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RESEARCH ARTICLE

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Susceptibility of Enterococcus Faecalis to the Antibacterial Effect of Various Metabolites of the Mammea Americana Seed

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Abstract

Background: Enterococcus faecalis has been identified as one of the main microorganisms that cause oral infections, especially those of endodontic origin, due to its broad multi-drug-resistant spectrum. Some studies have demonstrated antibacterial activity of Mammea Americana, most of them have focused on extracts obtained from leaves and bark but few indicate an antibacterial activity of plant seeds. **Objective:** To evaluate antibacterial activity of different metabolites present in Mammea Americana seed extracts against Enterococcus faecalis ATCC (51299). **Materials and methods:** previously crushed and cold macerated dry plant material was used, employing ethanol as solvent, which was concentrated under reduced pressure and of which four (4) additional organic extracts with solvents of different polarities were obtained. Extracts were solubilized in DMSO and antibacterial activity was measured using the 96-well plates broth microdilution technique in a concentration range of 50 to 500 mg/L, using kanamycin as a positive control and DMSO as a negative control. **Results:** All evaluated extracts showed inhibitory activity against Enterococcus faecalis. Hexanoic (BMI 100 mg/L) and chloroformic extracts (BMI 200 mg/L) showed higher inhibitory activity. **Conclusions:** Obtained extracts revealed a variety of secondary metabolites, principally coumarins. All extracts showed inhibitory activity. Hexanoic extracts (BMI 100 mg/L) and chloroformic extracts (BMI 200mg/L) stood out as extracts with the highest activity.

Keywords: Enterococcus faecalis, Mammea, Antibacterial agent, Sodium hypochlorite.

1 | INTRODUCTION

Numerous species of bacteria have been isolated from the oral cavity, most of them are inoffensive. Despite this, oral microorganisms are responsible for two common diseases in humans: dental caries and periodontal disease (1) . Endodontic treatment provides possibilities of preserving teeth in a patient´s mouth, overcoming disease prevalence. However, failure is a conceivable possibility and it has been usually related, among others, to microorganism's presence in root canals. Within many existing bacterial species, one of the most frequently found in teeth with necrotic pulp (without a previous history of endodontic treatment) and highly isolated from those with infection recurrence (indication of endodontic retreatment) is Enterococcus faecalis (E. faecalis). These are gram-positive cocci, facultative anaerobes, extremely resistant to conventional chemical-mechanical procedures (2). Such resistant has prompted exploration for new bacterial control alternatives that allow decontamination or elimination of said pathogen.

Using plants for healing purposes dates back many years and it is defined by the native flora of each territory. Today, curative principles of plants are subject to multiple scientific research in which some medicinal actions have been experimentally demonstrated. This provides an opportunity to find new active agents, from a renewable source (3).

Mammea Americana is a native tree from the Antilles and northern South America (4) it belongs to Clusiaceae (Guttiferae) family (5). Its fruit is edible and frequently consumed, it has an aromatic, yellow-reddish pulp, a slightly irregular or round shape, with a thick, brown skin. Its diameter ranges from 10 to 20 cm. It contains a single seed, largest ones can have up to four. Seeds are brown, harsh, oval-shaped and approximately 6 cm long (6).

Previously referred plant is widely used as a cure for some diseases due to its anti-parasitic and insecticidal effect. In this sense, seed latex is known to be used to eliminate Aedes aegypti larvae (7). Fruit effect to treat anemia has been also confirmed. On the other hand, resin and decoction of the cortex are usually employed against parasites, to treat fungal infections and eczema (8).

Extracts from certain plants have shown to have an antibacterial effect on Enterococcus faecalis and the studied plant in this investigation; Mammea Americana is one of them.

The main objective of this study was to evaluate antibacterial activity of different metabolites obtained from seeds extracts of Mammea Americana against Enterococcus faecalis ATCC (51299) strain, which in turn, allows preparing field for further research using other plants and bacteria that actively participate in endodontic pathologies development.

2 | METHODS

An in vitro experimental study was conducted.

ORGANIC MATERIAL

Mammea Americana fruit was obtained from rural areas of Atlántico Department, located on Colombian Caribbean coast. Specimens were prepared and identified in Antioquia University Herbarium (HUA 183928).

