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Obtaining salts of resin acids from Cuban pine by metathesis reactions

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ABSTRACT
The resin acids obtained from the Cuban pine rosin are the starting material for the development and application of metathesis reactions. These reactions allow the obtaining of salts with high added value which could be used in the development of biomaterials for dental use. The objective of this work was to obtain calcium, magnesium and zinc resinates from the resin acid obtained from the Cuban pine resin by metathesis reaction for possible use in the development of biomaterials. The products obtained were evaluated by elemental analysis, X-ray powder diffraction, infrared spectroscopy, scanning electron microscopy associated with electron dispersion spectroscopy, nuclear magnetic resonance and biological tests (antibacterial activity). The results showed the formation of the different resinates, observing the presence of cations in the salts obtained. The disappearance of the signal corresponding to the hydrogen of the carboxyl group was verified by nuclear magnetic resonance analysis due to the reaction between the resinic acid and the different metals studied. The biological analyzes showed that the best results are achieved with zinc resinate.
Keywords: *Resin acid; Metathesis reactions; Antibacterial assays; Biomaterials; Resinate salts.*

1. INTRODUCTION

The rosin of the conifers (Pinaceas, Pinus genus) is a renewable natural by-product. It is rich in diterpenic compounds of great interest for the chemical, pharmaceutical and cosmetic industries. It is characterized by being a volatile fraction constituted mainly by monoterpenes and sesquiterpenes. The terpenes, which constitute the non-volatile fraction of these resins, are mainly diterpenic acids with three molecular skeletons of the abietane, pimaran and labdan type, varying quantitatively among the different families of conifers [1-3].

It is the main source of diterpene resin acids such as abietic, levopimaric, palustric, neoabietic and dehydroabietic acid, along with other non-abietane compounds [4, 5]. Abietic acid is the most stable isomer and thus is the major component [6, 7]. In the specific case of the caribbaea species from the western zone of the Cuban archipelago, studies by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) showed that the rosin was mainly composed of a mixture of levopimaric acid (between 18 and 20%), palustric acid (18%), abietic acid (40%) and dehydroabietic acid (22%). Abietic acid is found in a greater proportion within the mixture. [2].

Metallic resinates are the product of the reaction between an organic acid (in this case, resin acid) and a metal ion. In general, they were produced by three different methods: fusion, solution, and the precipitation method [8]. The precipitation methodology occurs in two stages: First the saponification of the resin acid with sodium hydroxide is carried out, forming the sodium resinate, water-soluble carboxylate. Subsequently, the reaction between the sodium resinate and the salt containing the metal ion of interest is carried out. In this process the substitution of the sodium ion by the metal ion occurs, forming the corresponding resinate which is insoluble in aqueous medium. This process is known as metathesis reaction [9, 10]. Previous studies demonstrated the feasibility of obtaining resin acid from rosin from pine (var caribbaea) from the western part of Cuba [2, 11].

The development of biomaterials for use in tissue regeneration and dental implants is of great importance today and its demand increases daily, due to the increase in the aging population, the increase in expectations and quality of life, as well as the increase in accident rates (traffic and violence). The development of 3D printing has accelerated the search for new biomaterials. Guaranteeing materials that contribute to new properties is one of the lines of work of many research groups in the field of biomaterials. In this work, the main objective was to obtain resin salts of resin acids by metathesis reaction for possible use in the development of biomaterials.

2. MATERIALS AND METHODS

Pine resin was collected in the Forest Station Viñales, Pinar del Río, Cuba. It was stored at room temperature (30 ± 2 °C) and protected from sunlight until use. The quality control of the pine resin was carried out and subjected to a steam distillation process [11, 12].

2.1. Obtaining resin acids from rosin.

25% sodium hydroxide solution was prepared and placed on heating plate subjected to a heating-stirring process (temperatures between 80-85 °C at 500 rpm). Little by little the mass of rosin was added. After melting, the solution was vacuum filtered and cooled to 25 ° C. 18% cold hydrochloric acid solution was added. The precipitated resinic acid mixture was vacuum filtrated and dried at 100 °C [2].

