Asian Journal of Pharmaceutical Research and Development
(An International Peer-Reviewed Journal of Pharmaceutical Research and Development)

Open Access Available online at http://ajprd.com/index.php Research Article

TERPENOIDS AND PHYTOSTEROLS ISOLATED FROM *PLUMERIA RUBRA* L., FORM *ACUTIFOLIA* (AIT) WOODSON AND ITS FUNGUS PARASITE *COLESPORIUM PLUMERIAE*

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ABSTRACT

The hexane and ethyl acetate extracts of the leaves from *Plumeria rubra* L., form *acutifolia* (Ait) Woodson and the methanolic extract from the fungus *Coleosporium plumeriae* Pat. were analized by high resolution gas chromatography (HRGC) and by high resolution gas chromatography coupled to mass spectrometes (GC-MS). The identification of the chemical constituintes is these extracts has been made by the automatic comparison of the mass spectra obtained standards within the NBS, Wiley and Wiley 275T librarys supplied with the mass spectrometer and by analized of the principal fragmentations. Phytochemical studies of the hexane and ethyl acetate extracts of the leaves showed nine terpenoids (5-ergosten-3β-ol, 5,22-stigmastadien-3β-ol, 4-stigmasten-3-one, 5-stigmasten-3β-ol, 12-ursen-3β-ol, 12-oleanen-3β-ol, 12-oleanen-3-one-28-oic acid, ursolic acid and 3β-acetoxy-12-oleanen-28-oic acid). The compounds 5-ergosten-3β-ol, 4-stigmasten-3-one, 12-oleanen-3-one-28-oic acid, ursolic acid and 3β-acetoxy-12-oleanen-28-oic acid have been described for the first time from *Plumeria rubra* L., *forma acutifolia* (Ait) Woodson. From the methanolic extract of spores of the fungus *Coleosporium plumeriae* Pat were isolated 5-stigmasten-3β-ol, methyl esters of long chain fatty acids (C18:0, principally) and one aromatic compound, *N*-methyl toluenesulfonamide.

Keywords: *Plumeria rubra* L., form *acutifolia, Coleosporium plumeriae* Pat, terpenoids, phytosterols GC/ MS, HRGC.

Article Info: Received: 21 Sep 2018; **Review Completed**: 14 Nov 2018; **Accepted:**02 Dec 2018**; Available online**:15 Dec 2018

Cite this article as:
Barreto A. S., Resende, C. M.; Pinto, P.R.; Nascimento C. C. H. C., Vasconcelos, S. D. D. De, Azevedo, L.A.C., Stephens P. R. S., Nogueira R. I., Saranraj, P., Diré G. F.,Pinto, A.C., Terpenoids And Phytosterols Isolated From *Plumeria Rubra* L., Form *Acutifolia* (Ait) Woodson and Its Fungus Parasite *Colesporium Plumeriae,* Asian Journal of Pharmaceutical research and Development.**2018;6(6):15-22**

DOI: http://dx.doi.org/10.22270/ajprd.v6i6.428

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INTRODUCTION

Plumeria rubra L., form acutifolia (Ait) Woodson belongs to the subfamily Plumerioideae (Apocynaceae). In this species have been described iridoids[7,33, 35], terpenoids[35, 36], lignans[7], glycosylated flavonoids[6], many of them with antitumor, bacterial, fungicidal and anti-AIDS action[1-6, 12-15]. The species *Plumeria rubra L.*, is found throughout the tropical and subtropical world[33-36]. It has arboreal habitus and its size varies from 5 to 8 meters in height[33-36]. This species is susceptible to attack by *Coleosporium plumeriae* Pat.[7-11] [= *Coleosporium dominguensis* (Burk.) Arth], the rust fungus.

The genus *Coleosporium* belongs to the Family Coleosporaceae of the Order Uredinales. This family

has two other genera and nearly 80 cosmopolitans including the genus *Coleosporium*. The genus has numerous described species, many of which are doubtfully distinct morphologically[27]. The specimen *Colesporium plumeriae* was initially detected in *Plumeria obtusa* leaves and *P. rubra* L. varieties.

