International Journal of Science and Research (IJSR)
ISSN: 2319-7064
SJIF (2020): 7.803

In Vitro Antimicrobial Activity of Jamelao Seed Proteins (Syzygium Cumini (L.) Skeels)

Ana Catarina Viana Valle[1], Andrea Maria Lazzari[2], Francislete Rodrigues Melo[3]

[1]Professor – Departamentof VeterinaryHomeopathy, IDISLamasson Institute – Integrative Health and Medicine, Ribeirao Preto, SP, Brazil
Corresponding Author E- mail: *dranacatarina[at]gmail.com*

[2]Professor – DepartamentofMicrobiology, UPIS, Planaltina, DF, Brazil
E- mail: *lazzari[at]upis. br*

[3]Professor – DepartamentofBiochemistry, UniCeub, Brasilia, DF, Brazil
E- mail: *etemelo[at]gmail.com*

Running title: *Antimicrobial activity of jamelao seed proteins*

Abstract: ***The interest in all types of research related to medicinal plants has intensified with the increase in microorganism resistance to conventional drugs. Within this context, antimicrobial peptides found in plant seeds are of great importance due to their high antimicrobial potential. Several authors have already reported such antimicrobial action in jamelao (Syzygiumcumini (L.) Skeels). Therefore, this work aimed to test the antimicrobial properties of the protein fraction from the jamelao seeds. The S. cumini protein fraction was obtained by precipitation with ammonium sulfate. After dialysis against distilled water, this fraction was dried, measured by the Bradford method, and submitted to electrophoresis by the Laemmli method. Additionally, tests to determine the antimicrobial activity were performed according to the Kirby - Bauer modified methodology. The Minimum Inhibitory Concentration (MIC) was determined by the Muller Hinton dilution method. Protein bands between 10 and 75 kDa were observed on the SDS - PAGE gel. Such protein fraction was able to inhibit the growth of a considerable portion of the microorganisms tested. The MIC varied between 2.07 and 4.625 µg/mL of protein fraction. Based on the results of this study, additional chemical, pharmacologic, toxicology, and clinical tests must be performed with this plant due to the considerable antimicrobial effects recorded.***

Keywords: Antimicrobial peptides, Jamelao, Minimum Inhibitory Concentration, Seeds.

1. Introduction

The use of medicinal plants in health recovery has evolved from the simplest forms of local treatment to the technologically sophisticated forms of industrial manufacturing [1]. Considering herbs as the basis of phytotherapeutic medicines, they have one or more chemical substances with medicinal action capable of interacting with the human and animal organism, restoring its health and balance [2, 3]. Therefore, these substances present growing importance as clinical, pharmaceutical, and economic resources in various parts of the world. The observed trend is that phytotherapy, as in the past, will play an increasingly important role in human and animal health care [2, 4].

Microbial resistance

The medical area has identified the various infections caused by pathogenic microorganisms as the leading cause of mortality in immunocompromised patients. Thereby, the choice of an adequate medication to fight a given infection is a complex problem due to the development of resistance to the active ingredient [3]. The emergence of resistance to antibiotics and other antimicrobial principles was and probably will continue to be one of the major problems of medicine, agriculture, and livestock [5]. Within this subject, adverse reactions to antimicrobial drugs are also important restrictions to their therapeutic administration. However, substances that present selectivity of action may reduce their side effects [6].

Due to the wide gap between infection and conventional treatment, phytotherapy has been gaining prominence among conventional therapies, both as adjuvant and unique medicines capable of fighting the infection [7]. A better understanding of these mechanisms concerning antimicrobial agents is needed to control the problem. On the other hand, the current scenario of this problem causes a feeling of impotence facing the various microorganisms, showing that knowledge may be losing the race against multiresistant pathogenic organisms [5; 8].

Plant Defense Mechanism

The antimicrobial defense mechanism of plants consists of the production of several substances, including proteins. They may be indirectly involved in plant defense mechanisms against microorganisms through interactions with the cell wall [9; 10]. The functions of these proteins can be varied and seem to be related to the stages of seed maturation and germination and environmental conditions to which the plant was subjected [11]. They directly correlate with plant defense mechanisms against the pathogenic microorganisms'attack [12], such as infection by fungi, bacteria, and viruses. The synthesis of an important class of peptides (defensins or defense peptides), with antifungal, antibacterial, and antiviral actions, is induced in the plant by these proteins [7; 12].

Plant defensins are peptides of cationic nature [7; 11] due to their high lysine and arginine content. They are amphipathic and have a primary structure rich in cysteine. Defensins can be defined as innate components of the plant's immune

system and are classified as antifungal, antibacterial, and even antiviral. They may even act against protozoa, permeabilizingmembranes [5; 11].

The mechanism of action of plant defensins is still not well understood [7; 13]. It is known that a specific cell membrane response occurs when in the presence of defensins. This response is understood by the formation of pores (channels), resulting in increased membrane permeability and a change in its physiological state [11]. Consequently, increased calcium intake and potassium output from the cell are observed, disrupting the membrane balance [13].

It is supposed that the mechanism of action of antimicrobial peptides in Gram - positive bacteria occurs with the cytoplasmic membrane. In contrast, in Gram - negative bacteria, the interaction with the outer membrane and cytoplasmic membrane is necessary to realize the bactericidal activity. Cationic peptides possibly interact with the lipopolysaccharide (LPS) found in the outer membrane of Gram - negative bacteria, quickly creating a transport pathway for the peptide itself [14].

Jamelao

Jamelao, also known as jambolan, java plum, common plum, and Eugenia jambolana, belongs to the family *Myrtaceae*, one of the most characteristic of the Brazilian flora [15]. It is considered a tree originating in East India and also present in Australia and the Americas [16]. This plant is well known in Indian and Pakistani folk medicine [16].

