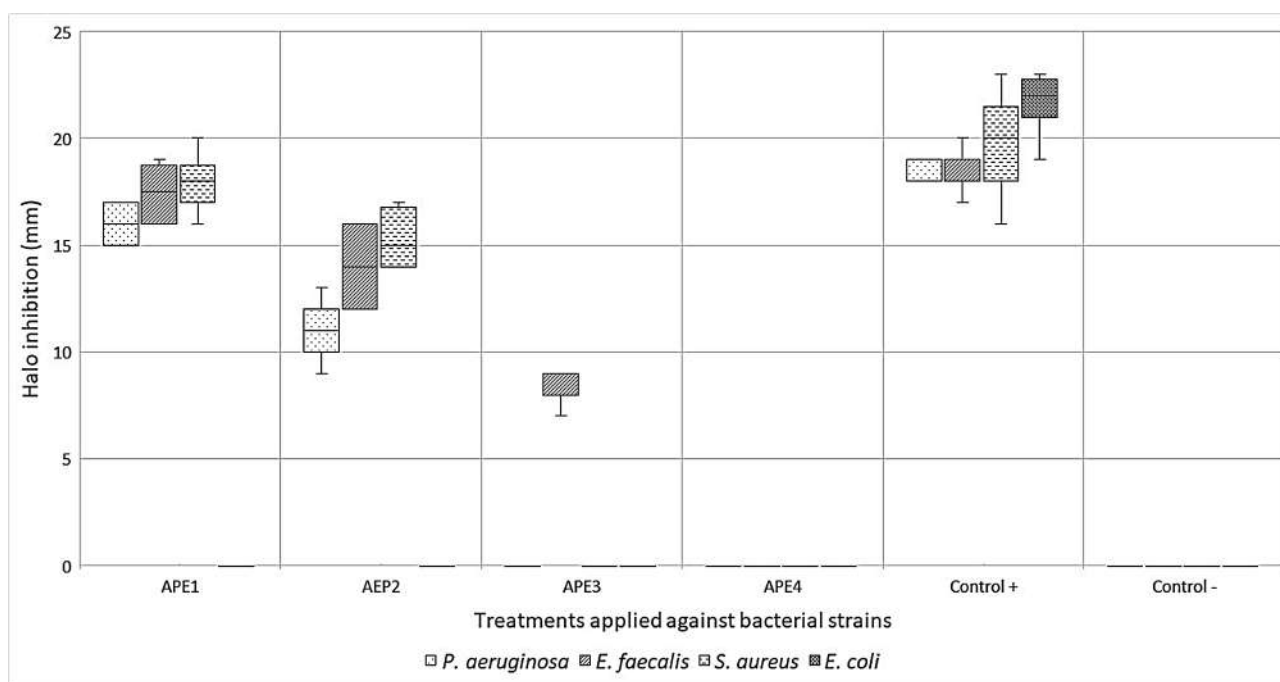
## DISCUSSION

The antibacterial activity of *A. pampeanus* was demonstrated from its ethanolic extract. This activity occurs in other species of the genus *Agaricus* throughout the world and provides an additional

value other than the nutritional one (Barros et al, 2008; Gamboa-Trujillo et al, 2014; Öztürk et al, 2011; Singdevsachan et al, 2017). The antibacterial activity on strains of *E. faecalis*, *S. aureus* and *P. aeruginosa* is important since there are species of the same genus to which a similar inhibitory effect against other strains has been attributed; this has been previously demonstrated when extracts of *A. bisporus* are used against *Bacillus subtilis*, *A. silvicolae-similis* against *B. cereus*, *B. subtilis* and *S. aureus* (Barros et al, 2008). Likewise, the activity of *A. bisporus* against *S. aureus*, *B. subtilis*, *K. pneumoniae* and *P. aeruginosa* was verified (Jagadish et al, 2009). Furthermore, Mazzutti et al. (2012) established the effectiveness of *A. brasiliensis* against *S. aureus* and *B. subtilis*. While Bernardshaw et al. (2005) determined that extracts of *A. blazei* have the possibility of being used against *Streptococcus pneumoniae* in certain treatments. It should be noted that the effectiveness against Gram-negative bacteria has a lower spectrum when identifying only the inhibitory effect on *E. coli* using extracts of *A. placomyces* (Pérez-Silva, 1959). In turn, the activity of the genus against Gram-positive bacteria is verified better when exposing

extracts of macro fungi such as *A. bitorquis* and *A. essettei* against strains of *Micrococcus luteus*, *M. flavus*, *B. subtilis* and *B. cereus* (Öztürk et al, 2011). Thus, the activity resulting from *A. pampeanus* on Gram-positive and Gram-negative bacteria is corroborated.