This disease occurs mainly in the rainy season. This fungus compromises the sale of the ornamental representatives of this family due to the fall of its leaves. Sometimes, this defoliant action can reach close to 100% [7-10]. This fungus is chemically controlled by foliar fungicides[11], or through the use of the fungus *Verticilium lecanii*[12] that feeds on the colonies of *Coleosporium plumeriae*. *Verticilium lecanii* was used, initially and commercially, in Europe in the biological control of different species of insects and other fungi that cause the rusts[8, 12]

[*Uromyces appendiculatus*, *Uromyces dianthi* (Pers) Niessl, *Puccinia recondita* f. sp.tritici Rob. ex. Desm. and *Puccinia striiformis West*]. The use of *Verticillium lecani*i proved to be more advantageous in combating rust in *Plumeria* sp. than the foliar fungicides most used, many of these toxic to other life forms[12]. However, there is still a need to quantitative determine in the field the number of applications of *Verticillium lecanii* needed to control this disease. Many investigators[7-10], mainly from phytopathology, are interested in the life cycle and in the ways of combating this fungus *(Coleosporium plumeriae* Pat.). However, there has not yet been a concern to identify what kind of substances are produced, absorbed and / or metabolized by these fungi.
This work aims to identify by CGAR - MS the major secondary metabolites in extracts in hexane and ethyl acetate from the leaves and to identify the main chemical constituents of the fungus *Coleosporium plumeriae* Pat., The main one responsible for the fall of the leaves of *Plumeria rubra* L.

EXPERIMENTAL

Extraction and isolation of substances from *Plumeria rubra* L., form *acutifolia* (Ait) Woodson and *Coleosporium plumeriae*. The species *Plumeria rubra* L., form *acutifolia* (Ait) Woodson was collected on the campus of the Federal University of Rio de Janeiro by one of the authors and deposited at the Institute of Biology of the Federal University of Rio de Janeiro under the number 24346. It was used 500g of fresh and healthy leaves of Plumeria rubra L., form acutifolia (Ait) Woodson. After concentration of the solvent on a rotary evaporator the extracts were extracted into hexane (18.5 g), ethyl acetate (21.5 g) and methanol (37.3 g). A part of the leaf hexane (**A**; 30mg) and ethyl acetate (**B**; 30mg) extracts were then methylated with 0.5mL of ethereal diazomethane solution in a separate vial, which were then capped and left at room temperature for 30 min to allow the methylation reaction to go to completion.

Then, the solvent were evaporated under nitrogen and analysed by GC (HP 5790 GC and HP 5880) and GC-MS using an equipament Hewlett Packard 5897 GC-MS and 6890 GC-MSD - model Quadrupole and MS operating at 70eV ionization energy.

The fungus *Coleosporium plumeriae* Pat. was collected from the leaves of *Plumeria rubra* L., form *acutifolia* (Ait) Woodson in the States of São Paulo Campinas and in Rio de Janeiro on the Oswaldo Cruz Foundation campus. It was classified according to literature data and in consultation with phytopathologists[7-10] specialists in diseases of *Plumeria rubra* L.

The pustules (*Colesporium plumeriae* Pat.) were collected from the adaxial part of *Plumeria rubra* leaves. They looked like a bright yellow orange powder arranged in an ellipsoid shape. The collection was made with the aid of dry cotton. Then, dry cotton were extracted (3 × 200 mL) with methanol, at room temperature. The yellow resulting solution was filtered and concentrated in vacuum to yield 90,5mg of the crude extracts methanolic.
A part of the methanolic extract (30mg) was submitted to preparative (1mm) silica-gel TLC (Thin-Layer Chromatography) purification, using EtOAc: C_6H_{14} 7:3 as eluent. The three fractions obtained from the preparative TLC (**C**- 5mg; **D**- 6mg and **E**- 5mg) were then methylated with 0,5mL of ethereal diazomethane solution in a separate vial, which were then capped and left at room temperature for 30 min to allow the methylation reaction to go to completion. Then, the solvent was evaporated under nitrogen and analysed by GC (HP 5790 GC and HP 5880) and GC-MS using an equipament Hewlett Packard 5897 GC-MS and 6890 GC-MSD - model Quadrupole and MS operating at 70eV ionization energy.

Chromatographic analysis

Samples **A**-**E** (Figure 2 - 3) dissolved in chloroform were run fully at a range of 40-500 m/z and the results were compared by using Wiley Spectral library search programme. We used an SE-54 [(5%-phenyl) (1%-vinyl)-methylpolysiloxane] used sílica capillary column, we used a 30m x 0,25mm i.d.; 0.25□m film thickness. Helium was used as carrier gas at a flow rate of 2mL/min. The HRGCMS oven temperature was kept at 60°C for 5min and programmed to 290°C at and then kept 290°C for 20min. The amount of injection was 1βL. Samples were injected using the split mode (split ratio 1:10).

The analysis by gas chromatography with flame ionization detector in and automatic injection system (HRGC-FID) and gas chromatography-mass spectrometer (GC-MS) were performed in duplicate. HRGC-FID analysis were performed on a HP 5790 GC and HP 5880, using the same column with the same temperature programming utilized for GC-MS analysis. The temperatures of the injector and detector was 260°C and 290°C respectively. The temperature of the oven, injector and detector were the same as used in HRGC-MS. Helium was used as carrier gas at a flow rate of 2mL/min split in either 10:1 ratio.