Jamelao (Fig.1A) is popularly known for its hypoglycemic [16, 17] and antidiarrheal properties. Different parts of the plant, such as leaves, flowers (Fig.1B), fruits (Fig.1C), and seeds, are used for these purposes [16].

The plant is rich in compounds that contain anthocyanins, glucoside, ellagic acid, isoquercetin, kaemferol, and myrecetin. The seeds contain alkaloid, jambosin, and jambolin or antitimelin glycoside, interrupting the diastatic conversion of starch into sugar [17].

Bark, seeds, and leaves are characterized by their astringent capacity. The leaves are rich in tannins and saponins (Table 1) [16; 18]. Such parts are widely used in folk medicine for preparing teas [16], aqueous and ethanol extracts, and raw plant juice to treat various conditions. Fruit juice can be used as a diuretic and stomachic. The astringent properties present in the barks are used against diarrhea, menorrhagia, and emetic episodes. The peel decoction is considered effective as a mouthwash for treating mouth ulcers, stomatitis, throat affections, and other oral [1; 16]. There are also reports of its use in the treatment of genitourinary candidiasis [8].

The protein fraction of the seeds has proved antibacterial and antifungal activity [19; 20]. The methanol extract of its seeds had a significant action on the central nervous system in albino rats that ingested a dose of 5mg/kg, for 14 days, through a gastric tube [21]. Sagrawatet al. [22] reported the pharmacological properties found in the raw seed extract of this plant, described as anti - inflammatory, antidiarrheal, gastroprotective, antidiabetic, antibacterial, antioxidant, anorectic, among others.

This study aimed to evaluate the antibacterial and antifungal effects of protein fractions from jamelao (*Syzygiumcumini* (L.) Skeels) seeds on microorganisms of animal importance.

2. Material and Methods

Obtaining the fruits

This study used jamelao (*Syzygiumcumini* (L.) Skeels) seeds from fruits harvested at different times of the year from regions in the surroundings of Brasilia, DF, Brazil (Table 1).

Table 1: Harvesting dates of the different samples used and climatic conditions registered at harvest day.

Samples	Harvesting Date	Climatic Conditions							
		$_{Max.}$ T (^{0}C)	$_{Min.}$ T (^{0}C)	$_{Avg.}$ T (^{0}C)	$_{Max.}$ Humidity (%)	$_{Min.}$ Humidity (%)	$_{Avg.}$ Humidity (%)	RD (mm)	Rad. (UV)
1st	Jun - 01	24	14	19.9	88	40	66	0	485.86
2nd	Aug - 01	27.8	13	20	75	25	49	0	490
3rd	Sept - 15	30	16.5	23.6	58	17	35	0	573
4th	Nov - 30	24.8	18.8	20.2	96	68	91	16.3	217.51
5th	Mar - 13	27.4	17.1	20	98	61	91	16	323.91
6th	Aug - 20	26.7	14.1	20	71	27	47	0	528.81

T – temperature; Max. - Maximum; Min. – Minimum; Avg. - Average; RD - Rainfall Density; Rad. – Radiation; UV – Ultraviolet.

Obtaining Seed Flour

The seeds (Fig.2A) were manually extracted from the fruits, washed with water, and dried in a forced - air - circulation oven at 35ºC for 48 hours. Afterward, they were crushed in a mill and sieved until obtaining a fine - textured flour (Fig.2B).

Flour Delipidation

The flour obtained was delipidated with 99.5% acetone (Fig.3A) in three washes at 4^{o}C for 24 hours. After the third change, the flour was left to dry for 12 hours at room temperature.

Protein Extract/Fraction Obtaining and Dialysis

The extract was obtained at a ratio of 1: 5 (w/v) in 0.1M HCl solution with 0.15M NaCl (extraction solution) at room temperature, for 2 hours under constant agitation. After this period, the extract was centrifuged at 12, 000 rpm for 30 minutes at 4^{o}C. The precipitate was discarded, and the supernatant had its pH adjusted to 7.0 with the addition of 1M NaOH. This supernatant was left for 12 hours at 4^{o}C for precipitation of the non - soluble material at pH 7.0 (Fig.3B). Then, the precipitate was separated by centrifugation at 12, 000 rpm for 15 minutes at 4°C. The resulting supernatant was precipitated with 0/70%

ammonium sulfate and allowed to stand for 12 hours in a cold room. Afterward, another centrifugation was performed at 12, 000 rpm for 30 minutes at 4°C. The supernatant was discarded, and the precipitate was submitted to dialysis against distilled water (Fig.3C) in a cold chamber. A total of six changes was accomplished in 48 hours, followed by centrifugation at 12, 000 rpm for 30 minutesat 4°Cto obtain the supernatant, which was subjected to lyophilization for 48 hours. The lyophilized protein fraction was thereby obtained.

Protein Dosing and Protein Pattern Visualization by SDS - Page

The lyophilized sample was subjected to protein dosage using the Bradford method in a spectrophotometer. Protein concentration was calculated from a standard curve obtained using bovine serum albumin. The experiments involving electrophoresis were all carried out according to the methodology described by Laemmli. The application gel was prepared to contain 5% acrylamide and 1.0% SDS in 0.5M Tris - HCl buffer, pH 6.8. The separation gel was prepared to contain 12% polyacrylamide and 1.0% SDS. The gel was stained by the silver nitrate method.

Evaluation of Antimicrobial Activity

Seventeen microorganisms were used: seven Gram - negative bacteria, nine Gram - positive bacteria, and one yeast. Table 3 lists the microorganisms with the respective locations and animals from which isolation was carried out. The microorganisms were tested against the protein fraction by the methodologies Kirby - Bauer modified and Minimum Inhibitory Concentration (MIC) (dilution in Muller Hinton broth).

Table 3: Microorganisms used in the "in vitro" tests.

