In vitro and *in vivo* antibacterial activities of *Rhizophora mangle* L. against *Helicobacter pylori*. Chemical compounds elucidation.

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Abstract

Ethno-pharmacological relevance: Rhizophora mangle L. is a vegetal species widely distributed in Cuba and other Caribbean countries with ethno- pharmacology relevance and preview reports as antiulcer and wound healing properties.

Aim of the study: In the present work, we describe the *in vitro* and *in vivo* antibacterial activities from dried aqueous extract of bark and polyphenol fractions from *R. mangle* against *Helicobacter pylori* and the identification of new compounds in the active extracts.

Materials and methods: Minimum inhibitory concentration (MIC) and Minimum bactericide concentration (MBC) were evaluated against reference and clinical *Helicobacter pylori* strains with total extract, High Molecular Weight and Low Molecular Weight fractions isolated from total extract. Positive active fractions *in vitro* test were evaluated *in vivo* using *H. pylori* C57BL/6 mice. Fractionation, isolation and structural elucidation of the compounds on High Molecular Weigh fraction and on Low Molecular Weight fraction were made using Chromatography methods and Mass and H^+ NMR spectrometry.

Results: Total aqueous extract from bark of *R. mangle* and some fraction shown promissory antibacterial activity on **in vitro** and **in vivo** models, for also it was possible to determinate the anti- *Helicobacter* properties of this plant by the treatment of

gastroduodenal ulcer. It were isolated and identified proantocyanidin, catechin and epicatechin derivates, cyanidin and other compounds in this promissory extract.
Conclusions: With these results we can to consider at total extract with a promissory active principle in the development of phytodrug, by it antibacterial effect as proton pump inhibitor, for example tablet by the treatment of gastroduodenal ulcer so as preventive of gastric cancer.

Key words: *Rhizophora mangle* L., *in vitro* and *in vivo* anti- *Helicobacter* effect, chemical compounds elucidation.

1. INTRODUCTION

Rhizophora mangle L. was widely distributed in Cuba and other Caribbean countries. This plant has several ethno botanical uses, as astringent, hemostatic, antipyretic, antifungal, angina, leprosy, antimicrobial properties in throat and it is use in skin ulcer (Roig, 1974). Some empiric use have made by population in internal and external ulcer, however any preview study have been as antibacterial against *Helicobacter pylori*, with primordial importance in gastritis, gastric and duodenal ulcers.

Gastric and duodenal ulcers affect a considerable number of people in the world and it is induced by several factors. *Helicobacter pylori* is a gram negative bacteria who habit the epithelial surface of human stomach. In 1994, WHO declared it as a principal causal agent of peptic ulcer. International Agency of Cancer Researching classifies this bacterium as a carcinogen of group 1 (IARC, 1994; NIH, 1994).

Many generic drugs are used in the treatment of gastroduodenal ulcers, but in the totally are necessary the application of combinatory therapies by the complexity of this etiology. These treatments are not effective in the 20 % of the case by apparition of resistant strains and adverse effects. For also, the treatment of *Helicobacter pylori* will be focus with a novel liberation system for fighting against the confrontation between biochemical and physical that present in the gastric mucosa infection place (Pajares, 2006).

Though, the finding of other new therapies is important, between it the traditional medicine has a high level.

Previous works report the cytoprotective effect of freeze - dried aqueous extract from red mangrove bark on gastric ulceration induced by ethanol – hydrochloric acid in rats and in mice (Sánchez et al., 2001; Sánchez et al., 2010). Also, we report the antiulcerogenic effect by other action's mechanism as antisecretor, inhibitor of depleting of PGE2 in the gastric mucous and antioxidant (Sánchez et al., 2004; Berenguer et al., 2006). However,

the significant in gastroduodenal ulcers of antibacterial drug joint to proton pump inhibitor is considerable by the treatment of this illness.

R. mangle L. has polyphenolic structures as major components, named tannins (Sánchez et al., 1998). Tannins have many biological actions as antimicrobial, antifungal, antiviral, antioxidant, etc. (Leinmmüller et al., 1991).

Other preview studied performance with this extract; it had shown the presence polyphenolic structures (54.78%) and other structural components (45.22%). Polymeric tannins were the major polyphenolic component 80 and 20% were hydrolysable tannins. Epicatechin, catechin, chlorogenic acid, gallic acid and ellagic acid were monomeric structures determined in this extract. Phytosterols (0.0285%): stigmasterol, β-sitosterol and likewise campesterol were present too (Sánchez et al., 2008b). This extract present semivolatil compounds (Pino et al., 2001); fatty acids and sugars (Sánchez et al., 2008b), for also it represent a complex mixture of secondary metabolites.

This plant with promissory pharmacological activity as antiulcer in gastro duodenal tract was studied its possible toxic effect in acute, sub-acute oral toxicology in rats and genotoxicological tests (Labienie and Gabryelck, 2003; Sánchez et al., 2008a). Any toxicological sign was shown in these studies.

The objective of the present work was to evaluate *in vitro* and *in vivo* antibacterial activities of *Rhizophora mangle* L. against *Helicobacter pylori* and chemical compounds elucidation in two fractions from total extract.

2. MATERIALS AND METHODS

2.1 Preparations of aqueous extract of R. mangle L. bark

R. mangle L. was collected from the western zones of Cuba in 2009. The identity of the plant was authenticated by a botanist and a voucher specimen has been deposited in National Botanical Garden's Herbarium (voucher sample 6539). The extract was prepared by the decoction of the bark in distillate water. The proportion of vegetal matter: water was 1:7; the decoction was made for 20 min at 90°C in lab reactor with 2 L of capacity. The plant material was separated by centrifugation and the aqueous extract was concentrated and freeze dried to preserve it and one part was dried by spray dried.

2.2 Fractionation of freeze- dried aqueous extract in high molecular weight polyphenols (HMWP) and Low Molecular weight polyphenols (LMWP).

The freeze- dried aqueous extract was fractioned by it high concentration of polyphenols in High Molecular Weight Polyphenols (HMWP) and Low Molecular Weight Polyphenols (LMWP) following the scheme described in Figure 1.

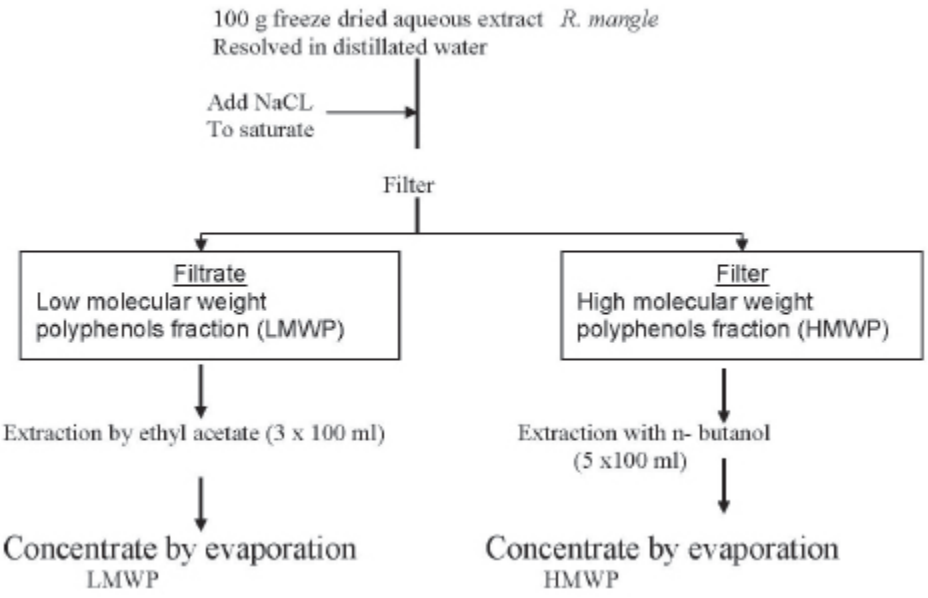


Figure 1. Low and High molecular weight polyphenols fractionation from an aqueous extract *Rhizophora mangle* L.

Antibacterial activity against *H. pylori* were determined with both fractions (LMWP and HMWP)

2.3 In vitro e in vivo antibacterial activities of Rhizophora mangle L. against Helicobacter pylori

2.3.1 Antibiotic susceptibility of total extract.

First, the freeze- dried aqueous total extract was evaluate against reference strain from *Helicobacter pylori* # CCUG 17874 proceeding Gotemburgo University collection, Sweden. Microorganism suspension was 10^5-10^7CFU /ml. The concentration range of total extract were 50 µg - 40 mg/ml.

Inhibitory activity was evaluate using minimum inhibitory concentration (MIC) in solid medium. After, the total extract was test for antibiotic susceptibility by plate dilution method against 30 lines of *H. pylori*. It were prepared 13 different concentrations, between 0.062ug/ml to 256 ug/ml. It were used plates with antimicrobial drugs in the same concentration: tetracycline, metronidazole, amoxicillin and clarithromycin. It were

selected 29 clinical isolated from *H. pylori* with quick growing and 1 reference line from *H. pylori* ATCC 43504. In each case was prepared an inoculum in saline equivalent at 2 in McFarland standard (10^7 – 10^8). Inoculum were placed in 8 control plates preparing with total aqueous extract of *R. mangle,* LMWP, HMWP and the 4 antibiotic in different concentrations using an automatized Robot. It was used a replication volume from 1.2 uL. Plates were incubated in micro aerobic conditions at 37°C, during 3 at 7 days. Plates were checked 3, 5 and 7 days after the incubation.

*2.3.2 Minimal bactericide concentration (MBC) from total aqueous extract dried with freeze dried or spray drier***.**

Six strain of *H. pylori* was using in the study. Two lines were of reference: 26695 (TG), SS1 (Sydney strain) and four clinical isolated from lab strain bank. Strain purity were evaluated by microscopic observation for typical morphology using Gram coloration and by the presence of catalase, urease and oxidase enzymes.

MBC were determinate using micro-dilution technique, where series dilution were made in micro-plates for products in analysis with 2 replicates. The initial concentration was 3 mg/mL in sterile water from freeze dried or spray drier extract. It added 10 uL of bacterial suspension equivalent at 12 x 10^8 ufc/mL. Plates were incubated in micro aerophilic condition for 48 hours at 37ºC and humid of 95%. MBC was consider as a minor concentration enable to produce a bactericide effect of 99.9% of the original inoculum.