RESULTS AND DISCUSSION

P. rubra L., form *acutifolia* (Ait) Woodson

The results of the chromatographic analysis of extracts in hexane and ethyl acetate of leaves of *P. rubra* L., form *acutifolia* (Ait) Woodson (Figure 1) evidenced the presence of fatty acid methyl esters and nine terpenoids [5-ergosten-3β-ol (**2**), 5,22-stigmastadien-3β-ol (**3**), 12-ursen-3β-ol (**4**), 5-stigmasten-3β-ol (**5**), ursolic acid (**6**) 12-oleanen-3β-ol (**7**), 4-stigmasten-3-one (**8**), 3β-acetoxy-12-oleanen-28-oic acid (**9**) and 12-oleanen-3-one-28-oic acid (**10**); Figure 1, 4 - 6]. The structural proposals for the various components of the extracts were carried out based on the automatic comparison of mass spectra of the components with the literature[13-18].
The survey of the terpenoid composition of the genus *Plumeria* revealed that its most frequent triterpene structures are pentacyclic[13-18]. The most abundant are hydrocarbons having one or two double bonds, monohydroxylated, mono or bicarbonylated and mono or bi-acetylated. In addition to molecular ions, the most frequent skeletal fragmentation routes were used in species of the Apocynaceae family.

These skeletons belong mainly to the group of ursans and oleanans[14, 15] which present in their mass spectra an intense ionic fragment of m/z 218 corresponding to C-12 unsaturation (Figure 1, 5 and 6). The presence of the peak m/z 262 in the spectra of substances **6**, **9** and **10** (Table-1; Figure 1, 5 and 6) indicates the presence of an acid group (in methylated form) bound to C-17. The m/z 205 fragment in substance **10** (Table 1) corresponds to the presence of a carbonyl at C-3. The water loss characteristic of steroids[17,18,32] was observed by the peak m/z 382 in the mass spectra of 5-ergosten-3β-ol no (**2**) and m/z 396 of 5-stigmasten-3β-ol (Table 1, Figure 2**)**. The mass spectrum of 5,22-stigmastadien-3β-ol [**3** (Table 1; Figure 3)] showed the m/z 351, 300, 271 and 255 fragments corresponding to fragmentation of the side chain.
The cleavage of the D ring to 5,22-stigmastadien-3β-ol (**3**) was observed by the fragment m/z 213, which is identical to the fragment proposed for 5-ergosten-3β-ol (**2**; Figure 1 and 4), since the skeleton is the same for the four steroids, with only the side chain, with the exception of substance **8** (Figure 1 -5), which has a ketone function in C-3. The presence of the m/z 124 base compound of the **8** [4-stigmasten-3-one compound (Figure 1 and 5, Table 1)] is indicative of B-ring cleavage of the m/z 412 molecular ion of alpha and beta unsaturated ketones.

Coleosporium plumeriae Pat

HRGC analysis (Figure 3) provided the chromatograms of the total ions of two fractions originating from the spores of *Coleosporium plumeriae* Pat. Four carboxylic acids were identified [hexadecanoic acid (**a;** C16:0), 9,12-octadecadienoic acid (**b;** C18:2), octadecenoic acid (**c;** C18:1), n-octadecanoic acid (**d;** C18:0)], an alcohol [*n*-octadecanol (**e**)], a steroid [5-stigmasten-3β-ol (**5**)] and an aromatic compound [*N*-methyl toluenesulfonamide (**1**); Figure 3]. The mass spectra obtained from the carboxylic acids and 5-stigmasten-3α-ol (**5**) were similar to those found in the extracts in hexane and ethyl acetate from the leaves of *Plumeria rubra* L., form *acutifolia* (Ait) Woodson of which the fungus was collected. The phytosteroids stigmasterol, sitostenone, squalene, ergosterol and ergosterol peroxide were isolated from *Colletotrichum gloeosporioides*, an endophytic fungus, isolated from *Virola michelli*, a typical Amazonian plant, used in popular medicine to combat skin infections[26]. The presence of phytosterols in fungi is rare and this is the first report of the isolation of the phytosterol 5-stigmasten-3β-ol, from *Colesporium plumeriae*. The mass spectrum of substance 1 revealed a molecular ion m/z 155 and an intense peak m/z 91 (100%). The latter corresponds to the ion tropylium. It also observed an intense peak in m/z65 (35%), possibly produced by the removal of a neutral acetylene molecule from the tropylium ion [19-21, 31, 32] (Table 1; Figure 3).