Microorganisms	Origin
Escherichia coli	Guts/Bird (SB 334/93) ***
Klebsiella pneumoniae	Eye/Rabbit (Project) **
Pseudomonas aeruginosa	Lung/Buffalo (SB 22/00) **
Pseudomonas aeruginosa	Surgical wound/Dog (SB 39/06) **
Bordetella bronchiseptica	Lung/Rabbit (Project) **
Salmonella enteritidis	Intestine/Swine (SB 56/02) **
Proteus mirabillis	Urine/Dog (SB 49/04) **
Corynebacterium pseudotuberculosis	Ear/Sheep Pavilion (SB 41/06) **
Staphylococcus aureus	ATCC*
*Streptococcus suis*type 2	Lung/Boar (SB 78/02) **
Nocardia spp.	Milk/Buffalo (Project) **
Bacillus cereus	Milk/Cow (SB 177/94) ***
Enterococcus faecalis	ATCC*
Micrococcus spp.	Prepuce/Dog (SB03/00) **
Rhodococcusequi	Feces/Horse (SB 04/06) **
*Streptococcus equi*subsp. *equi*	Lymph node/Horse (SB 99/94) ***
Candida albicans	Milk/Buffalo (SB 30/04) **

*ATCC - American Type Culture Collection Samples provided by the Sabin Laboratory, Brasilia, DF, Brazil.
**SB – Bacteriology Sector of the Pioneer Union of Social Integration – UPIS, Brasilia, DF, Brazil.
***Bacteria provided by the Laboratory of Bacteriology at the Federal University of Santa Maria – UFSM, Santa Maria, RS, Brazil.

Antibiogram (Kirby - Bauermodified method)

The antibiogram was performed according to the Kirby - Bauer method with modifications. Concentrations between 90 and 278 µg of the protein fraction of *S. cumini seeds* were used for each 30 µl of saline solution (NaCl 0.9%), the maximum amount supported on each diskof absorbent paper (CeforDiagnostica Ltda. E0024).

The bacteria and yeast used were stored in a freezer in a stabilizing substance. They were cultured on 5% sheep blood agar before testing. Young cultures were used to perform the antibiogram.

Controls used:
1) Negative vehicle control: disc inoculated with 30 µL of saline solution (0.9% NaCl);
2) Negative disk control: disk with no sample.

Determination of the Minimum Inhibitory Concentration (MIC) by the Broth Dilution Method

The MIC was determined using the broth macro - dilution method, as recommended by the National Committee for Clinical Laboratories Standards (NCCLS). The Muller - Hinton broth (MHB) was used. Dilutions were performed, obtaining concentrations ranging from 4, 265 to 0.0081µg/mL.

Controls used:
1) Positive control: MHB (no protein fraction; + 0.01 mL of bacterial inoculum);
2) Negative control: MHB (no protein fraction and no bacterial inoculum).

Microorganisms Tested: Due to the small amount of *S. cumini*protein fraction sample, only the microorganisms listed below were tested by this technique:
1) Gram - positivebacteria: *Micrococcus*spp., *S. aureus, B. cereus, E. faecalis*
2) Gram - negativebacteria: *S. enteritidis, P. aeruginosa, B. bronchiseptica*
3) Yeast: *C. albicans*

Preparation of Bacterial Inoculum: The tested isolates were subcultured in 5% sheep blood agar and incubated at 37ºC for 24 hours. Afterward, they were cultured in simple broth at 37ºC for 2 hours. Subsequently, the turbidity of the bacterial inoculum was adjusted to the 0.5 Mac Farland scale.

Sample Preparation: The protein fraction was weighed and diluted in saline solution, keeping the initial concentration of 300mg/mL of powder (4, 265 µL/mL of protein fraction).

Testing: The volume of 0.01 mL of bacterial inoculum was cultured in each tube containing different concentrations of the protein fraction of *S. cumini*seeds. The optical density reading was performed after a 24 - hour incubation at 37ºC. The MIC value was the lowest concentration of protein fraction capable of inhibiting bacterial growth on a blood agar plate.

Statistical Analysis
Data were subjected to analysis of variance (ANOVA), and means were comparedby Duncan's test at 5% of probability. Statistical analyses were performed using the Statistical Analysis System Software (SAS Institute Inc., Cary, North Carolina, USA).

3. Results

Protein Pattern Analysis

1) SDS - PAGE
The analysis of the protein pattern was performed using polyacrylamide gel electrophoresis with SDS and β - mercaptoethanol. The protein fraction of *S. cumini* presented protein bands between 10 and 75 kDa (Fig.4).

2) Quantification of proteins by the Bradford Method
The protein fraction of the *S. cumini* seeds was quantified by the Bradford Method. This sample was read in a spectrophotometer after adding Bradford's reagent, which resulted in 0.0024µg/µL in 1 mg of powder.

"In vitro" Sensitivity Tests

1) Antibiogram (Kirby - Bauer modified method)
After 24 hours of incubation, the plates were read, verifying the existence or not of an inhibition halo with the protein fraction samples of *S. cumini* seeds (Table 4). Among all bacteria tested, only *S. equi*subsp. *equi*, *S. suis* type 2, and *E. coli* did not show an inhibition halo.