2.2. Preparation of calcium, magnesium and zinc resinate salts.

The elaboration of calcium, magnesium and zinc resinate salts was carried out according to a modification made to the methodology described by Li *et al*, [10]. The calcium, magnesium and zinc resinate salts were prepared by a two-step process, saponification of resinic acid followed by salt metathesis reaction, according to the methodologies described below.

2.2.1. Calcium resinate salts (RCa).

0.2 mol of sodium hydroxide is dissolved in 5 mL of distilled water. 150 mL of ethanol and 0.1 mol of Resin Acids (AR) are added. The mixture is stirred for 30 min (IKA RW 20, Germany). Subsequently, a solution of 0.1 mol of calcium chloride (dissolved in 100 mL of ethanol and 100 mL of distilled water) was added and the solution was stirred for 3 h. The obtained suspension was left at rest for 24 h. After that time, it is filtered under vacuum and the precipitate is washed several times with distilled water. Finally, the material is dried at 60 ° C for 6 hours.

2.2.2. Magnesium resin salts (RMg).

The magnesium resinate salts were obtained by following the same procedure described above, with the modification that 0.1 mol of magnesium chloride dissolved in 50 mL of distilled water and 100 mL of ethanol was added.

2.2.3. Zinc resinate salts (RZn).

The zinc resinate salts were obtained by following the same procedure described above, with the modification that 0.1 mol of zinc sulfate dissolved in 100 mL of distilled water and 100 mL of ethanol was added.

2.3. Chemical characterization of salts.

2.3.1. Elemental analysis of hydrogen.

The elemental hydrogen analysis was performed on a Perkin Elmer; Model 2400 System CHNS / O Series II (United States).

2.3.2. X-ray powder diffraction studies (XRD).

The XRD spectra were recorded at room temperature (25 ºC) with a SIEMENS D5000, DIFFRAC PLUS XRD diffractometer (Germany) with BRAGG-Brentano geometry, Cu Kα radiation (λ=0.154 nm), Flicker detector and graphite monochromator. The scattering angle range from 4º to 80º with 2θ step interval of 0.02º was used. An operating voltage of 40 kV and current of 30 mA was utilized, and the intensities were measured in the range of $5° < 2\theta < 30°$. Crystallographic search match software was used to identify the crystal structure of samples.

2.3.3. FTIR Spectroscopy.

FTIR spectra of the samples were measured on a FTIR VERTEX 70/BRUKER spectrometer (Germany) to determine the characteristic chemical bonds present in the samples. Transmission mode was used with 64 cumulative scans and a resolution of 4 cm^{-1}, in the frequency range of 4000 to 400 cm^{-1}.

2.3.4. Scanning electron microscopy (MEV) and Electron dispersive spectroscopy (EDS).

The morphological study of the samples was performed by scanning electron microscopy (SEM-FEG-JEOL, model 7500F) equipped with X-ray energy dispersive spectroscopy detector (EDS). The EDS counting time was 20000 s per analysis at an acceleration voltage of 10 kV. Previously the samples were coated by carbon evaporation (Baltec SCD 050 Spputer Coater, USA).

2.3.5. Nuclear magnetic resonance (NMR).

1D and 2D NMR spectra were recorded on a Bruker Advance III HD 600 spectrometer (14.1 Tesla) using an inverse detection 5-mm (^{1}H, ^{13}C, ^{15}N) cryoprobe and a z gradient, as well as automated tuning and marching (ATM) in $(CD_3)_2SO$-$_{d6}$ (99.95%, Sigma-Aldrich) as solvent purchased from Sigma-Aldrich TM, chemical shifts were referenced to tetramethylsilane (TMS).

2.4. Biological tests.

2.4.1. Assessment the antibacterial activity for determination of Minimal Inhibitory Concentration (MIC) against bacteria causing dental caries.