The *N*-methyl toluenesulfonamide compound (**1**) is probably the degraded form of a herbicide. In recent years the level of detection of herbicides in the soil has reached alarming levels, and is currently one of the concerns of environmentalists. The residence time of these substances in the soil varies according to the chemical composition, pH, organic matrix and temperature[66-68]. Herbicides of the sulfonylurea type, such as "Chlorosulfuron" {2-chloro-*N*- [(4-methoxy-6-methyl-1,3,5-triazinyl)–amino] carbonyl} –benzene sulfonamide], are used in control of weeds[22]. This compound has already been detected in different layers of the soil and its residence time is extremely long[19-22]. Researchers have observed that acidic soils with (pH less than 7) tend to accelerate the degradation of Chlorsulfuron-like compounds due to the high concentration of H^+ ions that accelerate the cleavage of the sulfonylurea bridge.

Veeh[19-21] and collaborators believe that the degradation process of Chlorsulfuron like herbicides could also be achieved by micro-organisms. This is not surprising, since the literature is full of works that demonstrate the enzymatic versatility of microorganisms, such as fungi, for example, against the most different substrates. Hydrolytic enzymes from yeasts[23-25] (*Pseudomonas cepacia*) are capable of hydrolyzing esters of toluene sulfonamide derivatives in racemic alcohols. In addition, the literature cites work on the enantioselective hydrolytic capacity of fungi[21-25] on different substrates.

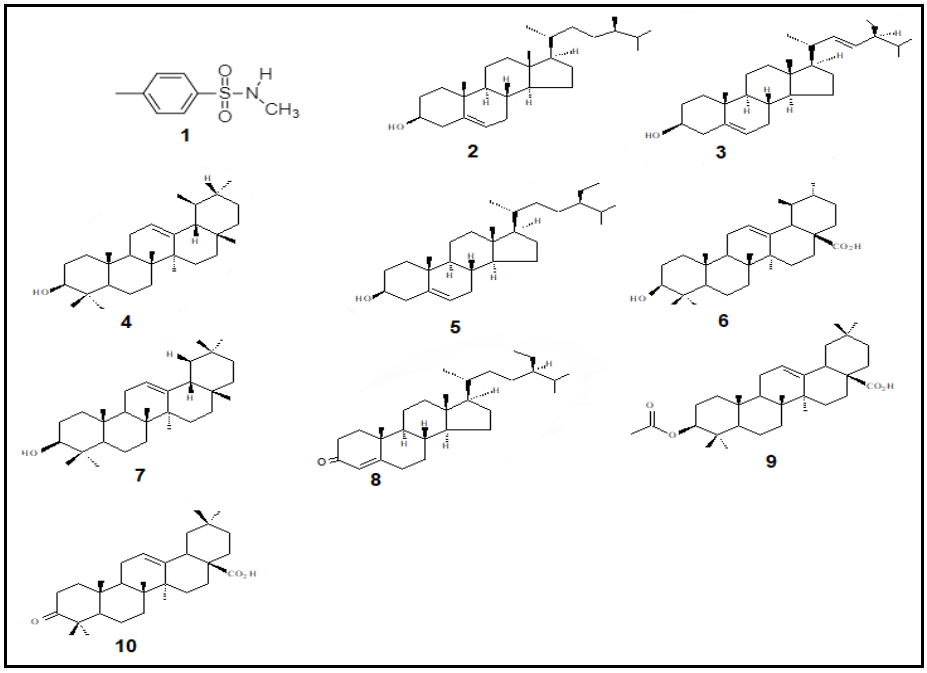
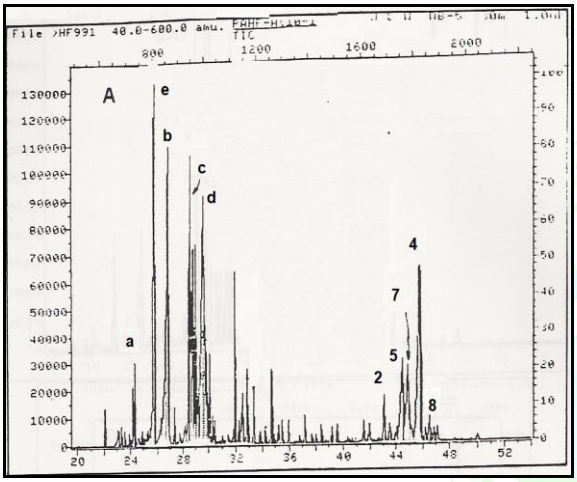
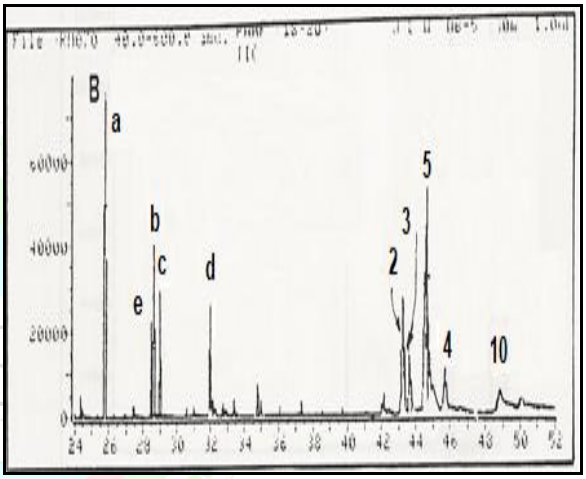