2.3.3 In vivo anti- Helicobacter pylori effect in experimental animals

Mice C57BL/6, 30 male animals, with 4– 5 week of age. They were maintain in collective box with conventional feeding and water ad libitum to start the experiment. Light – dark cycle of 12 hours and weather temperature of C57BL/6, with controlled humid.

SS1 strain was used growing on Agar Columbia plates so inoculum correspond at 6 or more than in Mc Farland scale for guaranty 10^9 ufc/mL from inoculum concentration.

Each mice were inoculated with 0.15 mL with SS1 strain, except control negative group. All animals were maintain in conventional feeding and water ad libitum by 12 days enough for mice stomachs were colonized.

After, animals were divide in group of five and it was proceeding to make two treatment by day in alternate days for a period of five days, by gavage, 0.15 mL. The dissolution medium was sterile water and the groups were the following:

Group I, negative control, within infection (PBS)

Group II, positive control, with infection from SS1 *Helicobacter*

Group III, with infection, treated with freeze dried extract of *R. mangle*, doses 500 mg/Kg bw.

Group IV, with infection, treated with freeze dried extract of *R. mangle*, doses 250 mg/Kg + 7.15 mg/Kg of clarithromycin.

Group V, with infection, treated with freeze dried extract of *R. mangle*, doses 500 mg/Kg + 7. 15 mg/Kg of clarithromycin.

Group VI, with infection, treated with LMWP, doses 250 mg/Kg

Group VII, with infection, treated with HMWP, doses 250 mg/Kg

Animals were observed the time of the experiment by clinical sign appearance. After the last treatment animal were sacrificed with ethyl ether anesthesia and cervical dislocation. Stomach were separated and washing with sterile saline and opening by major curvature and it was evaluated by lesion index follow the scale:

Fold loss ……1 point

Mucus discoloration …… 1 point

Edema …… 1 point

Bleeding ……. 1 point

Blood points……. 2 or 3 points

Ulcers to 1 mm …….. n x 2 points

Ulcers less than 1 mm …….. n x 3 points

Performance ulcers …… n x 4 points

Where n is the ulcer numbers.

Ulcer lesion index (ULI) were determinate as a sum of the lesions observed by group.

After these assessment, stomachs were divided in two fractions, one for bacterial culture analysis and another for histopathology.

Antrum were took for culture, because it is the major zone of *Helicobacter* colonization and the treatment could be less incidence and more recidivism. These fractions were weight for express the number of bacterial colonies by g of tissue.

Body of stomachs were took by histology.

Samples for culture were bring in BHI plates (bread- heart infusion stock). They were prepared in ram des- fibrinized blood and antibiotic in the same concentration as used in Agar Columbia plates. Plates were put into anaerobic jug with micro-anaerobe and were incubated at 37ºC for five days, after this time it was made the evaluation for bacterial growing and Gram test for verified appearance and number of bacteria from *Helicobacter pylori*.

2.4 Chemical compounds elucidation

2.4.1 Reagents

Reactive and solvents were pure grade for analysis (Sigma Chemical Co.) and HPLC solvents high pure (Fluka Chemical Co.).

Silica gel G, MN- Kieselgel G, Macherey – Ángel GMBH & Co. Düren Germany; Kieselgel 60 (0,040 – 0,063 mm), 230 – 400 mesh ASTM; Merck y DIAON HP -20, SUPELCO, USA. Bellefonte, PA, Sigma – Aldrich Chemie, GmbH, Germany, for Column Chromatography.

Aluminum plates (1.0554, DC – Alufolien. Silica gel 60, F254, 0.2 mm, Merck KGaA, DARMSTADT, Germany), for Thin Layer Chromatography. Vainillin/HCL (solution of vainillin 10% in methanol, acidic with 5 – 10 drops of concentrated chlorohydrin acid). Ceric sulphate/Amonium (12.5 g ammonium ceric sulphate, 350 g of ice and 22.2 ml of concentrated sulfuric acid). Anysaldehyde/H_2SO_4 (mix 2.5 ml of anysaldehyde, 50 ml acetic acid, 425 ml of methanol and 25ml of concentrated sulfuric acid).

Varian – Unity 300 mHz, Varian – Inova 500 mHz (Varian, LA, USA) and Bruker-Advance 300 mHz with DMSO, acetone and chloroform deutered were used by NMR proton and Carbon 13 NMR spectrum.

FAB + and electronic impact (EI +) were used by mass spectrum.

2.4.2 Fractionation and structural elucidation from major fraction, high molecular weight polyphenols (butanol extract).

2.4.2.1 Fractionation of butanolic extract by Sephadex LH- 20 Exclusion Chromatography.

Five gram of butanol extract was put into a Sephadex LH- 20 column, washed previous with methanol: water 1:1. Column was eluted with methanol: water 1:1, methanol: water 60%, methanol, acetone 70%. There were collected 37 fractions. Fractions were analysis by TLC with UV light, FeCL3 10% or vanillin/HCL (butanol- acetic acid- water, 4:1:5).

Fractions were made purification by preparative TLC, Sephadex LH-20 columns and crystallization in solid presence.

In case 20- 22 fraction were used DIAON HP – 20 column with water and mixture of water- methanol and methanol.

2.4.2.2 Fractionation of butanolic extract by Adsorption Chromatography.

Butanol extract, 30 g was put into Silica gel G chromatography column. Column was eluted with hexane, mixture of hexane- ethyl acetate, ethyl acetate, ethyl acetate-methanol, methanol, methanol- water and acetone 70%. Separation of diverse types of compounds were isolated with these system. Were collected 60 fractions each of 25 mL.

Other purification were made with consecutives Sephadex LH- 20 column chromatography, silica gel G column chromatography, preparative TLC, preparative HPLC.

Each compound isolated in this section were identified using NMR and Mass Spectrometry.

2.4.3 Fractionation and structural elucidation from minor fraction, low molecular weight polyphenols (ethyl acetate extract).

Ethyl acetate extract, 2 g, were put into a flash adsorption column chromatography, Kiesegel 60 (0,040 – 0,063 mm). Elution were made with hexane, hexane- ethyl acetate mixture, ethyl acetate and ethyl acetate – (MeOH: H_2O, 1:1) mixture, methanol and acetone 70%. Were collected 28 fractions each of 25 mL. Fractions were analysis by TLC with UV light and anysaldehyde or $FeCl_3$ 10% in ethanol.

Other purification of each fractions were made with consecutives adsorption column chromatography. Each pure compounds was identified by NMR and Mass spectrometry.

3. Results

3.1 In vitro antibacterial activities of Rhizophora mangle L. against Helicobacter pylori

Preliminary study from total aqueous extract of *R. mangle* against reference strain of *H. pylori* in the concentration range 50 ug/ml – 40 mg/ml shown a high activity between 50 – 100 % of inhibition.

Reference strain of *H. pylori* show a successful MIC accord at describe in Susceptibility Test. Total freeze-dried aqueous extract of *R. mangle* show a MIC 90% of 177.77 ug/ml (0.18 mg/ml); LMWP fraction a MIC 100% = 177.77 ug/ml (0.18 mg/ml) and HMWP fraction MIC 50%= 213.3 ug/ml (0.21 mg/ml) against at all strains (30 strains; one reference strain and 29 clinical strains). Antibiotic show MIC similar at report so some strain were resistant against clarithromycin.

Total freeze –dried aqueous extract of *R. mangle* show a MBC_{50}= 250 ug/ml (0.25 mg/ml), MBC_{90}= 450 ug/ml (0.45 mg/ml). In the case of polyphenol fractions were determined by LMWP a MBC_{67}=167 ug/ml (0.17 mg/ml) and by HMWP a MBC_{67}= 250 ug/ml (0.2 mg/ml).

These results shown that *R. mangle* has an antibacterial effect against *H.* pylori as inhibitory of the bacteria growing and as with bactericide properties. In either case, inhibitory or bactericide effect from to low molecular weight polyphenol (LMWP) is better than HMWP. However, both fractions give antibacterial activity obtained in total extract.

3.2 In vivo antibacterial activities of Rhizophora mangle L. against Helicobacter pylori

Table 1 represent the ulcer index *in vivo* model. We found that the treatment with total freeze dried aqueous extract in the doses of 500 mg/Kg b.w. decreasing considerable the ulcer index (UI) respect at positive group with SS1 strain *Helicobacter* infection within anymore treatment.

Similar results were obtained in the other two groups treated with the total extract more clarithromycin, not doses – dependence. Addition of clarithromycin at total extract not increase the effect, for also not additive or synergic effect was shown in this experiment. High molecular weight polyphenol (LMWP) has better activity, however it is not significant. Major activity was shown in the case of clarithromycin, within statistical significant.

Table 1. Ulcer index in the model in vivo in mice with inoculation of strain SS1 of *Helicobacter pylori*.

Groups	Ulcer index (mean ± SD)
I, Negative control (not inoculate)	1.6 ± 1.67 *
II, Positive control (with infection)	5.8 ± 1.64
III, Total extract of *R. mangle*, 500 mg/Kg m.c.	1.5 ± 1.73 *
IV, Total extract of *R. mangle,* 250 + clarithromycin	1.4 ± 0.89 *
V, Total extract of *R. mangle* 500 + clarithromycin	1.4 ± 0.89 *
VI, LMWP, 250 mg/Kg	1.2 ± 1.09 *
VII, HMWP, 250 mg/Kg	0.8 ± 1.09 *
VIII, clarithromycin, 7.15mg/Kg	0.4 ± 0.89 *

Statistical significant, $p < 0.05$

Table 2 shown the bacterial growing in the different treatments. *R. mangle* shown antibiotic effect with decreasing of bacterial growing. The use of clarithromycin join of *R. mangle* extract not increase the antibacterial answer, not additive effect in this combination.

HMWP and LMWP fractions did not shown antibacterial activity itself in these experimental doses, only it was appreciate a little antibiotic activity with HMWP fraction. For also, antibiotic effect in total freeze dried extract of *R. mangle* is due to at other compounds present in the extract as semi volatile compounds, phytosterols and fatty acids.

Table 2. Bacterial growing in inoculate plates with stomach contents from **in vivo** antibacterial experiment against Helicobacter pylori.