2.4.1.1. Bacterial strains.

The strains of the following bacteria were used in the present work: *Streptococcus mutans* (ATCC 25175), *S. mitis* (ATCC 49456), *S. sanguinis* (ATCC 10556), *S. sobrinus* (ATCC 33478), *S. salivarius* (ATCC 25975), *Lactobacillus casei* (ATCC 11578) and *Enterococcus faecalis* (ATCC 4082), all of them obtained from the American Type Culture Collection (ATCC) and were maintained on Brain Heart Infusion broth (Difco, Kansas City, MO) with glycerol (20%) at - 80 ºC in the Laboratory of Research in Applied Microbiology of the University of Franca.

2.4.1.2. Antibacterial assays.

The MIC values (the lowest concentration of the compound capable of inhibiting microorganism growth) of the Resinic acid, Calcium resinate, Magnesium resinate and Zinc resinate were determined in triplicate by using the microdilution broth method in 96-well microplates, according to Lima *et al*, [13] with some modification.

The samples were dissolved in dimethyl sulfoxide (DMSO) at 1 mg mL^{-1}, followed by dilution in Brain Heart Infusion broth (Difco); concentrations ranging from 0.195 to 400 μg mL^{-1} were achieved. The final DMSO content was 5% (v/v), and this solution was used as negative control. The inoculum was adjusted for each organism, to yield a cell concentration of 5 x 10^5 colony forming units (CFU).mL^{-1}, according to previously standardization by the Clinical Laboratory Standards Institute [14]. One inoculated well was included, to allow control of the adequacy of the broth for organism growth. One non-inoculated well, free of antibacterial agent, was also included, to ensure medium sterility. Chlorhexidine (Sigma-Aldrich, St. Louis, MO, EUA) - concentrations ranging from 0.115 to 59.0 μg mL^{-1}) were used as positive control. The microplates (96 - wells) were sealed with plastic film and the time needed to promote growth was 24 hours, incubated at 37ºC under appropriate gaseous conditions. After that, resazurin (Sigma-Aldrich) - 30 μL in aqueous solution (0.02%) was added to the microplates, to indicate microorganism viability, the blue and red color indicated absence and presence of bacterial growth, respectively, for the determination of minimal inhibitory concentration [13].

3. RESULTS

The pine resin showed the following quality parameters: Color 4A, Acid Value 168.7, Saponification Index 176.71, Softening Point 73 °C, Humidity 0.02%. These values satisfy the quality limits established by the analysis technique. By means of the metathesis reaction of each one of the metals evaluated, fine and crystalline powders with a darker color were obtained.

The elemental analysis showed that the content of the weight percentage of hydrogen in the resinates samples decreases with respect to the content of the resin acid sample (4.51%, 0.53%, 1.09% and 0.21%, for resin acid, calcium resinate, magnesium resinate and zinc resinate, respectively). This result indicates that during the reaction part of the hydrogen was replaced by the cation to form the corresponding salt.

Figure 1 shows the X-ray diffractogram of the resin acid and its salts. In the case of resinic acid, several characteristic peaks at 15.3, 27.2, 32.1, 45.0, 45.7 and 56.5 2θ were observed. While in its salts peaks at 29.9, 36.4, 39.9, 43.8, 48.4, 57.8, 61.8 and 65.2 2θ (calcium resinates); at 14.3, 37.5 and 59.8 2θ (magnesium resinate) and at 32.1, 35.0, 35.7, 48.1, 57.4, 63.7 and 69.1 2θ (zinc resinate) were observed. In general, all the salts present a structure mainly amorphous, being able to infer that they present a partially ordered structure, observing several sharp peaks.

The diffractograms of resinates were compared with diffractograms of the respective oxides of the metals studied according to powder diffraction file (PDF 00-001-1235; PDF 00-001-1160; PDF 00-003-0888) confirming that the acute peaks observed in most cases corresponded with peaks belonging to the presence of these metals in the sample. In general, there are few reported studies on the evaluation by X-ray diffraction of the resin acids and their respective salts.

