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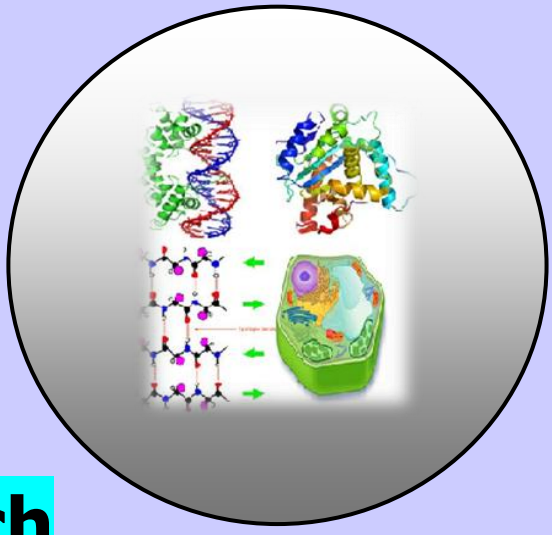

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RESEARCH PAPER

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Identification of Bioactive Compounds of *Ficus septica* Leaf Extract has Potential as Botanical Pesticides to Control Anthracnose Disease on Chili Pepper

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ABSTRACT

Based on the preliminary study as many as 20 species of plants extract found that the crude extract of the leaves of Ficus septica able to inhibit the growth of Colletotricum acutatum, its fungus the cause of anthracnose disease on chili pepper. Base on in vitro test on PDA with inhibition zone diameter of 30 mm, but it is not certain bioactive compounds. For this problem, the aim is research is conducted to determine the content of bioactive compounds potentially as botanical pesticides. The method used is the method of column chromatography and thin layer, and GCMS. The study states that the extracts of Ficus septica containing 4 secondary metabolites are terpenoids, alkaloids, flavonoids, and phenols. Result analysis using GCMS there are 14 active compound namely dl-glyceraldehyde dimer, 2,3,5 trimethyl heptane, Sulfurous acid cyclohexylmethylhexadecyl ester, guanosine, D-Allose, dodecanoic acid methyl ester, 1,2-Benzenedicar boxylic acid diethyl ester, 3-Deoxy-d-mannonic acid, cyclohexane tetraethyl 1,2,3,4, (Z) - 9-Tricosene, hexadecanoic acid methyl ester, octadecamethylcyclononasi-loxane, 1-Heptacosanol and 1,2-Benzene dicarboxylic acid mono (2-etilhexyl) ester. Based on the existing references, of 14 compounds and 8 of them have been known as antifungal compounds. Those are 2,3,5 trimethyl heptane, hexadecylcyclohexylmethyl Sulfurous acid ester, dodecanoic acid methyl ester, 3- deoxy-d-mannonic acid, hexadecanoic acid methyl ester, octadecamethylcyclononasiloxane, 1-Heptacosanol and 1,2-Benzenedicar boxylic acid mono (2-etilhexyl) ester.

Keywords: Antifungal, Ficus septica, Colletotrichum acutatum, Anthracnose Disease and Botanical Pesticides.

INTRODUCTION

Anthracnose disease in chili pepper is the most common disease and almost always occurs in every area of chili plants (Figure 1B). According to Suryaningsih et al. (1996), the most common cause of anthracnose in chili plants in Indonesia is *Colletotrichum capsici* and *Colletotrichum gloeosporioides*. According to (Sudiarta and Sumiartha, 2012; Sudirga, S.K. 2016) anthracnose disease on chili plants in Bali is mostly caused by *Colletotrichum acutatum*. Anthracnose disease can damage the aesthetic value of the chili and lowering the yield to 50% or more (Semangun, 2007). Anthracnose disease control today still relies on the use of synthetic fungicides. The use of synthetic fungicides continuously may lead to the emergence of pathogen resistance, pollute the environment and is harmful to consumers. Based on this, it is necessary to look for an alternative control of anthracnose disease on chili plants by utilizing the potential crop as botanical fungicide that is not dangerous for consumers and the environment. According Sudirga et al. (2014) in the preliminary study as many as 20 species of plants have been tested in terms of antifungal activity against *Colletotrichum acutatum* the cause of anthracnose disease on chili pepper, and found six kinds of plants that can inhibit the growth of *C. acutatum*. These 6 kinds of plants are *Ficus septica*, *Albizia saman*, *Piper nigrum*, *Piper crocatum*, *Piper retrofectum* and *Thitonia difersifolia*. Among the six species, *F. septica* leaf extract has the highest inhibitory activity with inhibition zone of 30 mm. Some plant species are reported to have antifungal activity such as *Ageratum conyzoides* has antifungal activity against *Penicillium italicum* (blue mold) the cause of fruit rot disease on Mandarin orange (Dixit *et al*., 1995); Origanum manjorona has antifungal activity against *Colletotrichum gloeosporioides* the cause of anthracnose disease on coffee (Silva *et al*., 2008); Albizia saman has antifungal activity against *Fusarium* sp. the cause of wilt disease in chili plants (Suprapta and Khalimi, 2012). *Awar-awar* (*Ficus septica* Burm.f.) is a wild plant and by the community it is only used as a traditional medicine (Figure 1A). Vital et al. (2010) reported a crude extract of *awar-awar* leaves can inhibit the growth of *Staphylococcus aureus*, *Canida albicans* and *Escerechia coli* with inhibition zones respectively 14 mm, 18 mm and 13 mm. Suspected chemical compound contained in the leaves, fruits and roots of *awar-awar* in the form of alkaloids, saponins, flavonoids, tannins and polyphenols (de Padua *et al*., 1999).