PREPARATION OF EXTRACTS

Seeds were extracted from ripe fruit and air-dried at 25 °C for 3 weeks, husks were removed and seed kernels were crushed using cutting instruments. 500 grams of crushed material were macerated using ethanol (50% w/v) as a solvent at room temperature for 72 hours under dark conditions. After filtering, ethanolic extract was concentrated in a rotary evaporator (Laborota 4001, Heidolph) under reduced pressure at 50 °C to obtain totality of the extract (86.12 g), from which 10 mg were used for biological activity. Remaining extract was mixed with alumina (Merck) in a proportion of 1/8 extract/alumina, using a mortar

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until a dry powder was obtained. Subsequently, it was allowed to dry in an incubator (Binder) at 45 °C for 24 hours in order to evaporate the remaining solvent. Previously referred alumina extract, gave rise to organic extracts of different polarities, each solvent flowed through the open chromatography column in increasing order of polarity. Solvents were hexane, dichloromethane, ethyl acetate and analytical grade methanol (9).

PRELIMINARY PHYTOCHEMICAL SCREENING OF M. AMERICANA EXTRACTS

Phytochemical screening of plant extracts was carried out through different procedures to detect and identify the following groups of secondary metabolites: alkaloids (Dragendroff's reagent test), tannins (ferric chloride test), coumarins (Borntrager's test), flavonoids (Shinoda's test and Citroboric reagent), triterpenes (Salkowski reaction), saponins (vanillin-sulfuric acid reagent), and cardiotonic glycosides (Kedde's reaction, Raymond-Marthoud, and Keller-Kiliani reagents) (9) .

PREPARATION OF SOLUTIONS OF M. AMERICANA EXTRACTS

Ten milligrams of extracts were weighed and dissolved in 1 mL of 99% Dimethyl sulfoxide (DMSO) until a stock solution of 10,000 mg/L was obtained. From this solution, intermediate dilutions were made until reaching concentrations interval to be evaluated (500-400-300-200-100-50 mg/L).

MICROORGANISM AND GROWTH CONDITIONS

Antimicrobial activity was evaluated using Gram-positive Enterococcus faecalis (ATCC-51299) bacterium, acquired from the American Type Culture Collection. Bacterial strains were kept in tubes that contained Mueller-Hinton agar (Merk) (inclined at 4 °C) before reactivation. Bacterial cultures were prepared in a tube along with Mueller-Hinton broth (Merck) and incubated at 37 °C in an incubator (Gemmy, LAB incubator model IN-010) to perform antibacterial activity test. The inoculum was done after 24 hours of incubation and optical density was adjusted to 0.5 (McFarland scale) using the Multiskans ascent spectrophotometer (Thermo Scientific, Germany) at a wavelength of 620 nm, which is equivalent to 1x108 CFU mL

ANTIBACTERIAL ACTIVITY TEST

Antimicrobial activity was determined by the 96-well plate microdilution method. With such technique, values of minimum inhibitory concentration against studied microorganisms were obtained.

DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC)

100 μL of microbial suspension, as well as 100 μL of extracts dilutions in Mueller-Hinton broth, were transferred to the microplates. Kanamycin was used as a positive control, and DMSO as a negative control, for both, effective concentration and MIC were determined. 200 μL aliquots were incubated for 24 hours at 37 °C. MIC was evaluated with the help of Multiskans ascent spectrophotometer (Thermo Scientific, Germany) at a wavelength of 620 nm at 0 and 24 hours and was defined as the lowest concentration that inhibited microbial growth.

Test was carried out twice, in two different days and it was performed in triplicate for each concentration.

STATISTICAL ANALYSIS

Results are presented as mean and standard error of mean (SEM). Statistical significance level was determined to carry out a one-way analysis of variance (ANOVA), using GraphPad Prism software 6.0. Differences between mean obtained for each concentration were evaluated by analysis of variance (ANOVA) and Tukey's test as Post-Test, employing it to make comparisons with control group as well. For all cases, p-values <0.05 are considered significant.

3 | RESULTS

ANTIBACTERIAL ACTIVITY

In order to determinate antibacterial activity of extracts, inhibitory percentages of each concentration were obtained, results are exposed in Table 1. Table 2 shows extracts with highest antibacterial activity (highest inhibitory percentage at lowest concentration). Table 3 shows minimum inhibitory concentrations of M. Americana seeds extracts, obtained from the minimum concentrations resulting from antibacterial activity tests. Additionally, average inhibitory

percentages for each extract are presented along with their standard deviation.