Table 4: Results of the antibiogram performed with the protein fraction. List of bacteria tested with the respective inhibition halos referring to the different samples used

Microorganisms	Samples Used Related to the Harvesting Periods					
	1st	2nd	3rd	4th	5th	6th
Concentrations Used	**90**µg	**130**µg	**190**µg	**210**µg	**278**µg	**270**µg
Gram - negative bacteria						
B. bronchiseptica (Fig.5C)	15mm	NT	18mm	12mm	13mm	17mm
K. pneumoniae (Fig.5E)	-	-	8mm	9mm	-	-
P. mirabilis (Fig.5G)	-	NT	8mm	12mm	-	10mm
P. aeruginosa (Fig.5K)	-	NT	-	11mm	-	8mm
P. aeruginosa (contaminated wound)	NT	NT	-	12mm	-	8mm
E. coli	NT	-	-	-	-	NT
S. enteritidis (Fig.5M)	8mm	-	-	10mm	-	8mm
Gram - positive bacteria						
B. cereus (Fig.5H)	10mm	NT	NT	13mm	11mm	18mm
C. pseudotuberculosis (Fig.5D)	NT	40mm	35mm	17mm	NT	32mm
E. faecalis	NT	9mm	9mm	-	-	NT
Micrococcus spp. (Fig.5F)	NT	20mm	19mm	19mm	20mm	28mm
Nocardia spp.	NT	20mm	-	-	-	NT
R. equi (Fig.5B)	14mm	22mm	15mm	21mm	14mm	18mm
S. aureus (Fig.5A)	10mm	NT	NT	13mm	11mm	18mm
S. equi subsp. *equi*	NT	40mm	35mm	17mm	NT	32mm
S. suis type II	NT	9mm	9mm	-	-	NT
Yeast						
C. albicans (Fig.5L)	NT	15mm	16mm	17mm	12mm	16mm
EFFECTIVENESS (%)	**62.2**	**58.3**	**56.25**	**75**	**40**	**91.66**

(NT) bacteria not tested; (-) negative.

2) Minimum Inhibitory Concentration (MIC)
Proof of microorganism growth in each tested dilution of the protein fraction was performed by culturing the microorganisms in 5% sheep blood agar. Turbidity could not be used for this purpose, as the sample of the protein fraction of *S. cumini* seeds has an intensepigment (Fig.6). The results of the concentrations are described in Table 5.

Table 5: Minimum inhibitory concentration of the protein fraction of *Syzygiumcumini* seeds of the different microorganisms tested.

Microorganisms	MIC (µg/mL)
Candida albicans	2.07
Bordetella bronchiseptica	133
Micrococcus spp.	530
Enterococcus faecalis	530
Staphylococcus aureus	2, 140
Bacillus cereus	4, 267
Pseudomonas aeruginosa	4, 267
Salmonella enteritidis	4, 267

Statistical Analysis
According to the analysis of variance, all groups showed a statistical difference ($P<0.01$). Table 1 compared the results among the Gram - negative bacteria; Table 2 compared the results among the Gram - positive bacteria; Table 3 compared the results among the Gram - negative bacteria, Gram - positive bacteria, and yeast; and Table 6 compared the difference among the MIC of the tested microorganisms. Once the statistical difference was verified by the analysis of variance (ANOVA), Duncan's mean test at 5% was performed to determine the differences among the results obtained.

The bacterium *B. bronchiseptica* differed from the other Gram - negative bacteria (Table 6) and was the most sensitive to their FPV. Among the Gram - positive bacteria, *C. pseudotuberculosis* showed a significant difference in relation to the others (Table 7). Comparing Gram - positive bacteria, Gram - negative bacteria, and yeast, the protein

fraction was remarkably more effective against yeasts and Gram - positive bacteria (Table 8).

Also, the MIC analysis demonstrated the higher inhibition efficacy of the protein fraction against the yeast *C. albicans* (Table 9).

Table 6: Mean inhibition halos (Gram - negative bacteria) produced by the protein fraction of *Syzygiumcuminis*eeds (Kirby - Bauer modified method).

Gram - negative bacteria	Mean inhibition halos
B. bronchiseptica	15, 000 (a)
P. mirabilis	6, 000 (b)
S. enteritidis	5, 638 (b)
P. aeruginosa	5, 000 (b)
*P. aeruginosa**	3, 800 (b)
K. pneumoniae	2, 833 (b)
E. coli	0.0 (b)

*Different letters indicate a significant difference (Duncan's test, P<0.05).

Table 7: Mean inhibition halos (Gram - positive bacteria) produced by the protein fraction of *Syzygiumcumini* seeds (Kirby - Bauer modified method).

Gram - positive bacteria	Mean inhibition halos
C. pseudotuberculosis	31, 000 (a)
Micrococcus spp.	21, 200 (b)
R. equi	17, 333 (b)
S. aureus	13, 167 (b, c)
B. cereus	13, 000 (b, c)
Nocardia spp.	5, 000 (c, d)
E. faecalis	4, 500 (d)
S. equi subsp. *equi*	0.0 (d)
*S. suis*type 2	0.0 (d)

*Different letters indicate a significant difference (Duncan's test, P<0.05).

Table 8: Mean inhibition halos (Gram - positive bacteria x Gram - negative bacteria x Yeast) produced by the protein fraction of *Syzygiumcumini* seeds (Kirby - Bauer modified method).

Microorganisms	Mean inhibition halos
Yeast	15, 200 (a)
Gram - positive bacteria	12, 897 (a)
Gram - negative bacteria	5, 343 (b)

*Different letters indicate a significant difference (Duncan's test, P<0.05).

Table 9: MIC (Gram - positive x Gram - negative x Yeast).

Microorganisms	Mean MIC
B. cereus	4, 267 (a)
S. enteritidis	4, 267 (a)
P. aeruginosa	4, 267 (a)
S. aureus	2, 140 (b)
Micrococcus spp.	530 (c)
E. faecalis	530 (c)
B. bronchiseptica	133 (d)
C. albicans	2 (e)

*Different letters indicate a significant difference (Duncan's test, P<0.05).

4. Discussion

Several scientific discoveries and technological advances have recently been reported in the Phytotherapy field. Medicinal plants were momently used empirically and intuitively. On the other hand, they also incorporated advances in different research related to proving the safety and efficacy of their use [23].