GRUPO	CULTIVO	GRAM
I, Negative control –	0/5	0/5
II, Positive control +	4/4	4/4
III, *R. mangle* 500 mg/Kg bw. –	0/4	0/4
IV, *R. mangle* 250 + clarithromycin	1/5	1/5
V, R. Mangle 500 + clarithromycin	1/4	1/4
VI, LMWP +	4/5	5/5
VII, HMWP +	2/4	2/4
VIII, clarithromycin +/-	2/5	2/5

For a also*, in vivo* model shown that *R. mangle* has a prominent antibacterial activity, for this reason it was consider with antibiotic properties in the treatment of infection by *Helicobacter pylori.*

3.3 Fractionation and structural elucidation from major fraction, high molecular weight polyphenols (butanol extract).

3.3.1 Fractionation of butanolic extract by Sephadex LH- 20 Exclusion Chromatography.

Fraction 4_1, 3.2 mg, white solid, Rf= 0.7 with fluoresce at $UV_{254\ nm}$ with the presence of other peak with $CeSO_4$. Its H^+ NMR characterized with chemical shits (δ, ppm): 8.54 (singlet), characteristic of hydrogen from carbon 4 in the antocyanidin; some signals in aromatic region: 6.56 ppm (s), 6.48 (d), 6.42 y 6.4 (d); sugar signals: 5.8 anomeric, 4.69

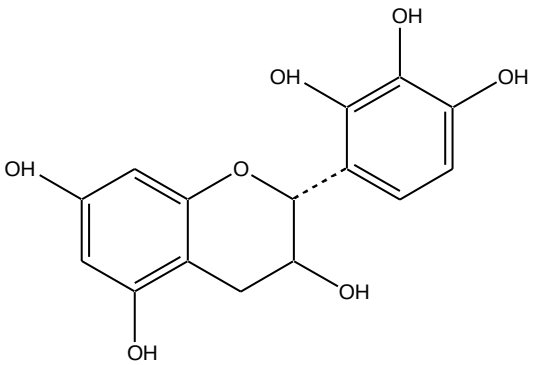
(d), 4.57 (s, sh), 4.21 y 4.20 (d) possible presence of glucose in the molecule. Mass spectrum (EI+) with M+= 576 Da, other fragments ions at m/e: 139, 257, 285, typical in antocyanidins. For also, this is a mixture of **proantocyanidin type A** and **cyanidin (delphinin glucose)**.

Compounds 4_2*, 4.2 mg, White crystals, Rf= 0.59, UV254 nm, H+ NMR, (δ, ppm): 8.54 (s), antocyanidin; aromatic regions: 7.4 (d), 7.34 (d), 7.32 (d), 6.78 (d), 6.75 (d), y 6.73 (s, m); sugar regions: 4.5 – 5.7 ppm. EI+ spectrum: M+= 576 Da, fragment ions m/e: 139, 257, 285 (catechin galate), m/e: 109 and 307 (antocyanidin), m/e= 550. For also, it shown presence of **cyanidin -3-O- glucoside (methyl and acetyl in the sugar)** mixture with **proantocyanidin A.**

Compound 51-1, 26 mg, white solid with H+NMR:

Proton	δ (ppm)
2	4.82
3	3.9
4 α	2.52
4 β	2.72
6	6.1
8	5.78
2´	6.51

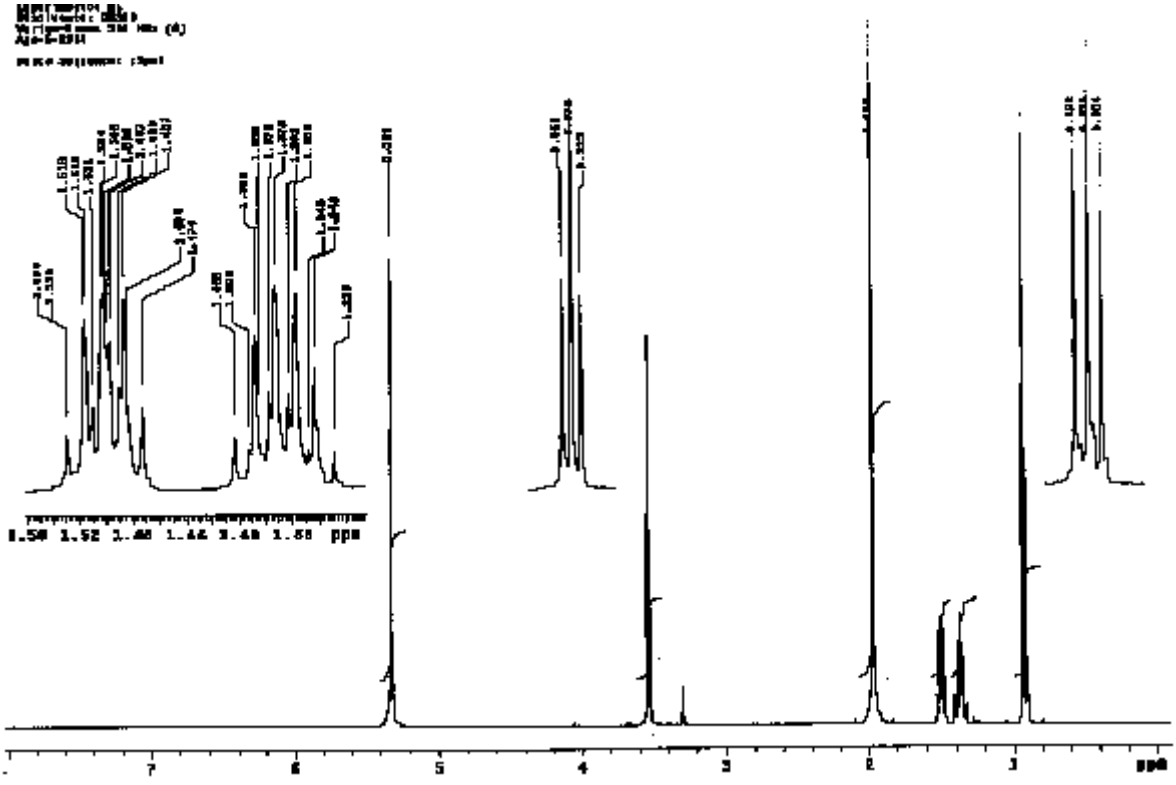
EI+ mass spectrum, present M+ = 306 and other fragment ions: 290, 273, 139.This compounds correspond at epigallocatechin (Figure 2).



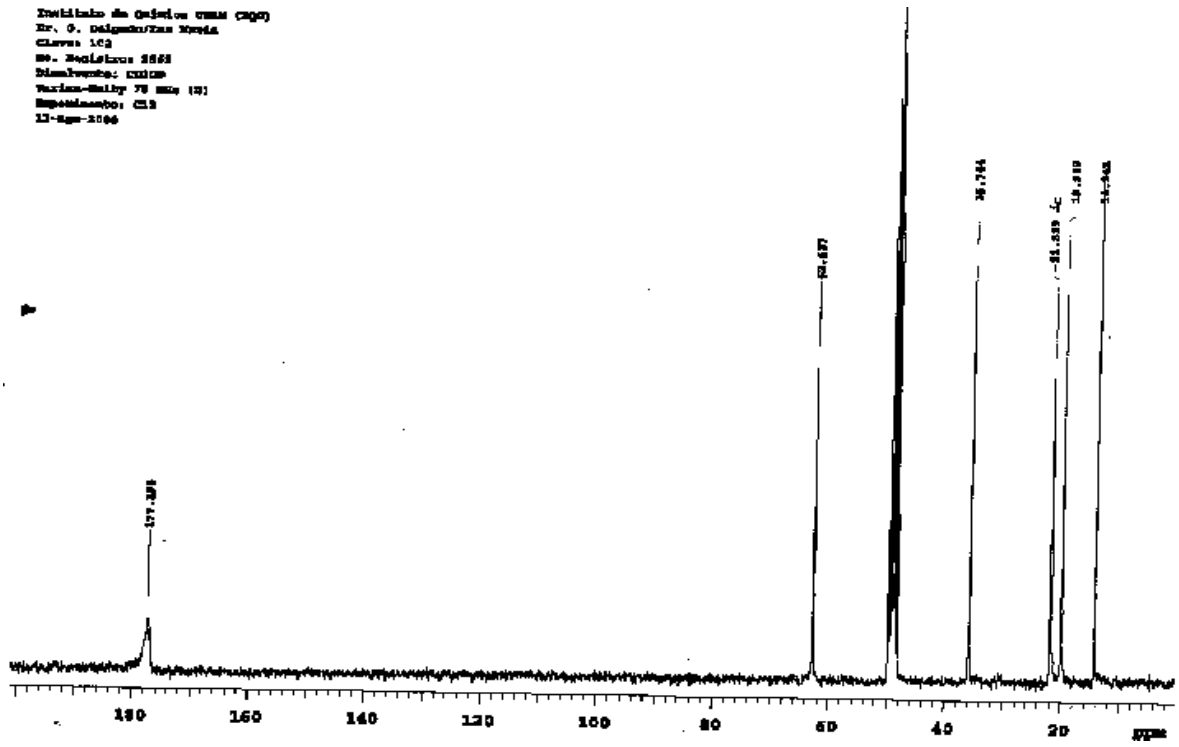
$C_{15}H_{14}O_7$
Exact Mass: 306,07
Mol. Wt.: 306,27
C, 58,82; H, 4,61; O, 36,57

Figure 2. Epigallocatechin structure.