A comparison of inhibitory percentages for each extracts with positive (Kanamycin) and negative (DMSO) controls are displayed in Figure 1.

Table 1. Inhibitory percentages for concentrations of each extract.

Ethanolic extract				
Concentration (mg/L)	Inhibitory percentages (%)			Standard deviation
	Test 1	Test 2	Mean	
50	28,64	28,14	28,39	0,36
100	54,86	53,85	54,36	0,71
200	51,93	50,34	51,13	1,12
300	48,49	48,07	48,28	0,30
400	52,01	50,67	51,34	0,95
500	59,97	61,98	60,97	1,42
Hexanoic extract				
Concentration	Inhibitory percentages (%)			Standard deviation
	Test 1	Test 2	Mean	
50	30,07	29,31	29,69	0,53
100	56,95	54,94	55,95	1,42
200	55,70	56,03	55,86	0,24
300	54,52	52,76	53,64	1,24
400	54,10	55,19	54,65	0,77
500	73,87	73,20	73,53	0,47
Chloroformic extract				
Concentration	Inhibitory percentages (%)			Standard deviation
	Test 1	Test 2	Mean	
50	25,63	25,71	25,67	0,06
100	61,64	62,14	61,89	0,36
200	71,78	75,80	73,79	2,84
300	65,16	68,51	66,83	2,37
400	61,89	62,73	62,31	0,59
500	68,09	65,08	66,58	2,13
Ethyl acetate extract				
Concentration	Inhibitory percentages (%)			Standard deviation
	Test 1	Test 2	Mean	
50	39,03	36,60	37,81	1,72
100	37,69	39,87	38,78	1,54
200	45,98	47,32	46,65	0,95
300	48,74	49,16	48,95	0,30
400	39,78	42,80	41,29	2,13
500	55,03	52,76	53,89	1,60
Methanolic extract				
Concentration	Inhibitory percentages (%)			Standard deviation
	Test 1	Test 2	Mean	
50	30,40	26,30	28,35	2,90
100	43,30	44,56	43,93	0,89
200	40,12	39,61	39,87	0,36
300	39,11	37,69	38,40	1,01
400	36,26	35,51	35,89	0,53
500	41,21	40,03	40,62	0,83

Table 2. Minimum inhibitory concentrations of M. Americana seeds extracts with the highest antibacterial activity.

Extract type	Minimum inhibitory concentration (mg/L)	Average inhibitory percentage
Ethanolic	**100**	54,36±0,71
Hexanoic	**100**	55,95±1,42
Chloroformic	**200**	73,79±2,84
Ethyl acetate	**300**	48,95±0,30
Methanolic	**100**	43,93±0,89

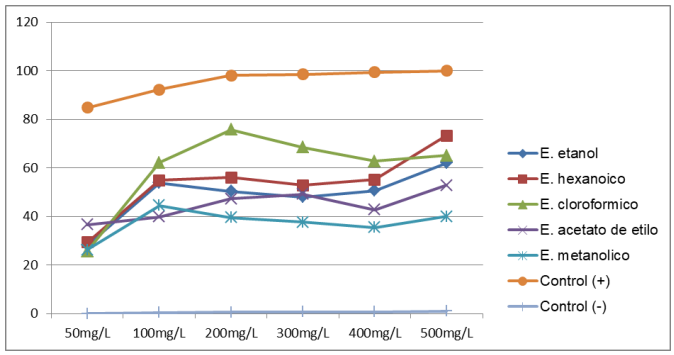

FIGURE 1: Inhibition of M. Americana seed extracts againstE. faecalis.

PRELIMINARY PHYTOCHEMICAL SCREENING OF M. AMERICANA SEEDS

Through preliminary phytochemical screening, presence of metabolites in total extract and in extracts of different polarities were identified. Results of Phytochemical Screening are shown in Table 3.

Table 3. Preliminary phytochemical screening of Mammea Americana species.