In this context, studies on *S. cumini* haveshown its different activities in distinct formulations. Leaves, flowers, fruit peels, and fruit mucilage are used. They are administered as crude extracts, such as ethanolic, alcoholic, hydroalcoholic, and aqueous extract. Such extracts are tested against a series of microorganisms of human and veterinary interest using various techniques, such as disk or well diffusion assay and minimal inhibitory concentration tests.

The results obtained in this work are promising since the growth of most tested microorganisms was inhibited. Various studies corroborate these findings. Louguercioet al. [16] stated that the 10% hydroalcoholic extract of *S. cumini*, leaves presented antibacterial and antifungal activity against 17 isolates, with no differences in sensitivity between Gram - positive and Gram - negative bacteria being observed. Oliveira et al. [8] confirmed the antimicrobial activity of the hydroalcoholic extract of this plant, reporting inhibitory effects against *C. albicans*, *C. krusei*, *E. faecalis*, *E. coli*, *Kocuriarhizophila*, *Neisseria gonorrhoeae*, *P. aeruginosa*, *Shigella flexneri*, *S. aureus*, and *K. pneumoniae*.

Nascimento et al. [24] evaluated the MIC of the ethanol extract of *S. cumini* leaves and obtained values of 50.0 µg/mL for a sample of *P. aeruginosa* and 300.0 µg/mL for *S. aureus.*

Chandrasekaran and Venkatesalu [25] studied the antifungal and antibacterial action of the aqueous and methanolic bark extract of *S. cumini*. They found that the methanolic extract had superior performance than the aqueous one. According to the authors, the methanolic extract was more effective against *Candida albicans*, *Aspergillus flavus*, *Aspergillus niger*, *Bacillus subtilis*, and *Staphylococcus aureus*. However, the growth of all other microorganisms tested, such as *Salmonella typhymurium*, *P. aeruginosa*, *Trichophytum mentagrophytes*, and*Microsporumgypseum* was also inhibited.

The present work corroborates the data already reported in the literature, but this study used the protein fraction extracted from the seeds of this plant, characterizing a refined solution compared to the other substances mentioned above. Nevertheless, it is important to highlight that the results were similar against bacteria and fungi regardless of the plant part used.

Furthermore, the protein fraction used showed an inhibitory action by the modified Kirby - Bauer technique against *E. faecalis*, *S. aureus* (Fig.5A), *Micrococcus* spp. (Fig.5F), *B. cereus* (Fig.5H), *Nocardia* spp., *C. pseudotuberculosis* (Fig.5C), *P. aeruginosa* (Fig.5K), *S. enteritidis*, *P. mirabilis* (Fig.5G), *K. pneumoniae* (Fig.5E) and *B. bronchiseptica* (Fig.5C), as well as against the yeast *C. albicans* (Fig.5L).

The existence of a capsule on *S. equi*subsp. *equi, S. suis*type 2, and *E. coli* could explain the lack of growth inhibition of these species by the protein fraction. Unfortunately, it is not possible to confirm the capsule presence, not even that the protein fraction does not have an inhibitory effect on these microorganisms, as additional tests, such as MIC, were not performed, and other concentrations were not tested. However, other microorganisms showed 100% inhibition in tests performed according to the modified Kirby - Bauer method, such as *B. cereus, C. pseudotuberculosis, Micrococcus* spp., *R. equi, S. aureus*, and *B. bronchiseptica*. Except for *B. bronchiseptica*, all other bacteria are Gram - positive. The protein fraction showed the best results against Gram - positive bacteria (Table 8).

For the percentage of inhibited bacteria in each harvesting, the highest value was found in the sixth harvesting (91.66%), followed by the fourth (75%), first (62.2%), second (58.3%), third (56.25%), and fifth (40%). The highest value was recorded in the August harvest, and the lowest value was observed in the March harvest. Both months correspond to the beginning and end, respectively, of the *S. cumini* harvest season. Environmental differences are directly related to the presence or absence of certain active principles, reaffirming the concept of environmental variation. Given the different harvesting dates, the material used may suffer such variation as several proteins in this fraction will not be synthesized at certain times due to adverse conditions. According to the climate data observed (Table 2), the different variations plants are subjected to cannot be individually considered responsible for the observed antimicrobial effects. However, seeds collected at the beginning of the harvest and before the heavy rain season were generally more active than seeds collected in different periods. It is believed that plants under stress can produce various defense compounds against various pests and diseases. Thus, defense proteins and peptides may be produced in greater diversity or quantity in this period when there is a water deficit.

The results obtained by the MHB dilution method demonstrated inhibitory activity against all microorganisms tested, with concentrations ranging between 2.7 and 4, 267 µg/mL of protein fraction, being the lowest and highest concentration, respectively. Only *C. albicans, P. aeruginosa, S. enteritidis, B. cereus, Micrococcus* spp., *S. aureus, E. faecalis*, and *Bordetella bronchiseptica*were tested due to the small amount available of the protein fraction sample, prioritizing the microorganisms that most affect animals.

The variation of the protein fraction activity (concentration used) against these microorganisms seems to derive from the cell membrane constitution and different activities of the proteins present in the fraction used, mainly from peptides. Plant defensins are potentially antifungal, thus demonstrating greater activity against fungi when compared to their antibacterial action. This fact becomes evident when observing the MIC results, in which the *C. albicans* growth was inhibited with a lower concentration compared to other concentrations necessary to inhibit bacteria (Table 9).

In its turn, no differences were detected between Gram - positive bacteria and the tested yeast (Table 8) regarding the mean inhibition halos produced by the protein fraction. The protein fraction inhibited the growth of *B. bronchiseptica* with a concentration of 133 µg/mL, the second - lowest MIC among the microorganisms tested. This result is relevant since *B. bronchiseptica* is a Gram - negative bacterium. Therefore, the protein fraction needs to first interact with the cell wall and, later, with the cytoplasmic membrane. In addition, this bacterium had its growth inhibited in all tests of the modified Kirby - Bauer antibiogram. These results suggest a particular sensitivity of *B. bronchiseptica* to this protein fraction.