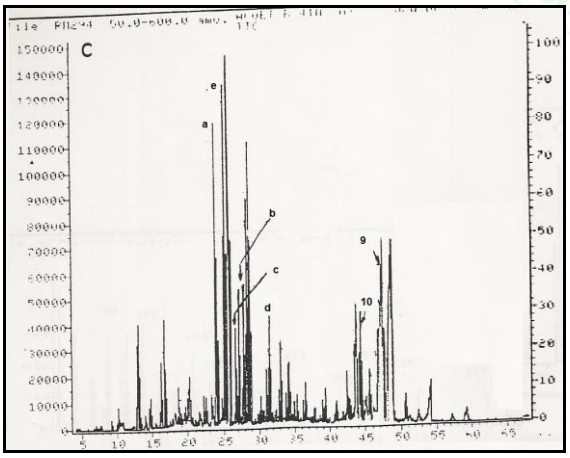
Figure 1- Chemical constituents detected in extracts in hexane and ethyl acetate of the leaves of Plumeria rubra L., form acutifolia (Ait) Woodson. The terpenoids acids were detected as methyl esters.



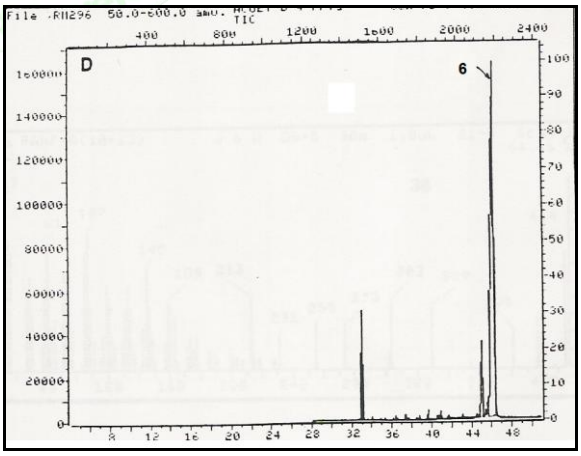
A



B



C



D

Figure 2- Chromatograms of total ions **A** - **D** (HRGC) of fractions from the hexane extract of leaves of *P. rubra* L., form *acutifolia* (Ait) Woodson. The initial peaks marked (**a** - **e**) correspond to methyl esters of fatty acid.