Plant name	*M. Americana*	EtOH	Hex	$CHCl_3$	AcOEt	MeOH
Metabolite	**Test**	**TLC**	**TLC**	**TLC**	**TLC**	**TLC**
Alkaloids	Dragendorff	+	+	+	-	-
Cardiotonic glycosides	Ballet	-	-	-	-	-
	Raymond-Marthoud	-	-	-	-	-
	Antimony trichloride	-	-	-	-	-
volatile coumarins	NaOH	+	+	+	-	-
	KOH	+	+	+	-	-
Flavonoids	Citroboric reagent	+	+	+		
Tannins	Ferric chloride test	+	-	-	+	+
Saponins	Vanillin-sulfuric acid reagent	-	-	-	+	+
	Antimony trichloride	-	-	-	+	+
Triterpenes and/or esteroids	Salkowski	+	+	+	-	-

EtOH (Ethanolic extract), He (Hexanoic extract), $CHCl_3$ (Chloroformic extract), AcOEt (Ethyl acetate extract), Me (Methanolic extract).
Presence (**+**); Absence (**-**); Doubtful (**+/-**)

Statistical analysis of antibacterial activity of M. Americana seed extracts versus E. faecalis, exhibited significant differences below 5%, after 24 hours of incubation. These results are shown in Table 1.

4 | DISCUSSION

Regarding antibacterial activity of M. Americana seed extracts, ethanol extract or any of its other fractions against Enterococcus faecalis no reports were found, however, some studies have shown that a main active component, Mammea A/AA, which has been isolated from plant stem bark and seed kernel can inhibit E. faecalis growth (10). Said compound also showed specific inhibitory activity on strains of Streptococcus pneumoniae, Clostridium difficile, and Campylobacter jejuni (11). Ethanolic extract according to scientific evidence showed activity against other bacteria. An ethanolic leaf extract provides inhibitory activity against Mycobacterium tuberculosis (12). Likewise, some xanthones and benzophenones isolated from M. Americana seed oils showed antibacterial activity (13).

Herrera et al found bactericidal and bacteriostatic activity in ethanolic extract against S. mutans, as well as bacteriostatic activity against P. gingivalis, referred extract also had presence of phenolic compounds such as tannin and coumarin (14). Remón et al evidenced inhibitory activity of M. Americana seeds ethanolic extract against Staphylococcus aureus (ATCC 29737); they also verified presence of various metabolites (alkaloids, coumarins, tannins, and flavonoids) that are similar to those found in this study. (3) On the other hand, Manzano et al described that the aforementioned ethanolic extract has an inhibitory effect on gram-negative bacteria (E. Coli, Pseudomonas spp, Salmonella spp) (15).

Phytochemical screening of M. Americana seeds allowed us to determine presence of alkaloids, coumarins, flavonoids, triterpenes and steroids in ethanolic, hexanoic, and chloroformic extracts; likewise, presence of saponins in ethanolic, ethyl acetate and methanolic extracts; and finally, presence of tannins in ethanolic, ethyl acetate and methanolic extracts, this information resembles data obtained and reported by Remón et al, who described presence of alkaloids, coumarins, tannins and flavonoids in staining of M. Americana extracts (3).

Additionally, for plant stem, results are also related to those reported by Manzano et al, who found presence of alkaloids and flavonoids in ethanolic and aqueous extracts, along with tannins in aqueous extracts (polar extract) (15). Finally, screening data coincide with those reported by Beltrán et al., Who informed presence of coumarins, tannins, flavonoids, triterpenes, and steroids in M. Americana seeds ethanol extract (9).

5 | CONCLUSIONS

Concentrates obtained from M. Americana seeds extracts revealed a variety of secondary metabolites, among which coumarins are mostly observed and usually reported as responsible for biological activity.

All Mammea Americana seed extracts that were evaluated in this work showed inhibitory activity against Enterococcus faecalis. Hexanoic (MIC 100mg / L) and chloroformic extracts (MIC 200mg / L) stood out as metabolites with the highest inhibitory activity.

Identified and reported metabolites, as well as their biological effects against different microorganisms, emphasize the utility of this plant for treatment of different conditions, however, it is suggested to carry out deeper studies that may allow using the plant with the purpose of identify compounds that may present antibacterial activity, it is advised as well to issue a wider criterion on compounds effectiveness, as a way to verify their applicability in traditional and natural medicine.

Conflict of interest: none declared

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