The Gram - positive bacteria tested were inhibited with concentrations ranging between 530 and 4, 267 µg/mL. This latter was the same concentration needed to inhibit the growth of *P. aeruginosa* and *S. enteritidis*, both Gram - negative bacteria. According to the statistical analysis, the bacteria *B. cereus, S. enteritidis, and P. aeruginosa* were classified in the same group and inhibited with the same concentration.

This study used a protein fraction containing different proteins with distinct binding sites. According to results observed in the SDS - PAGE gel, the fraction has different proteins with molecular mass between 75 kDa and 15 kDa, in addition to a major band with peptides below 10 kDa that may play an antimicrobial role. As Simone and Souza [14] stated, the determining factors of the microbicidal mechanisms of these peptides are related to the cell membranes characteristics of microbial cells and the physicochemical nature of the peptide. The activity and selectivity of cationic peptides are determined by the way they interact with the cell membranes of microorganisms. Most have relatively high cationic charge and hydrophobicity at physiological pH, which facilitates bidding and insertion into the membrane of microbial cells.

It is assumed that the mechanism of action of microbial peptides in Gram - positive bacteria will occur with the cytoplasmic membrane. In Gram - negative bacteria, the interaction occurs with the outer and the cytoplasmic membranes [14]. The peptides possibly interact with the lipopolysaccharide (LPS) found in the outer membrane of Gram - negative bacteria, quickly creating a transport pathway for the peptide itself [14].

In contrast, the mechanism of action in fungal cells follows the Shai - Matsuzaki - Huang model, demonstrating that defensins cover the membrane as a mat, causing destabilization with the displacement of lipids and thinning its thickness. Therefore, cell death occurs either by breaking the cell wall integrity or by diffusion of peptides into the cell's cytoplasm [7].

According to Oliveira et al. [26], the protein fraction of plant seeds may contain proteins such as lectins, which have intense hemolytic activity. The authors describe the hemolytic activities present in the protein fraction of Ingá seeds (*Inga edulis*), which produced a halo of hemolysis when cultured on a 5% sheep blood agar culture medium, ensuring this protein in that fraction. In the present study, the absence or even the small amount of such peptides was

verified since no noticeable hemolytic effect was observed in any test performed on a 5% sheep blood agar medium.

The fact that protein fraction has shown an inhibitory effect against Gram - positive and Gram - negative bacteria, as well as against yeast, justifies the mechanisms of action mentioned above.

5. Conclusions

The molecular mass of the protein fraction of *S. cumini* showed bands ranging from 10 to 75 KDa, demonstrating significant variation between the sizes of the protein bands. The given fraction proved to be effective against most of the microorganisms tested, having an expressive intensity of action when tested "in vitro". According to statistical analysis, protein fraction was more effective against the yeast and Gram - positive bacteria tested compared to the Gram - negative bacteria group. This sample did not show hemolytic potential when cultured on a 5% sheep blood agar culture medium. Therefore, there is a slight possibility of existing hemolytic lectins in the *S. cumini*protein fraction. Since the protein fraction of *S. cumini*seeds contains different proteins, the different activities found may be due to one or more bioactive molecules or even a synergistic effect among them. Based on the results obtained in this work, other pharmacological, toxicological, chemical, and clinical studies should be carried out with the protein fraction of *S. cumini*seeds, as this plant has demonstrated a considerable antimicrobial effect.

References

[1] Lorenzi H, Matos FJA. Medicinal Plants inBrazil: nativeandexotic [Plantas Medicinais no Brasil: nativas e exóticas].2nd Ed. Nova Odessa; Plantarum Press; 2008. p.11 - 25.

[2] David JPL, Nascimento JAP, David JM. PhytotherapeuticProducts: a business perspective for theindustry, a fieldlittleexploredbypharmacists [Produtos Fitoterápicos: uma perspectiva de negócio para a indústria, um campo pouco explorado pelos farmacêuticos]. Infarma.2004; 16 (9 - 10): 71 - 76.

[3] Perez E. Phytochemicaldiagnosisoffruitsof*Caryocar brasiliense*sCAMB., Caryocaraceae [Diagnose Fitoquímica dos Frutos de *Caryocar brasilienses* CAMB., Caryocaraceae]. Master Thesis. Parana, Brazil; Federal University of Parana; 2004.

[4] Colalto C. What phytotherapy needs: Evidence - based guidelines for better clinical practice. Phytother. Res.2018; 32 (3): 413 - 425.

[5] Srivastava S, Dashora K, Ameta KL, Singh NP, El - Enshasy HA, Pagano MC, Hesham AE, Sharma GD, Sharma M, Bhargava A. Cysteine - rich antimicrobial peptides from plants: The future of antimicrobial therapy. Phytother. Res.2021; 35 (1): 256 - 277.

[6] Sartori MRK. AntimicrobialActivityof Pure ExtractsandCompoundsObtainedfromAcmelia Brasiliensis SPRING Flowers (*Wedelia paludosa*) (Asteraceae) [Atividade Antimicrobiana de Extratos e Compostos Puros Obtidos das Flores de *Acmelia Brasiliensis* Spring (*Wedelia paludosa*) (Asteraceae)]. Master Thesis. Itajai, Brazil: University of Vale do Itajaí; 2005.

[7] Lobo DS. Analysisoftheproteininteractionof Psd 1 defensinfromPisumsativumwithproteinsfromthefungus *Neurospora crassa* [Análise da interação protéica da defensina*Ps*d 1 de *Pisumsativum*com proteínas do fungo *Neurospora crassa*]. PhD Dissertation. Rio de Janeiro, Brazil; Federal Universityof Rio de Janeiro; 2006.