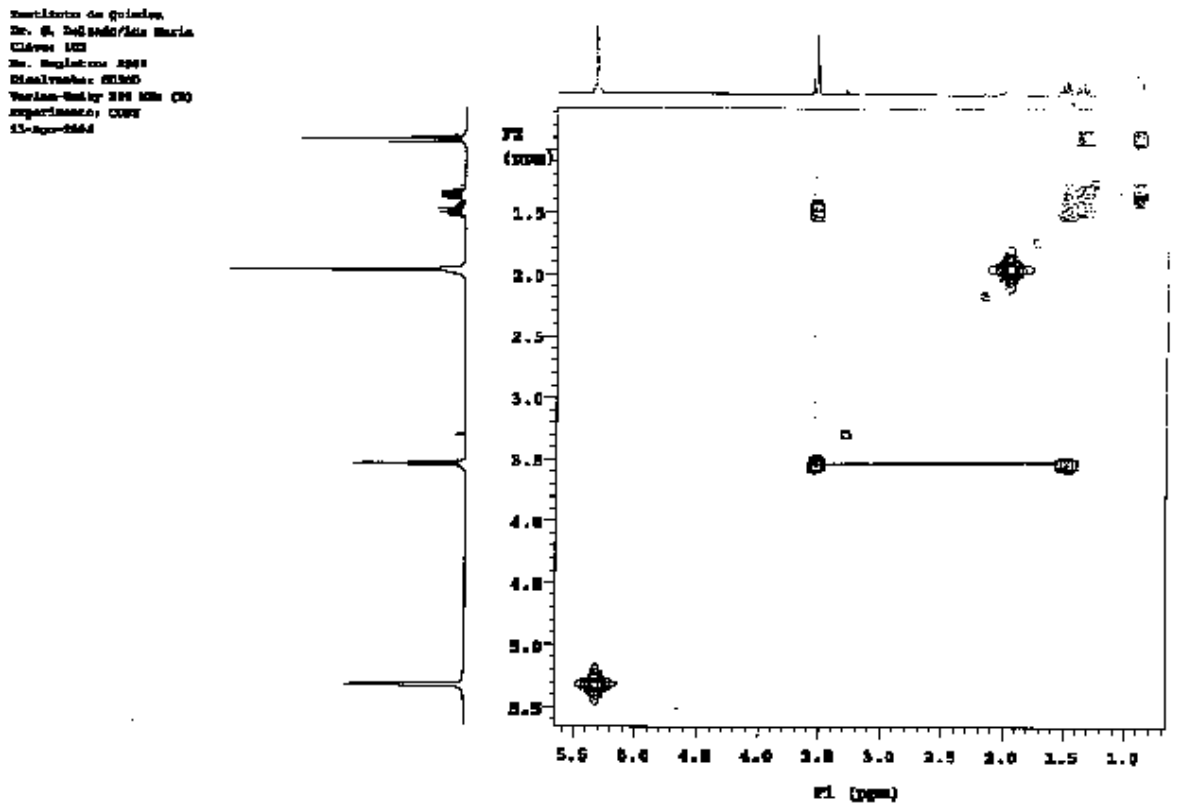
From fraction 10- 19 were purified two compounds: 1C2, 50 mg, Rf= 0.25 intense yellow with $UV_{366\ nm}$, in butanol: acetic acid: water system. These compounds no sprayed with FeCl3 nor vanillin. H^{+} NMR, C^{13} NMR, COSY, DEPT, HETCOR and FLOCK spectral analysis define this compound was **buthylcarbamate, CH_3-CH_2- CH_2-CH_2-0-CO-NH_2** (M.F. $C_5H_{11}NO_2$)



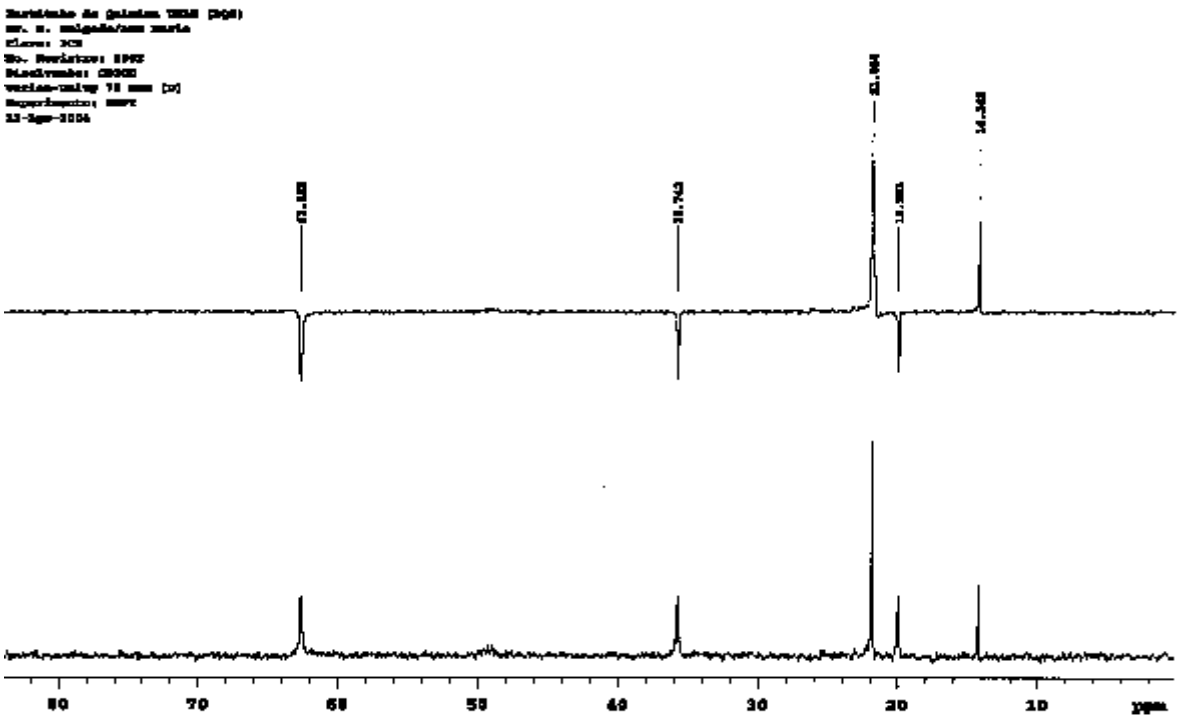
H+ NMR spectrum of buthylcarbamate.



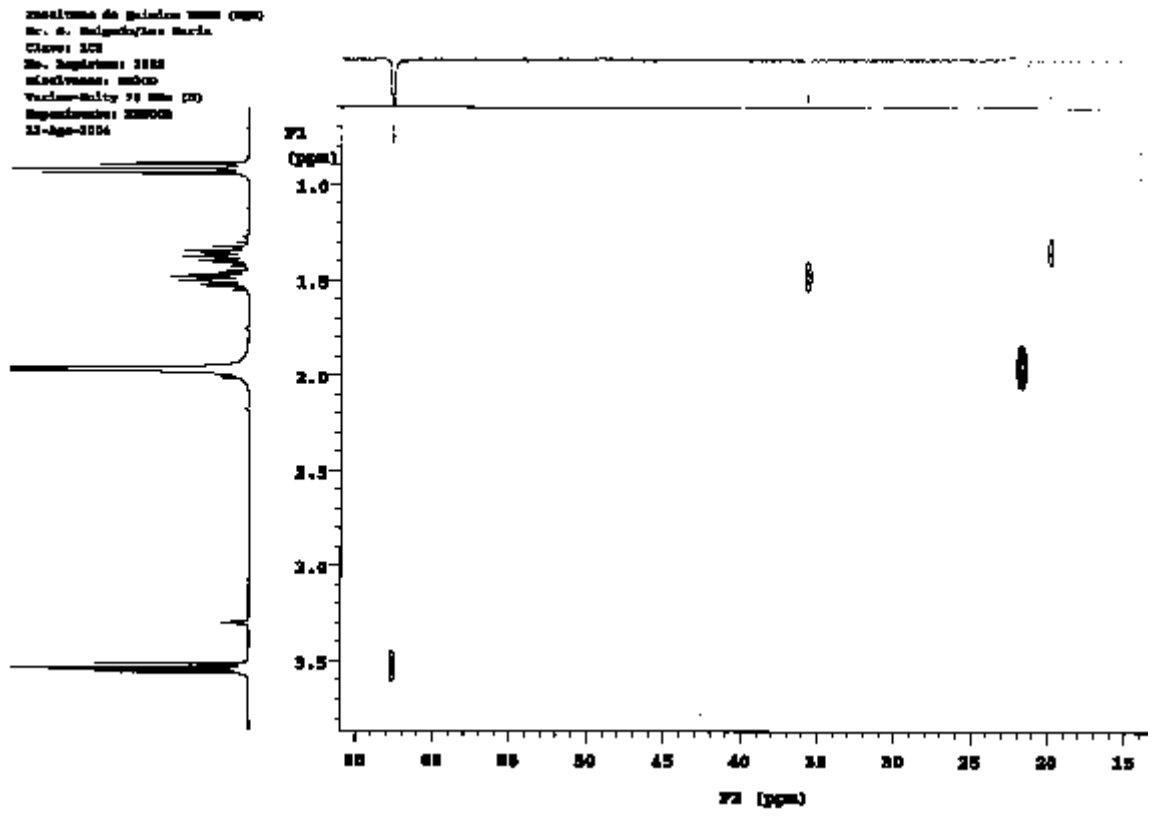
C13 NMR spectrum of buthylcarbamate.



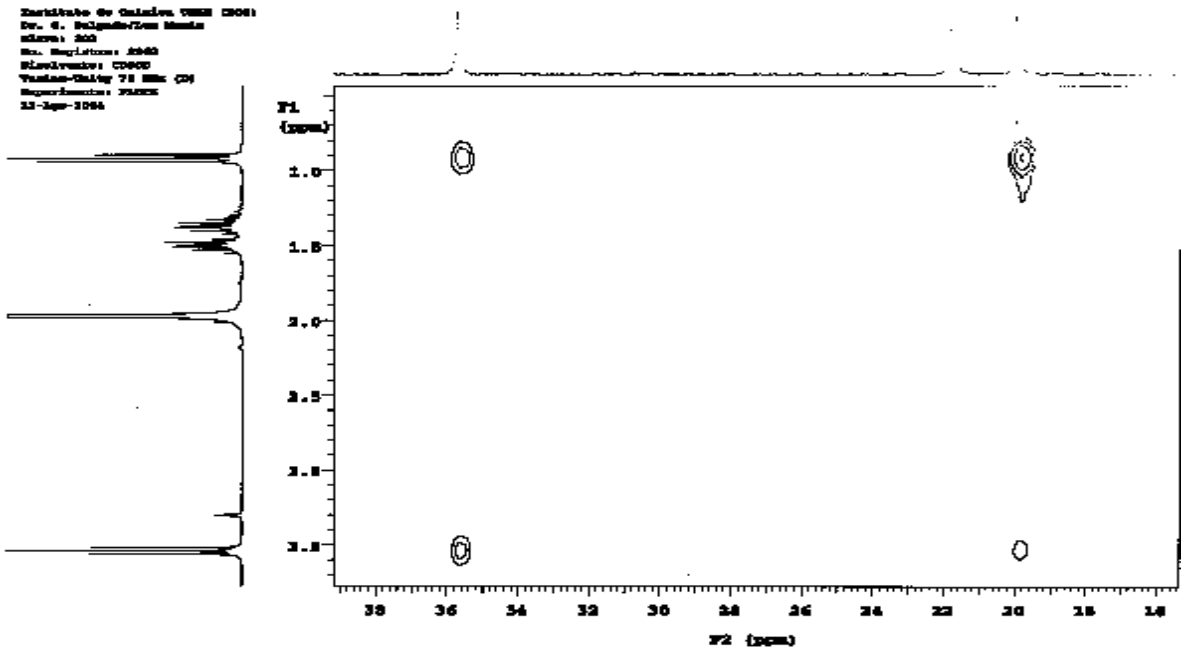
COSYspectrum of buthylcarbamate .