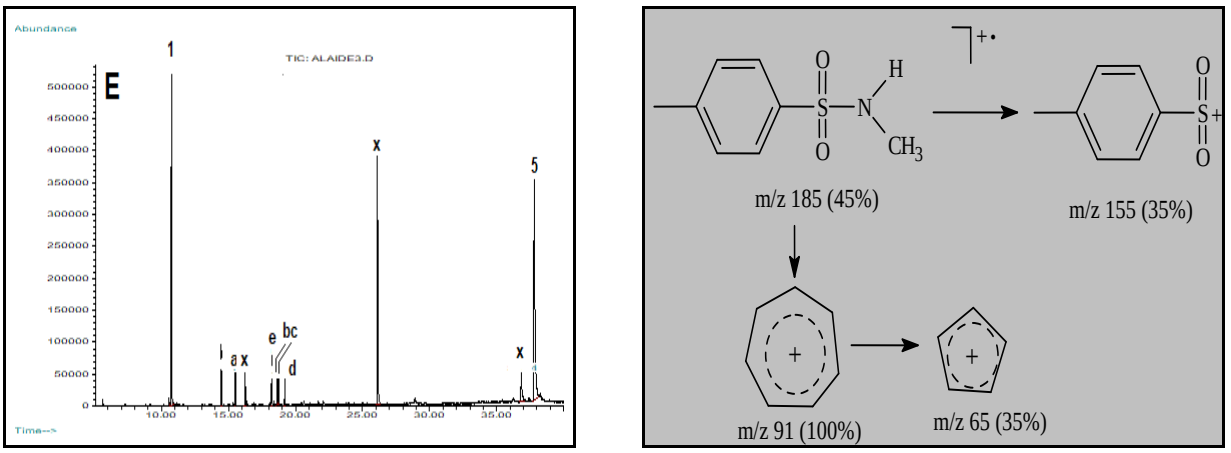


Figure 3- Chromatogram of total ions (HRGC) of the E fraction from the methanol extract of *Coleosporium plumeriae* Pat. (HP-5 stationary phase; entrainment gas: Helium). The vowels (a-e) correspond to the methyl esters of fatty acids and alcohols (e). Compound 1 corresponds to *N*-methyl-*p*-toluenesulfonamide and the number 5 corresponds to 5-stigmasten-3β-ol. Unidentified substance (x). Proposed fragmentation pathway for $M^{\cdot+}$ of 1.

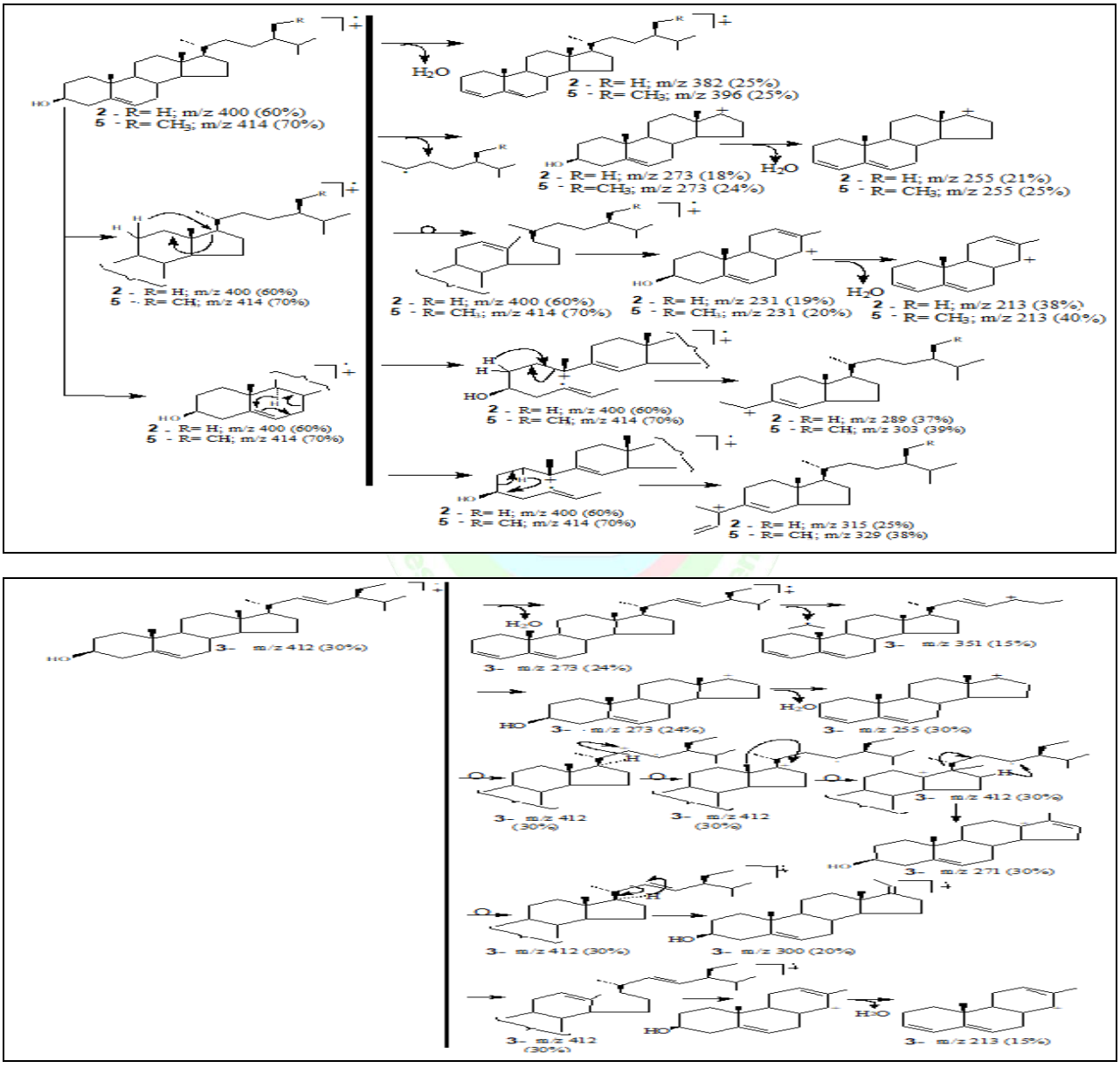


Figure 4- Proposed fragmentation pathway for $M^{\cdot+}$ of **2**, **5** and **3**.