Figure 2 shows the infrared spectrum of each of the obtained resinates. The disappearance of the characteristic band of the carboxylic acid (1691.1 cm^{-1}) and the appearance of corresponding signals to the stretching of the acidic group of the salts (1300 cm^{-1} to 1500 cm^{-1}) was observed, which indicates the formation of the corresponding resinates by metathesis reaction.

Figure 3 presents the morphological image of each of the samples evaluated. The chemical mapping obtained by EDS demonstrates the presence of the metal cations used in each of the samples. This result confirms the formation of the respective resinates obtained from the resin acid through the different reactions used.

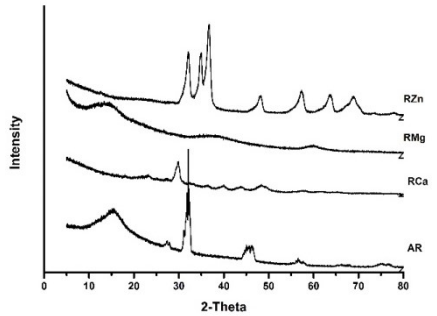

Figure 1. XDR patterns of AR, RCa, RMg and RZn samples.

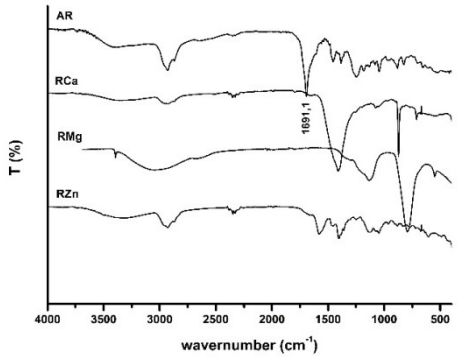

Figure 2. FTIR spectra of AR, RCa, RMg and RZn samples.

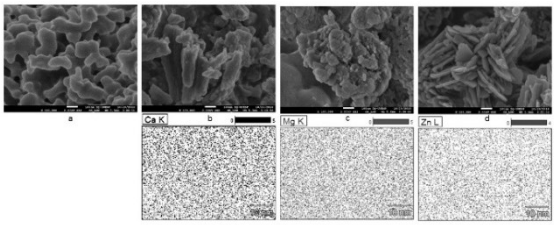

Figure 3. Morphological image (100000X) and EDS mapping of different samples. (a: AR; b: RCa; c: RMg and d: RZn).

The results of the NMR evaluation of the resin acid mixture are shown in Table 1. The typical signal at δ_H 12.19 (COOH, s) which corresponds to the carboxylic group hydrogen signal was observed in the ^{1}H NMR spectrum (Figure 4). When the reaction between the metals studied and the resin acid mixture was carried out, the loss of this signal was observed (Figure 4), which confirms that the formation of the corresponding salts occurred in the reaction conditions studied.

Antimicrobial assays of the resin acid mixture (majority abietic acid) obtained from Cuban pine rosin showed activity against the gram-positive bacteria *Streptococcus mutans* (ATCC 25175), *Streptococcus mitis* (ATCC 49456), *Streptococcus sanguinis* (ATCC 10556), *Streptococcus sobrinus* (ATCC 33478) and *Lactobacillus casei* (ATCC 11578), but not against the bacteria *Streptococcus salivarius* (ATCC 25975) and *Enterococcus faecalis* (ATCC 4082) up to 400 µg/ml (Table 2).

Table 1. ^{1}H, ^{13}C – NMR data of Resinic acid.