DEPT spectrum of buthylcarbamate.



HETCOR spectrum of buthylcarbamate.



FLOCK spectrum of buthylcarbamate.

Compounds 1-IC2, 5 mg, Rf = 0.26 blue at UV $_{366\ nm}$, with similar structure with 1C2, in H^+ NMR a signal at 3.33 ppm and in C^{13} NMR at 49.85 ppm. It is possible by an additional aliphatic group, **ethyl propyl carbamate, CH3-CH2-CH2OCO- NH-CH2-CH3.**

Compound 2C2, 69 mg, Rf= 0.71 UV 254 nm and with $FeCL_3$, in ethyl acetate: methanol: water, defined as **proantocyanidin A** (dimer type A).

Compounds from 20- 22 fractions. They were named E_1, 100 mg, UV max spectra (MeOH) with broad at: 208, 264, 495, 533 nm. Broad band at 533 nm is typical of cyaniding. Mass spectra EI+ with M+= 287, typical of cyanidin and other characteristic fragments from loss of 18, 46 and 76 mass unit at m/e: 269, 241 and 213. Proton NMR with the follow chemical shits:

De esta fracción se caracterizaron los compuestos purificados.

Proton	δ (ppm)
OH	8.8 (br s)
H-4	8.29 (s)
H-2´	6.96 (s)
H-5´	7.30 (d)

H-6´	7.13 (d)
H-8	6.57 (m)
H-6	6.17 (s)

In the Figure 3 represent cyanidin structure propos by this compound.

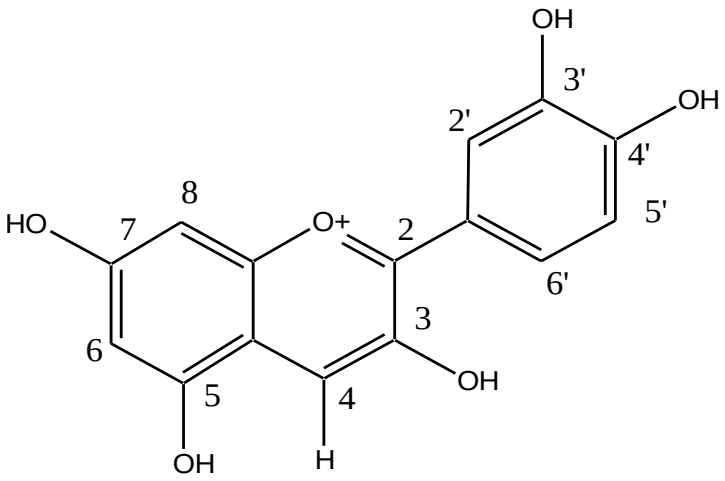


Figure 3. Cyanidin structure.

E_2 compound in this fraction, Rf= 0.27, orange with anysaldehyde. Mass spectra FAB+ with (m+1)+= 576 and other fragment ions at m/e: 551, 449, 369, 355, 306, 283, 267, 249, 139. This compound is define as **epigallocatechin methyl dimer**.

E_3 compound in this fraction, Rf= 0.13, orange with anysaldehyde. UV max (MeOH): 211, 249, 279, 398, 454 nm. FAB+ spectra (DMSO + D2O) present (M+1)+ = 603 and fragments ions at m/e: 576, 549, 411, 369, 306, 273, 257, 255, 235, 227, 209, 173 y 139. For this characteristic this compounds is similar structure at E2, some **epigallocatechin dimer** (Figure 4).

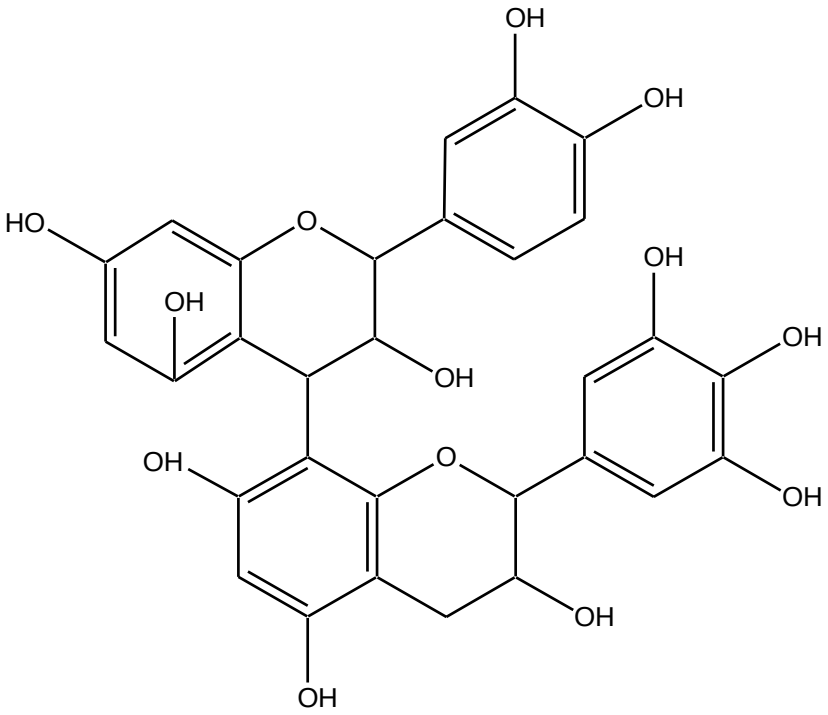


Figure 4. Epigallocatechin dimer.

Compound isolated from fraction 23- 28, 177 mg, Rf= 0.5 blue with UV 366 nm in butanol: acetic acid: water, was identified as **Dimer type A.**

Other compound named 23 sob, 60 mg, red solid, Rf= 0.7 in butanol: acetic acid: water. It tr= 1.59 min by analytical HPLC. UV spectra of this peak was characterized by signal at: 239, 279, 392, 457, 484 nm (signal at 279nm is typical of flavan-3-ol). Mass spectra, FAB^+ $(M+1)^+$= 376 and $(M+1+Na)^+$= 399, other fragment ions at 139 and 257 typical of catechin gallate and fragment ion at 289 typical of catechin. Proton NMR and 13C NMR analysis (carbonyl -carbon signal at 178 ppm; aromatic group at 145.5, 119.45 and 116.13; C-O signal at 73 and 72 ppm from ; 62.67 ppm signal from methoxyl; between other signals. All these date suggest the probable structure as **3- methyl- 4β - methylcarboxymethyl – gallocatechin** (Figure 5)

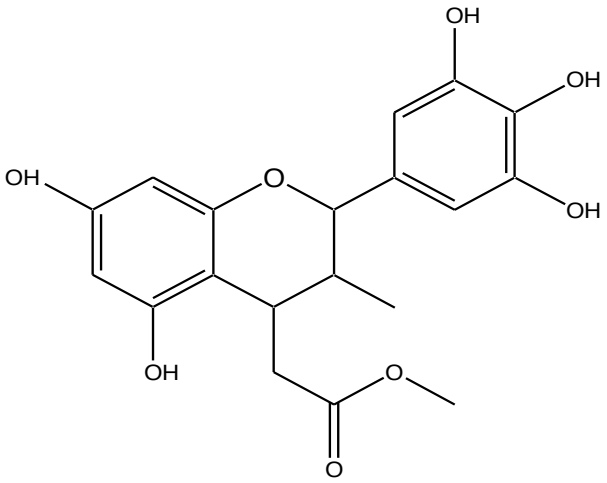


Figure 5. Structure of 3- METIL 4 β METHYLCARBOXYMETHYL – GALLOCATECHIN.

Compounds named 28 sob, 70 mg, red solid, Rf= 0.6. HPLC peak at tr= 1.65 min. It UV max spectra (MeOH): 237, 277, 394, 457 and 484 nm. FAB+ spectra, $(M + 1)^+ = 413$. IR spectra with signal at 3423 cm-1 (Figure 6). NMR analysis suggest as probable structure **3-metoxi (4 propyl carboxymethyl- gallocatechin)** (Figure 7).

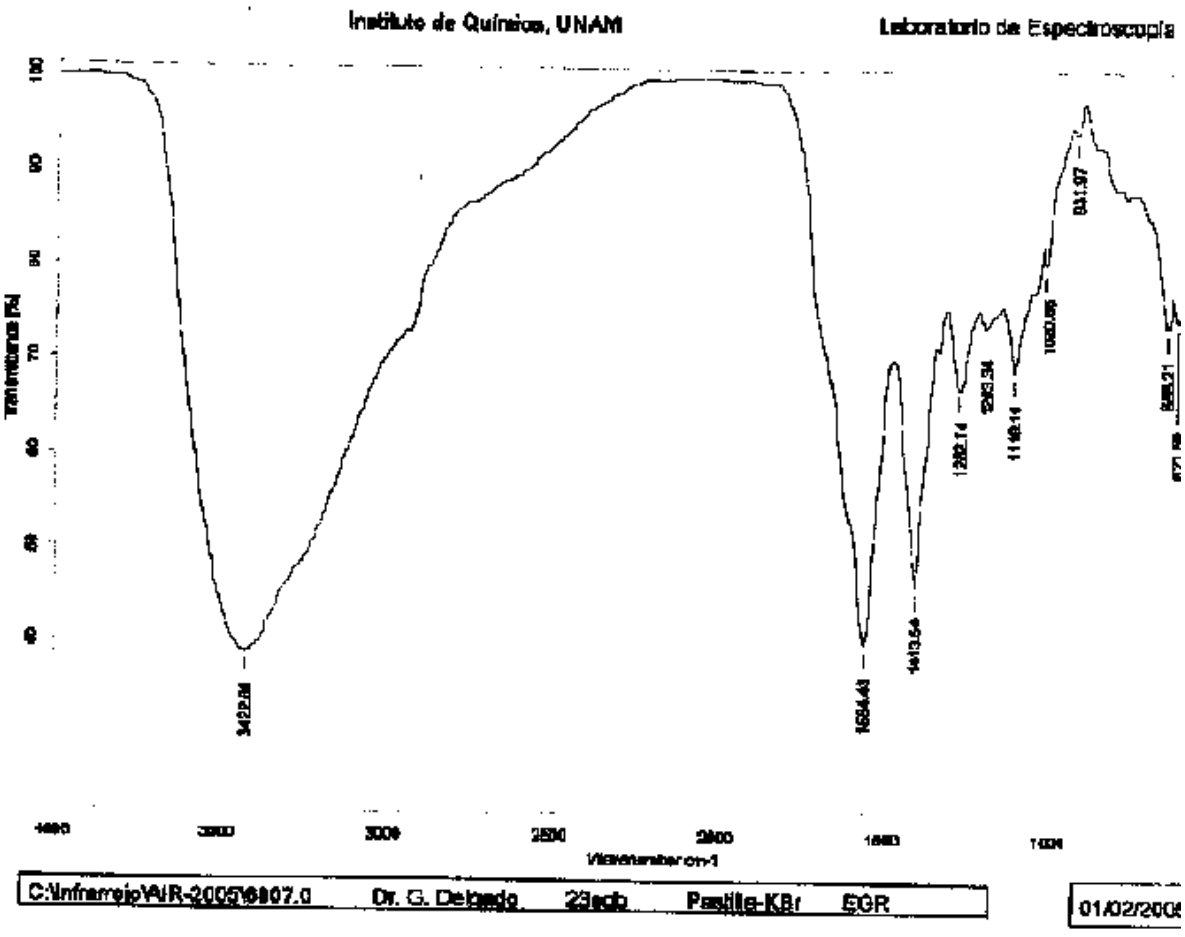


Figure 6. IR spectra of 28 sob.