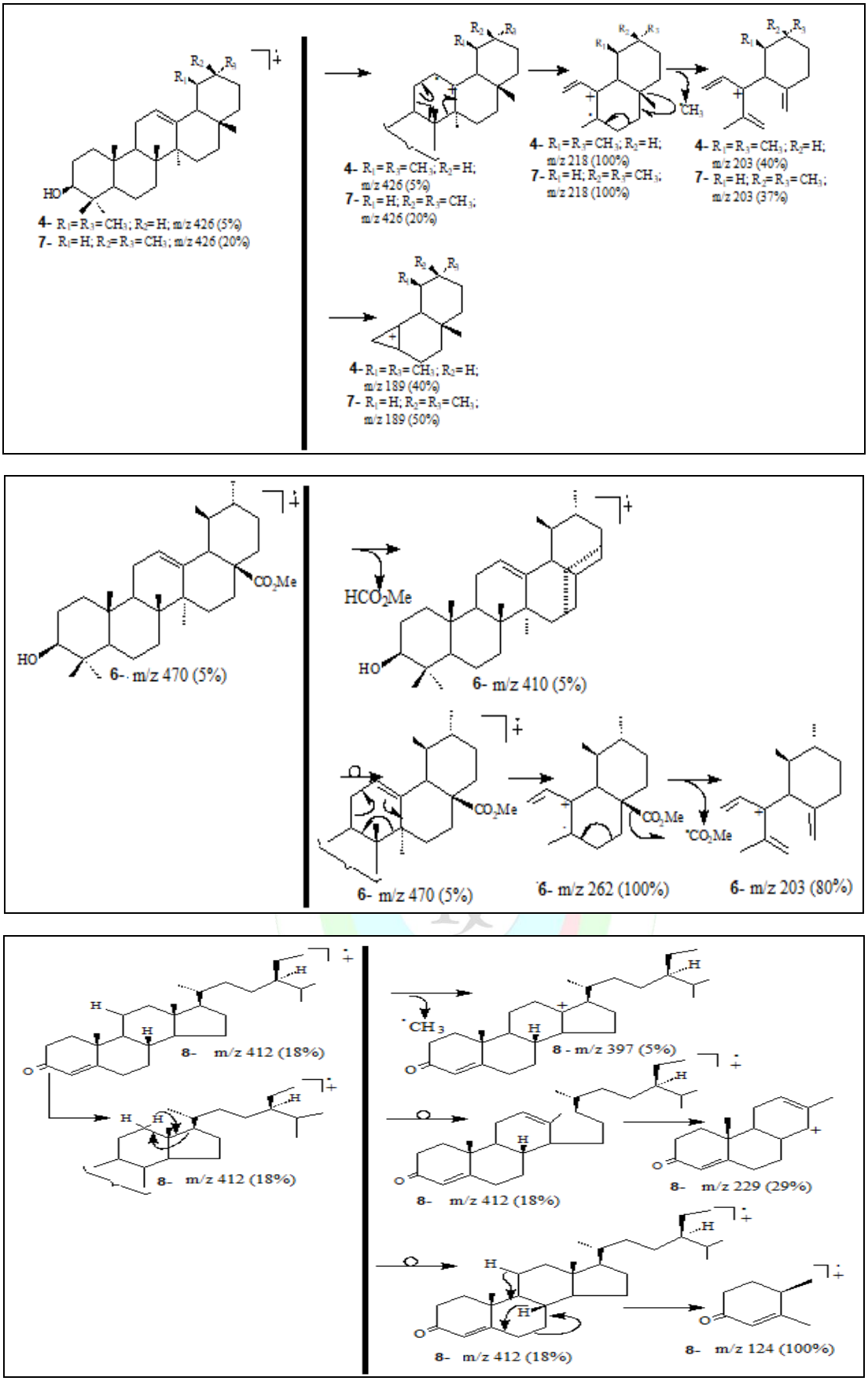


Figure 5- Proposed fragmentation pathway for M^{+} of **4**, **7**, **6**, and **8**.