The antimicrobial activity presented by an extract is due to the initial uptake of the metabolites generated by the organisms studied; therefore, it is important to use a suitable solvent that allows these metabolites to be extracted. Mazzutti et al. (2012), determines that the use of different solvents to obtain extracts of the same fungus, exhibits opposite results against the same bacterial strains, so when using *A. brasiliensis* against *S. aureus*, *B. cereus*, *E. coli* and *P. aeruginosa* different results were obtained. In the same way, the use of different solvents in extracts of *A. bisporus* differ in effectiveness compared to *S. aureus* (Barros et al, 2008; Jagadish et al, 2009). Therefore, the viability of extracts of *A. pampeanus* can be verified by using a solvent such as ethanol and obtain optimal results against three of the four bacterial strains evaluated.



**Figure 2.** Mean of inhibition halos by bacterial strain against the treatments of ethanolic extract of *A. pampeanus* (APE1, APE2, APE3, APE4, Positive Control +, and Negative Control -).

**Table 2.** Classification of the diameter of the inhibition halos exhibited by bacterial strains against different concentrations of the *A. pampeanus* ethanolic extract

Bacteria		APE1 (50.32 mg/mL)		APE2 (40.24 mg/mL)		APE3 (30.16 mg/mL)		Gentamicin (10 $\mu$ m)	
		Inhibition halo average (mm)	Classification*	Inhibition halo average (mm)	Classification	Inhibition halo average (mm)	Classification	Inhibition halo average (mm)	Classification
Gram negative	<i>Pseudomonas aeruginosa</i>	16	S	11	R	-	-	19	S
	<i>Escherichia coli</i>	-	-	-	-	-	-	22	S
Gram positive	<i>Staphylococcus aureus</i>	18	S	15	S	-	-	20	S
	<i>Enterococcus faecalis</i>	17	S	14	S	8	I	19	S

\*S = Sensitive, I = Intermediate, R = Resistant.

Through the disk diffusion test applied in this study, the effectiveness of the extract was verified, it was observed that *S. aureus* was inhibited to a greater extent by APE1 (18 mm), characterized as sensitive. Likewise, Barros et al. (2008) highlight positive results for methanolic extracts of highlight the positive results for methanolic extracts of *A. silvicolae-similis*, which shows effectiveness with halos greater than 9 mm, and the boiled ethanol extracts of *A. bisporus* have an average halo of 18 mm (Jagadish et al, 2009). While methanolic extracts of *A. bitorquis* and *A. essettei* presented halos of 12 and 11 mm respectively (Öztürk et al, 2011). The inhibition halos of the extract against *E. faecalis* (17 mm) and *P. aeruginosa* (16 mm) demonstrated the sensitivity that APE1 possesses against them, and are measures that approximate methanol extracts of *A. bisporus*, however, some authors differ from the results for the use of different solvents (Jagadish et al, 2009; Öztürk et al, 2011). Jagadish et al. (2009) and Öztürk et al. (2011), also characterized *E. coli* as resistant from extracts from species such as *A. bisporus*, *A. bitorquis* and *A. essettei*. However, Pérez-Silva (1959), in his study demonstrates that the crude extract of *A. placomyces* is able to inhibit the growth of *E. coli* with halos of 35 mm in diameter, and 25 mm in purified extracts, and mentions that bioactive fungal compounds can remain trapped during filtration processes for purification.

The extracts obtained showed the presence of Eugenol and acetic acid that reported antibacterial, antifungal and bacteriostatic properties, it should be noted that acetic acid was possibly generated by the oxidation of ethanol at the time of making the extract, so the primary compound already evaluated is Eugenol (NCBI, 2020). However, Dornberger et al. (1989), mentions that the antibacterial activity in the genus *Agaricus* is due to the presence of the bioactive compound called Agaritin. Despite this, after analysis by gas chromatography said compound was not found in the extract, we can assume that it suffered a rupture or physical change in its structure by the high temperature employed during the analysis. For this reason, *A. pampeanus* could be considered a new source of active principles with antimicrobial action and should be taken into account for future studies against other microorganisms. Therefore, after

the analysis of fungi such as *A. blazei*, *A. bisporus* and *A. silvicolae-similis* some studies express that these types of bioactive compounds can be used in medicine as prophylactic and therapeutic agents or simply be supplied in the diet of a person as nutraceuticals (Barros et al, 2008; Bernardshaw et al, 2005).

Finally, through this study it was determined that the ethanol extract of *A. pampeanus* has antibacterial activity against Gram-positive and Gram-negative bacteria. However, the effect against Gram-negative bacteria tends to decrease as in *P. aeruginosa* while with *E. coli* it lacks activity, possibly attributed to the difference in the constitution and complexity of the cell wall and some constituents such as phenols and flavonoids that could act differently in these organisms (Cavaliere et al, 2005; Jagadish et al, 2009; Öztürk et al, 2011; Soković and Van-Griensven, 2006). Based on the data reported in previous studies, it could be considered that eugenol favors the antibacterial activity of the extract.

## ACKNOWLEDGMENTS

We thank the Teaching Laboratory, Laboratory of Applied Mycology of the Faculty of Biological Sciences; Catalyst Laboratory of the Faculty of Chemical Engineering and the Biology Center of the Central University of Ecuador. Finally, thanks to Paul Gamboa-Trujillo and all leaders of the centers mentioned.

## CONFLICT OF INTEREST

The authors declare no conflict of interest, due to that the study was self-financed.

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