[8] Oliveira GF. Evaluation of the *in vitro* Antimicrobial Activity of the Crude Hydroalcoholic Extract of *Syzygiumcumini* (L.) SkeelsLeaves [Avaliação da Atividade Antimicrobiana, *in vitro*, do Extrato Hidroalcóolico Bruto das Folhas de *Syzygiumcumini* (L.) Skeels (Jambolão)]. Master Thesis. Franca, Brazil; University of Franca; 2005.94p.

[9] Nawrot R, Barylski J, Nowicki G, Broniarczyk J, Buchwald W, Goździcka - Józefiak A. Plant antimicrobial peptides. Folia Microbiol. (Praha).2014; 59 (3): 181 - 96.

[10] ParkIY, ChoJH, KimKS, KimYB, KimMS, KimSC. Stability helix confers salt resistance on helical antimicrobial peptides. J. Biolog. Chem.2004; 279 (14): 13896 - 13901.

[11] Salas CE, Badillo - Corona JA, Ramírez - Sotelo G, Oliver - Salvador C. Biologically active and antimicrobial peptides from plants. Biomed. Res. Int.2015; 2015: 102129.

[12] Tang SS, Prodhan ZH, Biswas SK, Le CF, Sekaran SD. Antimicrobial peptides from different plant sources: Isolation, characterization, and purification. Phytochemistry.2018; 154: 94 - 105.

[13] Hancock REW, Chapple DS. Minireview: Peptides Antibiotics. Antimicrob. Agents Chemother.1999; 43 (6): 1317 - 1323.

[14] Simone SG, Souza ALA. PeptidesMicrobicides [Peptídeos Microbicidas]. Biotecnol. Cienc. Desenvol.2002; 24: 12 - 16.

[15] Pepato MT, Mori DM, Baviera AM, Harami JB, Vendramini RC, Brunetti IL. Fruit of the jambolan tree (*Eugenia jambolana* Lam.) and experimental diabetes. J. Ethnopharmacol.2005; 96 (1 - 2): 43 - 48.

[16] LouguercioAP, Battistin A, Vargas AC, Henzel A, Witt NM. Antibacterial activity of hydroalcoholic extract leaves of jambolan (Syzygiumcumini (L.) Skells) [Atividade antibacteriana de extrato hidro - alcoólico de folhas de jambolão (*Syzygiumcumini* (L.) Skells)]. Cienc. Rural.2005; 35 (2): 371 - 376.

[17] Ayyanar M, Subash - Babu P. Syzygiumcumini (L.) Skeels: a review of its phytochemical constituents and traditional uses. Asian Pac. J. Trop. Biomed.2012; 2 (3): 240 - 246.

[18] Ahmed R, Tariq M, Hussain M, Andleeb A, Masoud MS, Ali I, Mraiche F, Hasan A. Phenolic contents - based assessment of therapeutic potential of *Syzygiumcumini* leaves extract. PLoS One.2019; 14 (8): e0221318.

[19] Valle ACV. Antimicrobial activity of proteins from jambolan (*Syzygiumcumini* (l.) Skeels) seeds. Master Thesis. Brasilia, Brazil; União Pioneira de Integração Social, 2008.

[20] Santos CA, Almeida FA, Quecán BXV, Pereira PAP, Gandra KMB, Cunha LR, Pinto UM. Bioactive

Properties of *Syzygiumcumini* (L.) Skeels Pulp and Seed Phenolic Extracts. Front Microbiol.2020; 11: 990.
[21] Kumar A, Padmanabhan N, Krishnan MRV. Central Nervous System Activity of *Syzygiumcumini* (L.) Skeels Seed. Pak. J Nutr.2007; 6 (6): 698 - 700.
[22] Sagrawat H, Mann AS, Kharya MD. Pharmacological potential of *Eugenia jambolana*: A review. Pharmacogn. Mag.2006; 2 (6): 96 - 105.
[23] Leite JPV. Phytotherapy: ScientificandTechnological Bases [Fitoterapia: Bases Científicas e Tecnológicas].1st ed. Sao Paulo, Brazil; Atheneu Press; 2008, p.3 - 20.
[24] Nascimento GGF, Locatelli J, Freitas PC, Silva GL. Antibacterial activity of plant extracts and phytochemicals on antibiotic - resistant bacteria. Braz. JMicrobiol.2000; 31 (2): 247 - 256.
[25] Chandrasekaran M, Venkatesalu V. Antibacterial and antifungal activity of *Syzygiumjambolanum* seeds. J. Ethnopharmacol.2004; 91 (1): 105 - 108.
[26] Oliveira LDR et al. HemolyticEffectofIngaSeedProteins (Ingaedulis) [Efeito Hemolítico de Proteínas de Sementes de Ingá (*Ingaedulis*)]. In: Anais of X International Conference ofEthnopharmacologyand XX Simposium of Brazilian Medicinal Plants. Sao Paulo, Brazil; 2008.

Figure 1: A) *Syzygiumcumini*tree (Brasilia Zoo, Brasilia, DF, Brazil). B) *Syzygiumcumini*flowers (Brasilia Zoo, Brasilia, DF, Brazil). C) *Syzygiumcumini*fruits (Brasilia Zoo, Brasilia, DF, Brazil).