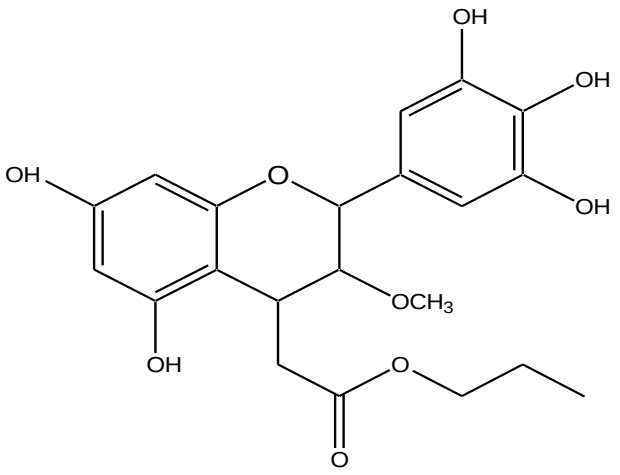


Figure 7. 3-methoxi (4 propyl carboxymetyl- gallocatechin) structure.

3.3.2 Fractionation of butanolic extract by Adsorption Chromatography.

Using column adsorption chromatography similar to Kandil et al.2004 [15] with leaves of *R. mangle* permit the isolation and elucidation of new structures in this extract. For example:

Fatty acid: Stearic acid

Esther- phytosterol: Sitosterol with a lateral chain of fatty acid.

p- methoxibenzoic acid, from fraction 17. This acid is precursor of polyphenol compounds.

Cathechin and epicatechin, from 10- 15 fraction.

From fraction 32, eluted with ethyl acetate- MeOH 1:1, was purified 93 mg of cream color crystal, Fusion Melting= 220- 225°C, pure by HPLC. Rf= 0.68, Ethyl acetate: MeOH: water, UV 254 nm, CeSO4 and with vanillin typical of flavan-3-ol. λ max= 265.3 nm, tr= 3.21 min. FAB+ $(M + 1)^+$= 338 other fragments m/e: 309, 257, 242, 235, 139. Ions at Ions 257 and 139, characteristic of catechin gallates (Figure 8).

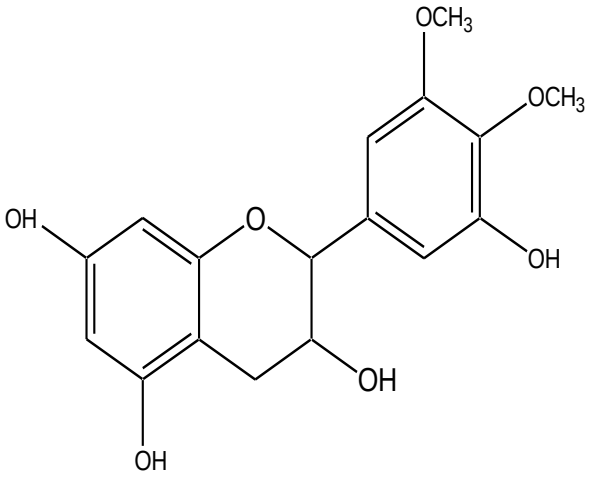


Figure 8. Methyl Epigallocatechin.

Compounds identified in 38- 40 fractions (named 10 C4, 13C4 y 15C4).

Compound 10C4: tr= 1.58 min, λmax= 237, 282, 448 nm; Rf= 0.63 (butanol: acetic: water, 5:4:1). $FAB^+ = (M+1)^+ = 482$ fragment pattern similar at 13C4. Monomeric structure no identified.

Compounds 13C4, tr= 1.42 min; λmax= 237, 277, 394, 457, 484 nm; FAB+: $(M + 1)^+ = 482$, (M + 1 + Na)+= 505. The probable structure of this compound is **epicatechin propilgallate** (Figure 9).

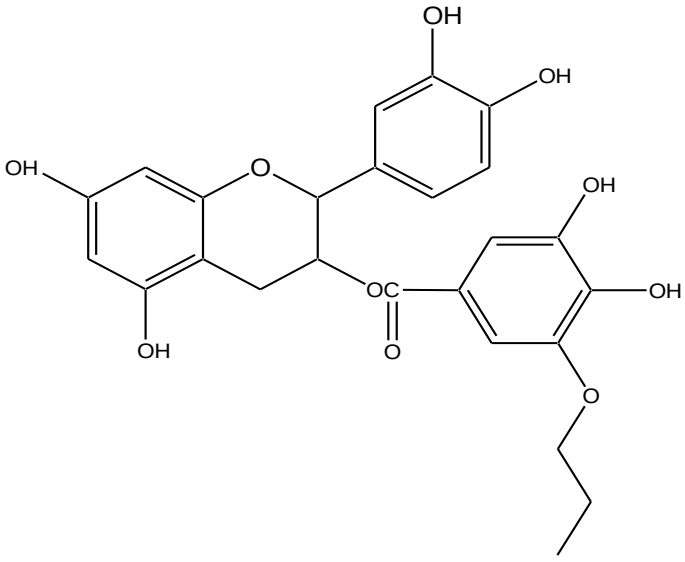


Figure 9. Epicatechin propilgallate structure.

Compound 15C4, tr= 1.47 min, λmax= 251, 395, 450 nm, Rf= 0.8 (butanol: acetic: water, 5:4:1), FAB^+= $(M+1)^+$ = 939, Trimmer no identified.

3.4 Fractionation and structural elucidation from minor fraction, low molecular weight polyphenols (ethyl acetate extract).

Proton NMR analysis from 4 – 6 fraction, 4 mg, Rf= 0.83 purple with anysaldehyde has a basic structure describe in Figure 10.

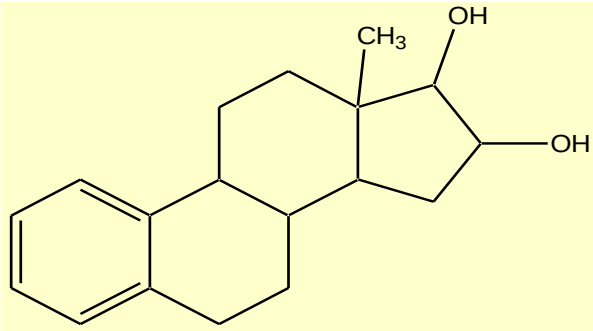


Figure 10. Basic structure by steroidal compound isolated from 4-6 fraction of ethyl acetate extract.

Fraction 12- 13 was observed Gallic acid by comparison by TLC with Gallic acid standard, Rf= 0.61, similar coloration and form of spot with anysaldehyde and FeCL3.

In this fraction were isolated other compounds as II_3, 15 mg, RF0 0.39, tr= 1.816 min (HPLC/Mass). Mass spectra with $(M+1)^+$= 485 and fragment ions at m/e: m/e: 470, 453, 437, 291, 159, 139, 128. Presence of fragment at m/e 291 y 139 from catechin and m/e 139 type gallate. UV max (MeOH): 226, 257 y 287 nm. Proton NMR spectra with sugar signal. The most probable structure by this compound was methoxi- gallocatechin glucose (Figure 11).

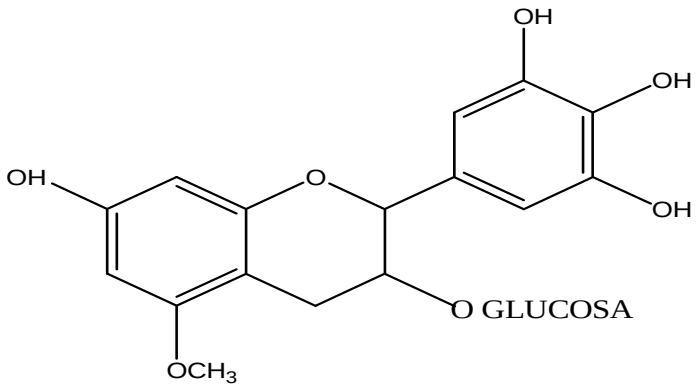


Figure 11. Methoxi- gallocatechin glucose structure.

Compound II_4, White needle, 12 mg, Rf= 0.12, brown color with anysaldehyde and blue with FeCl3; tr= 1.48 min. Proton NMR at 300 and 400 mHz are characteristic of gallocatechin. 13C NMR with presence of carbonyl and aromatic groups. Mass spectra (M+1)+= 462, fragment ions at m/e: 445, 257, 159 y 139. This compounds correspond at gallocatechin gallate (Figure 12).

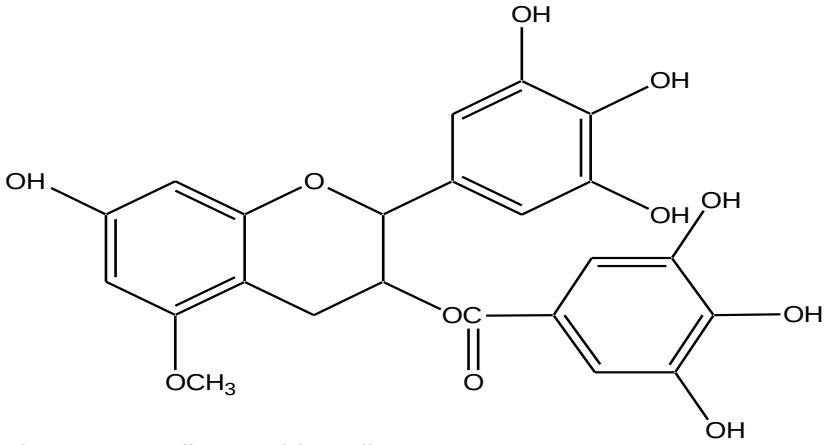


Figure 12. Gallocatechin gallate structure.