Figure 1. A = Leaf of *Ficus septica*, B= Anthracnose disease on chili pepper. (Source : private collection , 2014)

Sukadana (2010) reported the extracts of the root bark of awar-awar (*Ficus septica* Burm.f.) contains flavonoid compounds from the class of flavanones and these compounds can inhibit the growth of bacteria *Vibrio cholerae* and *Escherichea coli*. Damu et al. (2005) reported the extracts of stem awar-awar containing alkaloids compounds from the class of alkaloids phenanthro-indolizidine consisting of ficuseptines BD (1-3), 10R, 13aR-tylophorine N-oxide (4), 10R, 13aR-ylocrebrine N-oxide (5), 10S, 13aR-tylocrebrine N-oxide (6), 10S, 13aR-isotylocrebrine N-oxide (7), and 10S, 13aS-isotylocrebrine N-oxide (8). The alkaloid classes of compounds are cytotoxic. According to Nugroho et al. (2011) results from the fractionation of ethanol and hexane of awar -awar leaf extract has potential as an anticancer compound. Besides, leaves and roots awar-awar contains saponins and flavonoids, fruits contain alkaloids and tannins, and roots contain polyphenols (de Padua et al., 1999).

Crude extract of leaves of *Ficus septica* able to inhibit the growth of *Colletotrichum acutatum* fungus in vitro on PDA with inhibition zone diameter of 30 mm, but it is not certain bioactive substances. For these conditions, this study is a follow-up study, conducted to determine the content of bioactive substances potentially as botanical pesticides from leaves extract of *F. septica*.

MATERIAL AND METHODS

Methods of Extraction

Extraction of *awar-awar* leaves was done by chopping the leaves, and then dried at room temperature, and after the dry material was made into powder by means of a blender. *Awar-awar* leaf powder (100 grams) was then macerated with 1000 ml of methanol PA (Pro Analysis) for 72 hours at room temperature and dark place. The filtrate was obtained by filtering and the residue obtained was then macerated again with 1000 ml of methanol as much as two times. The filtrate obtained are combined and then evaporated using a vacuum rotary evaporator (Iwaki, Japan) at 40°C, to obtain a crude extract that was used for further testing.

Antifungal Activity Test

Antifungal activity test of crude extract of the leaves of *awar-awar* against *Colletotrichum acutatum* was done in well diffusion method. According to Ardiansyah (2005), if the diameter of inhibition zone is ≥ 20 mm the inhibitory activity is very strong; 10-20 mm the inhibitory activity is strong; 5-10 mm the inhibitory activity is moderate; and ≤ 5 mm the inhibitory activity is poor or weak.

Analysis of Phytochemicals

Phytochemical analysis was conducted to determine the compound of the active fraction obtained by using reagents for specific classes of compounds. The compounds of the active components tested included: terpenoids, alkaloids, flavonoids, phenols, saponins, and tannins. Analysis was performed on fractions which showed the highest antifungal properties (Harborne, 1989).

Separation and Purification of Active Extracts

The crude extract of *awar-awar* was partitioned with n-hexane and methanol to obtain the extract phase of n-hexane and methanol phase. Furthermore, both the extracts were tested for the antifungal activity.

The separation and purification was performed by column chromatography using silica gel (60 from 0.063 to 0.200 mm) as stationary phase, while the mobile phase is a mixture of various kinds of solvents which are based on differences in polarity. From the chromatography column, it produced several fractions and each fraction was tested for antifungal activity. Some active fractions were fractionated again using the same eluent as the previous fractionation. Each fraction obtained in the second fractionation was tested for antifungal activity and further active fractions were analyzed by KLT to determine the spot patterns generated from each of the fractions. Fractions that produce the same spot pattern were incorporated as a combined fraction of and tested for antifungal activity. The most active fraction was then analyzed by GC-MS to know the types of chemical compounds contained in this fraction.