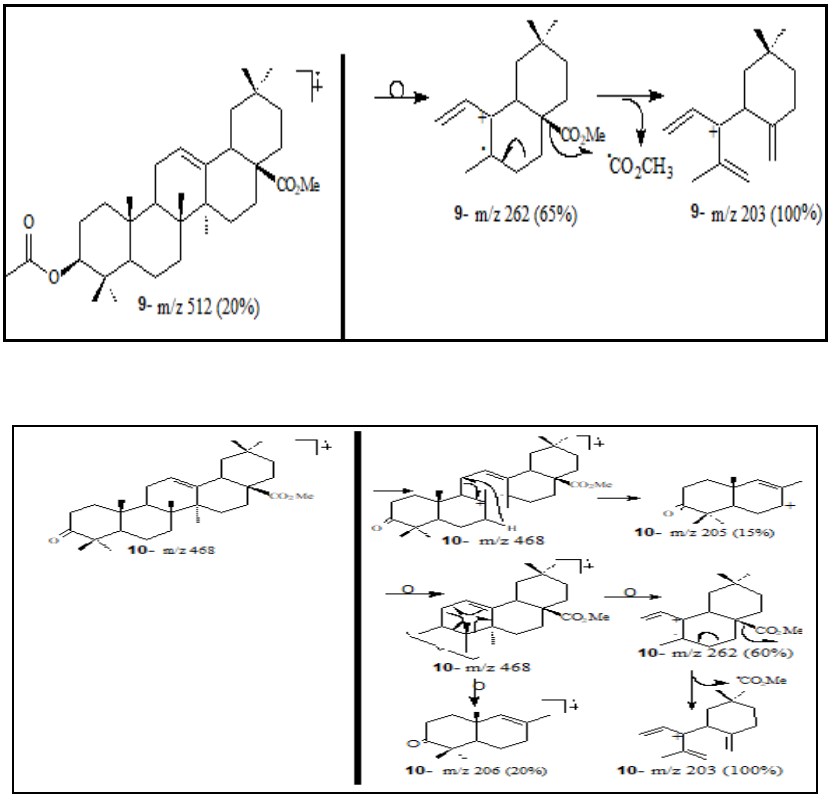


Figure 6- Proposed fragmentation pathway for M^{+} of **9** and **10**. The acids are methylated.

Table: 1. Principal peaks observed in the mass spectra (m/z and relative intensity in parenthesis) of the substances isolated

Peak	R_t	$[M]^{+\bullet}$; m/z	Principal peaks, m/z (%)	Principal peaks Compound
1	10.76	185 (45)	155 (35); 121 (20) 91 (100) 65 (35)	*N*-methyl toluenesulfonamide
2	43.02	400 (60)	382 (25); 315(25); 289 (37); 273 (18); 255(21); 43(100)	5-ergosten-3β-ol
3	43.62	412 (30)	351 (15); 273 (24); 271 (30); 213 (15); 55 (100)	5,22-stigmastadien-3β-ol
4	44.04	426 (5)	218 (100); 203 (40); 189 (40)	12-ursen-3β-ol
5	44.05	414 (70)	396(25); 363 (39); 329 (38); 277 (24); 255 (25); 231 (10); 43 (100)	5-stigmasten-3β-ol
6	45.00	470 (5)	410 (5); 262 (100); 203 (80)	ursolic acid
7	45.75	428 (20)	218 (100); 203 (37); 189 (50)	12-oleanen-3β-ol
8	46.47	412 (18)	397 (5); 229(29); 124 (100)	4-stigmasten-3-one
9	48.13	512 (20)	262 (65); 203 (100)	3β-acetoxy-12-oleanen-28-oic acid
10	48.78	468 (-)	262 (60); 205 (15); 203 (100); 189 (15)	12-oleanen-3-one-28-oic acid

CONCLUSION

The phytochemical study of the leaves of *Plumeria rubra* L., form *acutifolia* (Ait) Woodson revealed the presence of some terpenoids, and steroids not yet identified in this species. Four carboxylic acids, an alcohol, a steroid and an aromatic compound were identified in the methanol extract of the spores of *Coleosporium plumeriae* Pat. Phytosterols and lipids have presented important biological activities are broadly found in plants. Although isolation of phytosterols in fungi to be relatively unusual, some studies have demonstrated the isolation of these compounds from some fungi species[28-30] which opens up new possibilities to the isolation and obtaining this important class of compounds. This is the first report of the isolation of carboxylic acids, alcohol and stigmasterol from the *Coleosporium plumeriae* Pat. The isolation of substances from the fungus *Coleosporium plumeriae* Pat. corroborates with the theory of transfer

of skills between fungus and host plant, also contribute to knowledge secondary metabolites from this fungi.

ACKNOWLEDGMENTS

The authors are grateful to the National Council for Scientific and Technological Development (CNPq), the Foundation for Research Support of the State of Rio de Janeiro (FAPERJ) for grants and fellowships (CNPq) and Prof. Dr. Angelo da Cunha Pinto, who with remarkable coherence and courage lived intensely and we, his students and admirers, taught us to love science.

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