II_5, 8 mg, Rf= 0.10 UV 254 nm, tr= 1.8 min. Mass spectra (M+1)+ = 453 and fragment ions at m/e: 437, 291, 259, 159 y 128. UV max (MeOH): (M+1)+= 453 e iones fragmentos a m/e: 437, 291, 259, 159 y 128. The structure for this compounds was catechi-3-O-glucose (Figure 13).

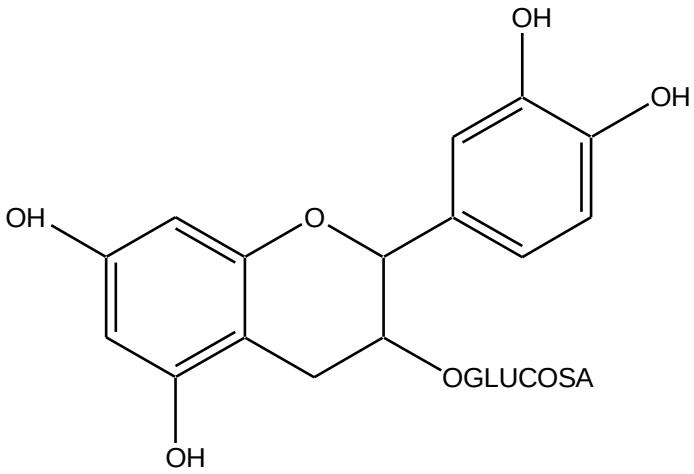


Figure 13. Catechin -3-O- glucose.

Fraction 18 – 19a, Rf= 0.66 UV 254 nm and orange with anysldehyde typical of proantocyanidin; tr= 1.844 min. and (M+1)+= 489 other ions at m/e= 415, 173, 127, 113, **cyanidin 3-acetylglucoside.**

Fraction 18 – 19b, Rf= 0.37 orange with anysldehyde typical of proantocyanidin; tr= 1.94 min. UV max= 211, 277, 392, 492, 525 nm and (M+1)+= 593 other ion at m/e=284, H+ NMR with a signal at λ= 8,44 ppm (s); **cyanidin 3-rutinoside** (Fig. 14). With other ion (M+1)+= 355, tr = 7.51 min, correspond with **chlorogenic acid.**

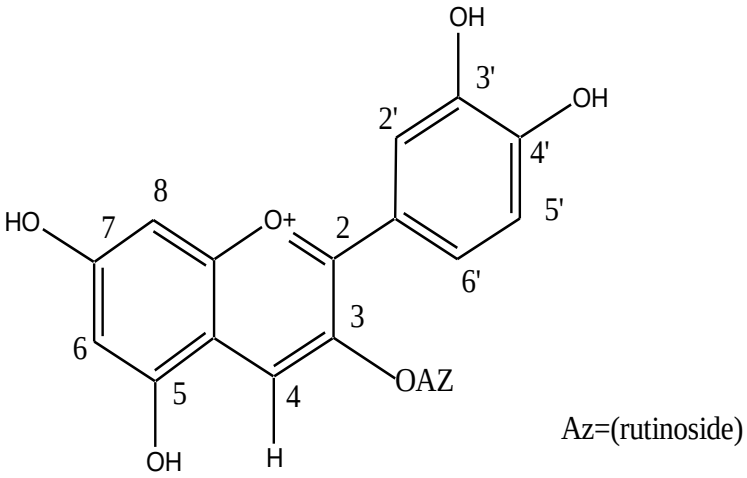


Figure 14. Cyandinin 3- rutinoside.

In fraction 20 was identified **epigallocatechin gallate**, with mass spectra (M+1)+= 459 and fragment ions at m/e= 291, 290, 289, 273, 257, 169, 125, 139 (Fig. 15).

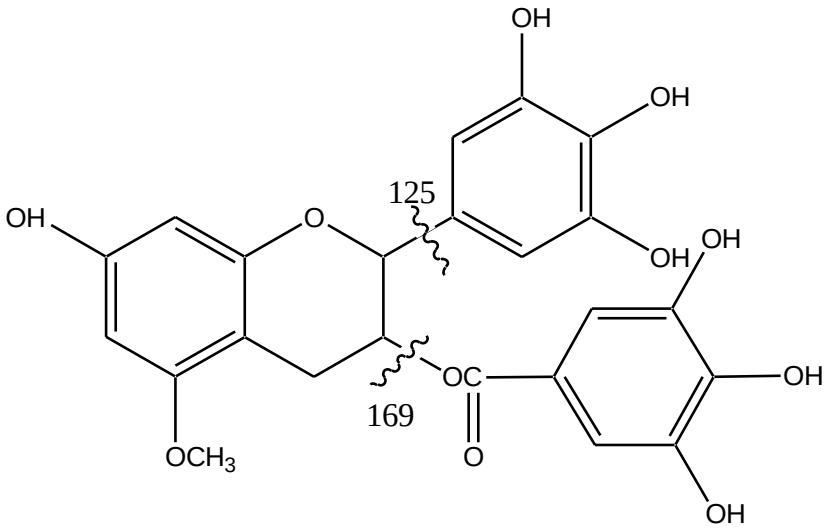


Figure 15. Epigalocatechin gallate.

In fraction 21 was identified glucoside of **Proatocianidin A**, Rf= 0 0.43 orange with anysaldehyde (M+1)+= 737 other fragments m/e= 577, 575, 453, 437, 419, 291, 249, 181, 127. (Fig. 16). In the following table was described the chemical shits of H+ NMR.

Ring	H	δ (ppm)
C	3	4.07 d
	4	4.25 d
A	6	5.93 d
	8	6.07 d
B	10	7.13 d
	13	6.71 d
	14	7.01 d
F	2´	4.78 d
	3´	4.13 m
	4´α	2.57 dd
	4´β	2.78 dd
	6´	6.07
	10´	6.94 d
	13´	6.81
	14´	6.81

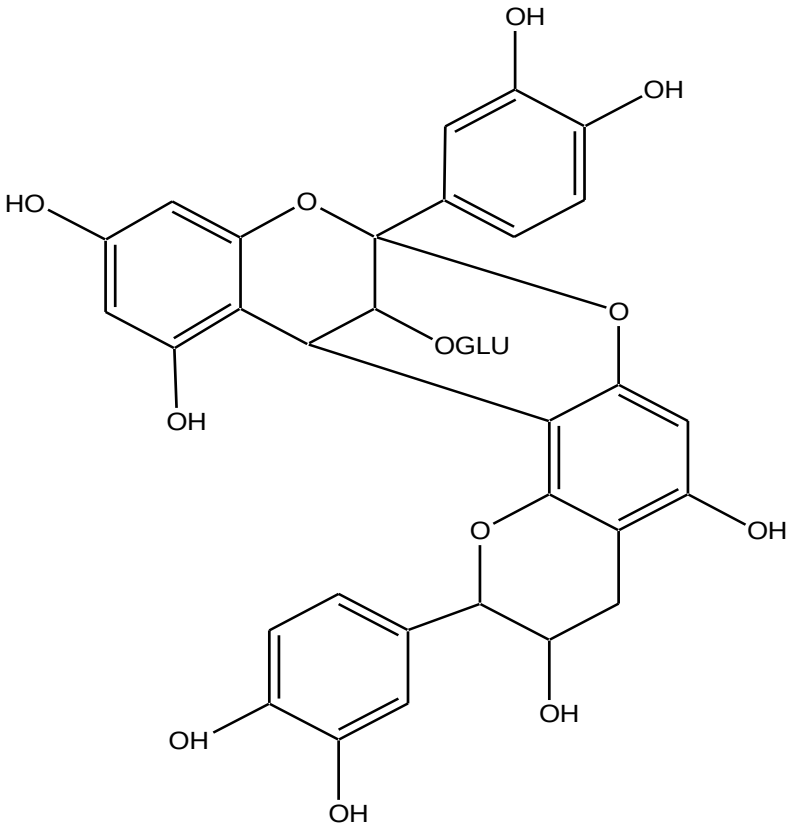


Figure 16. Glucoside of Proatocianidin A

From 22 – 24 fraction were isolated different compounds: IV 6(1) band, 50 mg, red color, Rf= 0.35, positive with anysaldehyde, UVmax (MeOH)= 220, 281, 400, 479 nm. Mass spectra EI+ (M+1)+= 446, of (epi)catechin gallate (442). HPLC/Mass tr1= 1.845 min. (major peak) with (M+1)+= 577 and other ions at m/e= 570, 533, 490, 453, 437, 425, 369, 301, 289, 249, 211, 163, correspond with **Proantocianidina A-1**, H+ NMR: δ 8.9 ppm brs de OH y δ 9.31 ppm brs de OH.

IV 6(2), 30 mg, Rf= 0.29 positive with anysaldehyde, UVmax (MeOH)= 228, 278, 432. HPLC/Mass tr= 1.861 min (major peak) with (M+1)+= 621, dimer. In the sample was remaning of malvidinin (m/e= 331) and peonidin (m/e= 301). Major compound was **delfinidin 3-galactoside**.