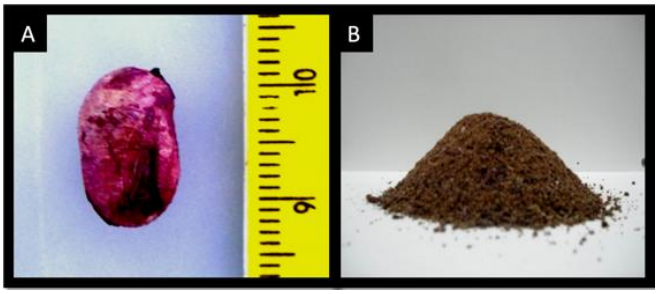

Figure 2: Seed (A) and flour from the seed (B) of *Syzygiumcumini.*

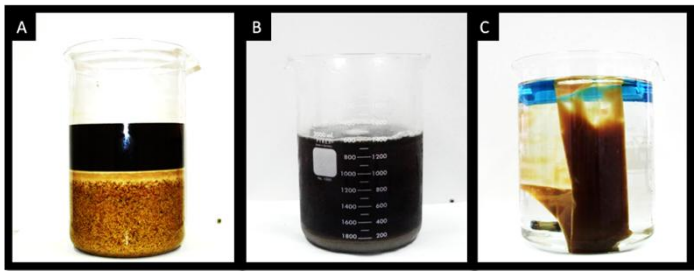

Figure 3: A) Delipidation of the *Syzygiumcumini*seed flour in acetone. B) Correction of the pH solution to 7.0. Precipitation of unstable proteins at this pH. C) Dialysis of the protein fraction of *S. cumini*seeds against distilled water after precipitation with ammonium sulfate in the range of 0/70% saturation.

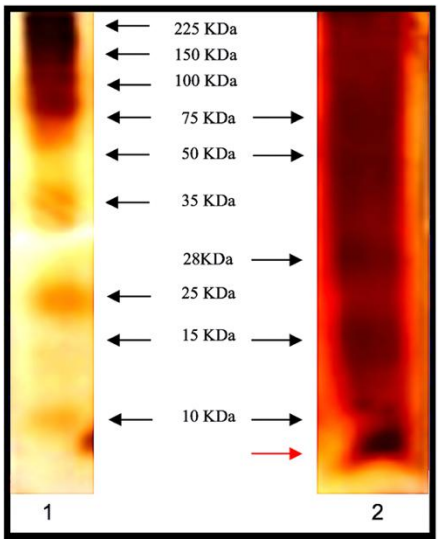

Figure 4: SDS - PAGE. Samples: 1 – Molecular marker; 2 – Protein fraction of *Syzygiumcuminii*seeds. Black arrows indicate protein bands with their respective sizes on the side. Red arrows indicate peptides likely to be smaller than 10 kDa.

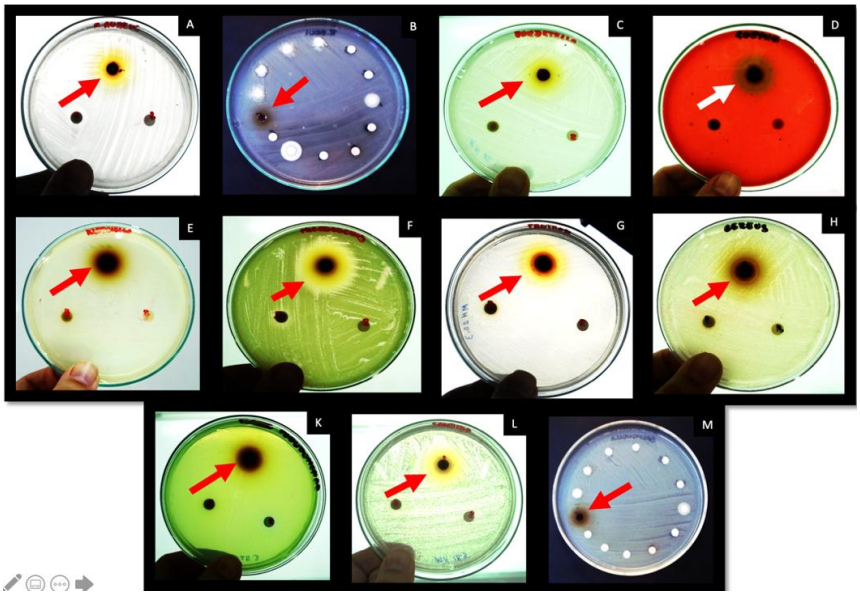

Figure 5: Inhibition halos formed by the protein fraction of *Syzygiumcuminii*seeds indicated by the red arrow. A) *Staphylococcus aureus* (14mm halo); B) *Rhodococcusequi* (22mm halo); C) *Bordetella bronchiseptica* (18mm halo); H) *Bacillus cereus* (13mm halo). D) *Corynebacteriumpseudotuberculosis* (17mm halo); E) *Klebsiellapneumoniae* (9mm halo); F) *Micrococcus* spp. (20mm halo); G) *Proteusmirabillis* (12mm halo). H) *Bacillus cereus* (18mm halo). K) *Pseudomonas aeruginosa (*12mm halo); L) *Candidaalbicans* (17mm halo); M) *Salmonella enteritidis* (10mm halo).

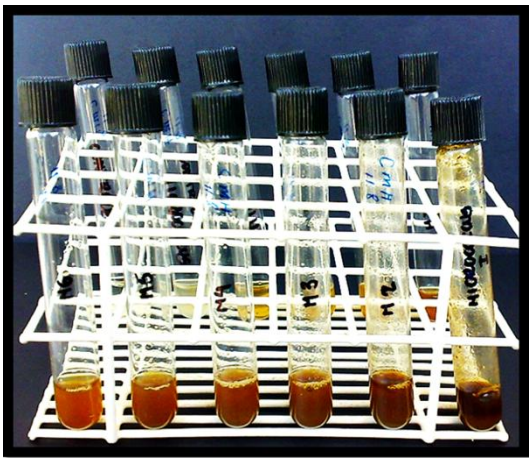

Figure 6: Tubes used for the MIC test. Visualization of the intense pigment of the protein fraction of *Syzygiumcumini* seeds.