^{1}H – NMR (δ ppm, m, *J* (Hz))	**^{13}C - NMR**
0.71 (3H, *s*)	24.4 (CH_3)
0.75 (3H, *s*)	25.3 (CH_3)
1.05 (3H, *d*, 7.0)	16.8 (CH_3)
1.06 (3H, *d*, 7.0)	17.3 (CH_3)
1,3 - 2,0	18.0 - 39.0 (CH_2)
4.14 (1H, *s*)	120.9 (CH)
4.15 (1H, *s*)	120.9 (CH)
4,40 (1H, *s* (sh))	122.8 (CH)
5.33 (1H, *s*)	122.8 (CH)
5.72 (1H, *s*)	124.6 (CH)
6.85 (1H, *d*, 0.7)	124.2 (CH)
6.97 (1H, *dd*, 0.7, 8.2)	124.6 (CH)
7.16 (1H, *d*, 8.2)	127.0 (CH)
12.19 (1H, *sh*)	194.0 (COOH)

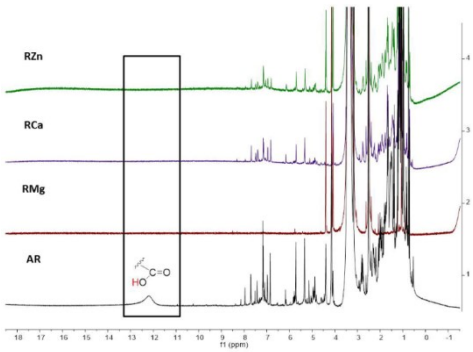

Figure 4. ^{1}H NMR analysis of AR, RCa. RMg and RZn samples.

The results of the antibacterial evaluation of the resinates obtained from the resinic-acid mixtures showed that calcium resinates and magnesium resinates do not show significant activity against the strains evaluated in this study (Table 2). However, the results obtained for the zinc resinate showed an increase of approximately 50% in the antibacterial activity for the bacteria *Streptococcus mutans, Streptococcus mitis, Streptococcus sanguinis, Streptococcus sobrinus and Lactobacillus casei. In the evaluation against Streptococcus salivarius* and *Enterococcus faecalis,* no antibacterial activity was observed.

The synthesis of the respective resinates occurs in two steps; First, through a saponification process, the sodium ion displaces hydrogen from the carboxylic group present in the resin acid molecule. Later in the second stage, the metal cations used in each of the reactions studied, displace the sodium ion present in the saponified resin acid molecule that forms the different resinates, which precipitate due to their poor solubility in the medium.

During the characterization process this reaction mechanism was demonstrated when the loss of the hydrogen of the carboxylic group of the resin acid molecule was verified by means of infrared spectroscopy and nuclear magnetic resonance. On the other hand X-ray diffraction analysis showed characteristic peaks of the presence of the metal cations studied in each of the evaluated samples and by means of the qualitative analysis by MEV-EDS the presence of these metals in said molecule was confirmed. All these results confirm that by means of the established synthesis process, the respective resinate salts are obtained.

Studies reported in the literature for other pine species have shown that abietic acid and dehydroabietic acid have a strong antimicrobial activity against gram-positive bacteria, being dehydroabietic acid the most effective of all simple resin acids [4, 15-20]. The differences in antibacterial efficacy detected among the different reports found in the literature are due to the fact that the studied rosins come from different species and the growth conditions, the recovery processes, the concentrations of the different resin acids, the neutral fraction, the storage and age are factors that affect the quality of rosin. On the other hand, the observation that some pure resin acids were more effective than others indicates that the structure, molecular weight and stereochemistry of the resin acid may be important [15].

Soderberg et al, [15], studied the antibacterial effect of the combination of zinc oxide and rosin (Portuguese rosin) observed that the mixture of these two components increased antibacterial activity against gram-positive strains (*Staphylococcus aureus, S. epidermidis, Enterococcus sp* and *S. pyogenes*). This author suggested that this increase could be associated with a synergistic effect between zinc and resin acids (specifically dehydroabietic acid and abietic acid) present in the evaluated rosin. According to these authors, resin acid molecules have a polar and non-polar part, which acts as a detergent for the bacterial cell wall.