IV 7, 90 mg, Rf= 0.29, positive with anysaldehyde. UVmax (MeOH)= 219, 286, 324(Majors), 439, 532nm (minor). HPLC/Mass, tr= 1.829 min. (major), (M+1)+= 477 (480), other fragments at m/e= 429, 411, 353, 335, 313, 255, 211, 154, 141, 127, 109. Presence in UV spectraof maximum at 532 nm is typical in antociadinin. However, molecular ion M+= 479 correspond with **petunidin-3-glucoside** , Figure 17, H+ RMN:

δ 8.12 (1H, s, H-4)

δ 7.18 (1H, d, H-2´)

δ 6.84 (1H, s, H-8)

δ 6.72 (1H,s, H-6´)

δ 6.56 (1H, s br, H-6)

δ= 5.931 (anómero) glucosa (s)

δ= 4.44 m
3.79 d
3.74 d
} azucar: glucosa

3.50 m

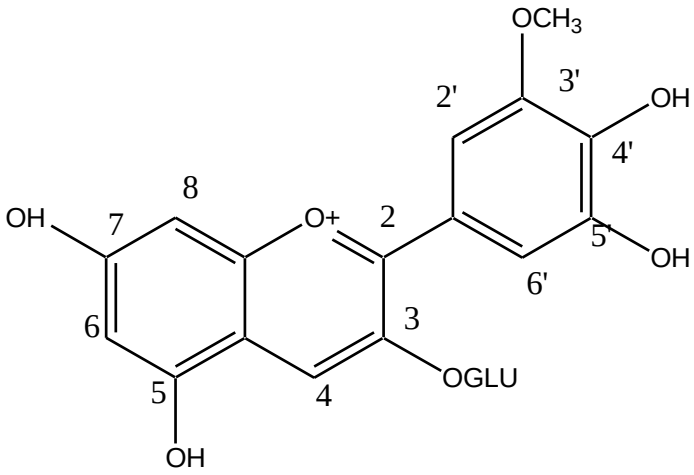


Figure 17. Petunidin-3-glucoside structure.

IV 7 Resin (2), 35 mg, UVmáx (MeOH)= 218, 282, 445 nm. HPLC/Mass tr= 1.899, 1.94, 2.006, 2.05 min. El+ (1.7 – 1.8 min) dio: (M+1)+= 564 fragment ions at m/e= 547, 413, 369, 353, 321, 255, 154, 141, 127, 109, **dimero: epi (catechin)- 4-epi(afzelechin)** (Fig. 18).

564 Por Retro-Diels-Alder m/e= 412

(RDA) (- 152 u) (loss of dihidroxi- α- hidroxisterene)

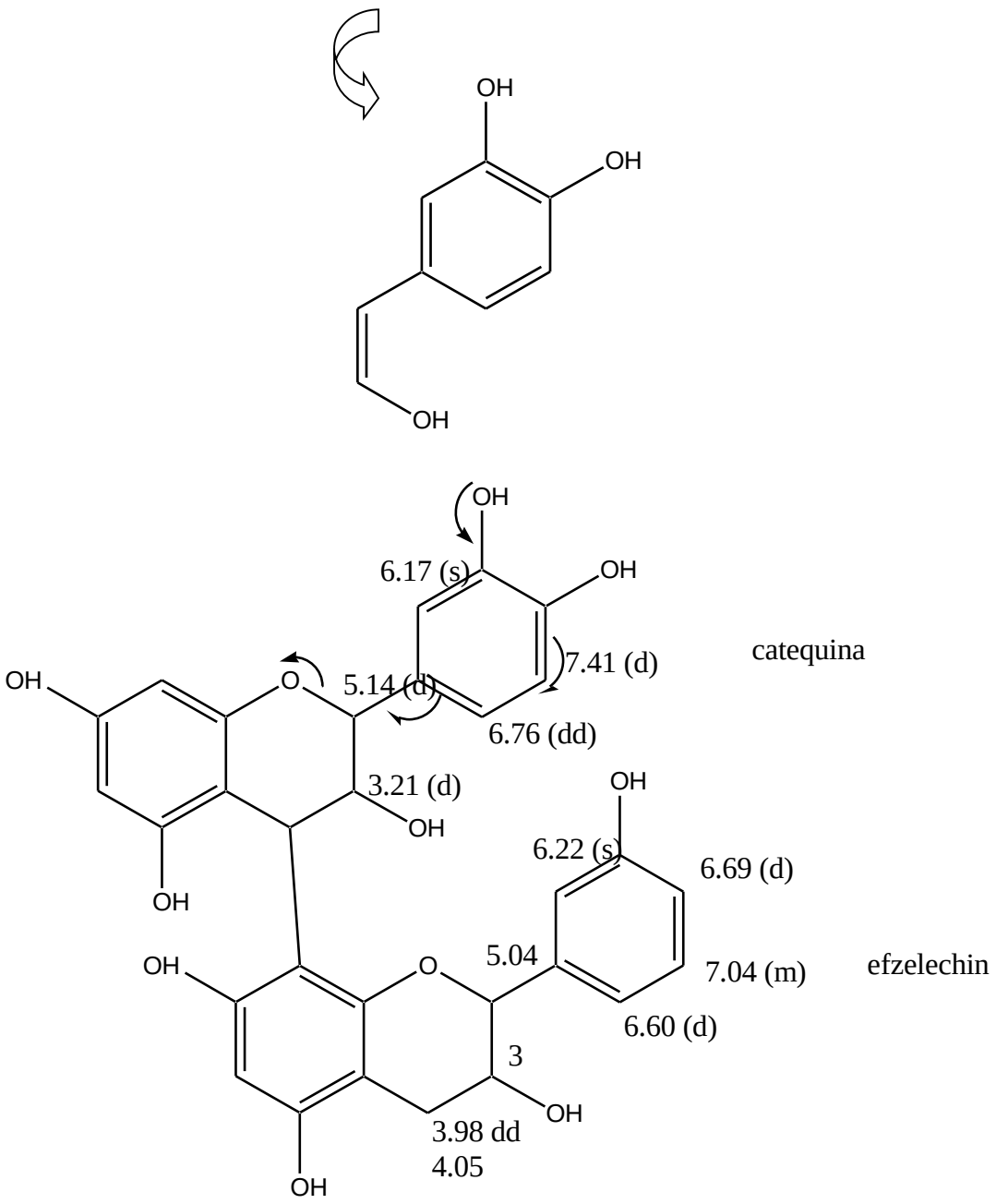


Figure 18. Epi (catechin)-4-epi (afzelechin).

4. Discussion

The development of gastric ulcers is a complex and multi- factorial process including bacterial infections, the increase of acid secretion, generation of reactive oxygen species (ROS), inhibition of the endogenous PGs, and the degradation of the extracellular matrix (ECM). Research during the last decade has offered new insights in the preventative

therapy and the healing of gastric ulcers and the synergistic efficiency of a multi-target approach based on individual mechanisms of action could be the new perspective for treatment of this disease (Pinheiro et al., 2015).

Helicobacter pylory is a Gram- negative, helical rod that colonizes human gastric epithelium. It plays a causal role in chronic gastritis and peptic ulcer, and is an important factor in the ocurrence of gastric cancer and gastric mucosa- associated lymphoid tissue lymphoma (MALToma). Thus, the eradication of *H. pylor*i can contribute to the treatment and prevention of these diseases. *H. pylori* eradication accelerates pecti ulcer healing, reduces the recurrence of gastric cancer after resection, and leads to regression of low-grade gastric MALToma. Currently, new triple therapies consisting of two antibiotics and a proton pump inhibitor show high eradication rates. However, some problems remain. *H. pylori* rapidly acquires resistance to some antibiotics. *H. pylori* strains resistant to clarihromycin and metronidazole are now increasing, which will reduce eradication rates. In the future, antibiotic resistance will be the greastest obstacle in the treatment of *H. pylori* infection. Furthermore, new triple teraphies upon occasion cause side effects; nausea, vomiting, epigastric pain, abdominal discomfort, diarrhea and another point, the significant cost of combination therapy . Therfore, a new antibacterial agent, which isboth highly effective and safe, is required for the treatment of *H. pylori* infection (Funatogawa et al., 2004).

As phytomedicine has proved to be an untapped treasure for the discovery of lead compounds to cure gastrointestinal disorders. Hence several studies have been aimed to evaluate the anti-helicobacter pylori activity of medicinal herbs (Kiranmai et al., 2012).

Rhizophora mangle L. is a tree high distribute in tropical countries. We report preview antiulcer effect of the total extract from bark of this tree, by proton bomb inhibitor. In the present work, we found an antibacterial activity *in vitro* e *in vivo* against *Helicobacter pylori.* The MIC and MBC of total aqueous extract is similar or better at the report with other species with anti- Helicobacter properties (MIC 90%= 0.18 mg/ml and MBC 90%= 0.45 mg/ml); for example, *Terminalia catappa* L. with a presence of some phenolic compounds present a MIC of 0.125 mg/ml) (Pinheiro et al., 2015), a flavonoid rich extract of *Glycyrrhiza glabra* Linn, which is commonly known as Licorice shown a MIC of 0.1 mg/ml against a ATCC reference strain of *H. pylori* (Patel, 2014). *Ageratum conyzoides, Scleria striatinux, Lycopodium cernua, Acanthus montanus, Eryngium foetidium, Aulutandria kamerunensis, Tapeinachilus ananassae, Euphorbia hirta, Emilia coccinea and Scleria verrucosa* in methanol extracts shown MIC and MBC btween 0.032 – 12.5 mg/ml (Ndip et al., 2007).

Presence in this extract of a very high prorportion of polyphenols compounds: proantocianidin and cyanidin join with other compounds terpenoid, catechin, epicatechin, EGC, EGC methyled, GCG, EGCG, chlorogenic acid, gallic acid and buthyl carbamate made of this extract very rich in compound with antibacterial activity.

Aqueous Extract from *Peumus boldus* Mol. has a potent anti-urease activity and anti-adherent effect against *H. pylori*, properties directly linked with the presence of catechin-derived proanthocyanidins (Pastene et al., 2014).

Funagotogawa et al., 2004 described the effect of hydrolizable tannins isolated from different extract of plants on lipid bilayer membranes, therefore, they have potential as new and safe therapuethic regimens against *H. pylori* infection, especially monomeric compounds with a strong activity.

Other phenolic compound, Curcumin from *Curcuma longa* has recently been shown to arrest *H. pylori* growth. The anti-Helicobacter pylori activity of curcumin against 65 clinical isolates of *H. pylori in vitro* was examined. Minimum inhibitory concentration ranging from 5-50 µg/ml, showing its effectiveness against *H. pylori* growth *in vitro* irrespective of genetic makeup of strains (Kiranmai et al., 2012).

Aqueous and butanol fractions of *Punica granatum* L. showed good activity on *H. pylori* clinical isolates with MICs of 156 and 195.12 µg/mL, related to tannin and phenolic compounds as proantocianin and ellagic tannin (Mahboobe, 2011).

Ilex paraguariensis A. and *Chamomila recutita* L., were capable of inhibiting the in vitro growth of H. pylori associate at the presence of phenolic compounds, triterpenes, flavonoids and essential oils (Cogo et al., 2010). Other natural compounds with anti-Helicobacter pylori activity are described quinones, coumarins, terpenoids and alkaloids (Wang, 2014).

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