Zinc acts as antimicrobial agents through various mechanisms, the most important being the generation of reactive oxygen species within the cell [21-26]. When resin acids react with zinc cations, resinates are formed that have a greater solubility. This facilitates the ability to penetrate the cell wall of bacteria and the excess inside the cell generates an alteration of the cellular metabolism. This synergism makes the antibacterial activity significantly increase [15, 27].

In the case of calcium resinate and magnesium resinate in the literature consulted there are no reported studies evaluating the antibacterial activity of the same. Only studies have been reported on the evaluation of calcium and magnesium activity in the form of hydroxide against Gram-positive bacteria.

Evaluation studies of magnesium hydroxide against strains of *S. mutans* and *E. faecalis* showed no antibacterial activity because the pH of the medium was not sufficient to create an alkaline medium capable of inhibiting the development of these bacteria [28]. On the other hand, studies conducted by Krishnamoorthy *et al*, [29] showed that magnesium oxide showed antibacterial activity against Gram-positive bacteria; *S. aureus* in concentrations of 1000 μg/mL. The mechanism by which this inhibition occurs has been attributed to the presence of oxygen in the molecule of magnesium oxide. This active oxygen like superoxide (O^{2-}) formed by the alkalinity of the surface of the magnesium oxide particles damages the cell membrane of the bacteria causing death [30-33]. Vázquez-Olmos *et al*, [27] concluded that the bactericidal action of nanoparticles of magnesium oxide is due to the union of oxygen to the cell wall, whereas the surface area of the particles increases, the concentration of oxygen in the surface increases resulting in the destruction of the cytoplasmic membrane and therefore the cell wall of the bacteria.

In the case of calcium, studies conducted by Barquero *et al*, [34] showed that calcium hydroxide did not show significant antibacterial activity against strains of *Escherichia coli*, *S. aureus*, *Bacteroides fragilis* and *S. pyogenes*. According to these authors the pH of the medium was between 11 and 11.9, so the alkalinity was low, causing poor diffusion and solubility of the material in the medium. These results corroborate that, as in the case of magnesium hydroxide, the oxygen present in the molecule is responsible for the bactericidal activity, influenced among other things by the alkalinity of the medium.

In this study, calcium resinate and magnesium resinate were used. These molecules, due to their chemical structure, do not present hydroxyl groups that allow the formation of superoxides on the surface of the cation. On the other hand, both molecules cannot generate the lysis of the cell wall of bacteria due to their chemical composition, shape and size.

Of the species studied in this work (caribbaea pinus) no studies were found in the literature where the antibacterial activity of the resin acids from rosin and its salts was evaluated. The results obtained in this study showed that, as the literature suggests

for other pine species, the resin acid obtained from species caribbaea pinus and its respective zinc salt show antimicrobial activity demonstrated for a group of strains evaluated. While the calcium and magnesium salts do not exhibit this activity.

Table 2. Antibacterial activities (MIC) of AR, RCa, RMg and RZn against caries bacteria.

Bacteria	MIC - µg/mL				
	AR	R Ca	R Mg	R Zn	Chlorhexidine (positive control)
Streptococcus mutans **ATCC 25175**	200	>400	>400	100	0.5
Streptococcus mitis **ATCC 49456**	200	>400	>400	100	1.8
Streptococcus sanguinis **ATCC 10556**	200	>400	>400	200	0.9
Streptococcus sobrinus **ATCC 33478**	400	>400	>400	200	0.5
Lactobacillus casei **ATCC 11578**	400	>400	>400	200	0.9
Streptococcus salivarius **ATCC 25975**	>400	>400	>400	>400	0.9
Enterococcus faecalis **ATCC 4082**	>400	>400	>400	>400	3.7

4. CONCLUSIONS

The results of this study showed that by means of the metathesis reaction calcium resinate, magnesium resinate and zinc resinate can be obtained from resin acid coming from Cuban pine rosin. The biological tests showed the strong antimicrobial activity of zinc resinate, which makes it a material with a good possibility of being used as biomaterials for dental use.

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