RESULTS AND DISCUSSION

Inhibitory Activity of Partitioned Extract

Based on the results of partition using counter-current distribution method with two types of solvents are hexan and methanol phase showed that the methanol extract could inhibit the growth of *Colletotrichum acutatum* with the diameter of inhibition zone of 30 mm, whereas hexane extract phase could not inhibit the growth of this fungus (Figure 2). These results indicate that the active compounds in the leaf extract of *awar-awar* that are antifungal against *C. acutatum* is in the phase of methanol and is polar.

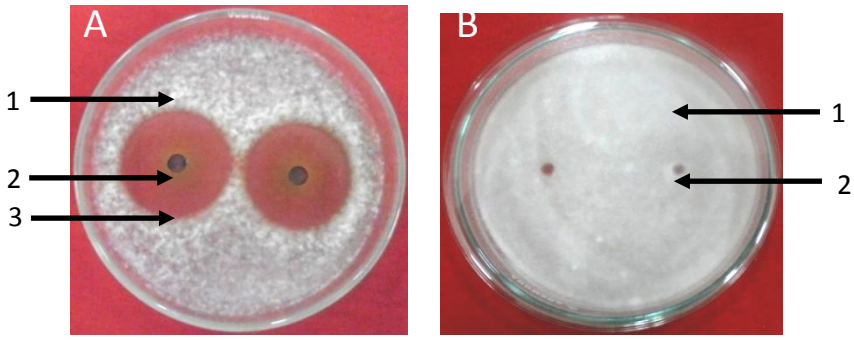

Figure 2. Photos of inhibition zone formed around the well diffusion filled with partitioned leaf extract of *awar-awar* of methanol phase (A) and hexane phase (B). (1 = mycelium of *C. acutatum*, 2 = well diffusion, and 3 = inhibition zone)

Gawade at al. (2014) reported the leaf extract of *Aegle Marmelos* (L). can inhibit the growth of the fungus *Colletotrichum acutatum* with inhibition zone diameter of 22 mm. According to Nogodula et al. (2012) crude extract of leaves of *awar-awar* is able to inhibit the growth of mold *Canida albicans* inhibition zone with a diameter of 16.67 mm, but has not been any report on the bioactive compound of leaf extract of *awar-awar* has potential as botanical fungicides to control anthracnose disease cause of *C. acutatum* on chili pepper.

Inhibitory Activity of Fractionated Extract

Methanol phase fractionation by column chromatography produces 44 fractions. All fractions tested for inhibitory activity against C*. acutatum* of on PDA media with well diffusion method. Five active fractions were found to inhibit the growth of *C. acutatum* namely fractions 40, 41, 42, 43, and 44 with the diameter of inhibition zone respectively 20 mm, 25 mm, 29 mm, 29 mm and 25 mm (Figure 3).

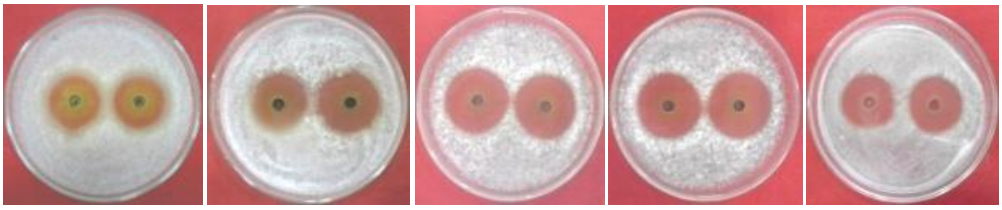

Figure 3. Inhibition zone of 5 active fractions of 44 fraction results of methanol phase fractionation of leaf extract of *awar-awar* (A = fraction 40, B = fraction 41, C = fraction 42, D = fraction 43 and E = fraction 44).

All five active fractions were combined into combined fractions and were fractionated again by column chromatography using eluent same as the previous fractionation. Eighteen fractions which showed strong antifungal activity against *C. acutatum* was analyzed using thin layer chromatography (TLC) to determine the pattern of the spots in each fraction. The results showed that of the 18 fractions were tested showed a pattern of spots and with almost the same Rf value *i.e.* between 0.7 and 0.8 so it can be presumed that the active compounds contained among the 18 active fraction possibilities belong to a group or class of similar compounds. The size of the inhibition of a plant extract against fungus varies greatly depending on the type and concentration of compounds (Suprapta, 2001). Castillo et al. (2012) reported *awar-awar* contain active compounds antofine and ficuseptine. Antofine compound has potential as an anticancer compound while the compounds ficuseptine potential as antibacterial and antifungal compounds. Results fractionation of ethanol and hexane *awar- awar* leaf extract has potential as an anticancer compound, in addition to leaves, fruits and roots *awar - awar* contain alkaloids, saponins and flavonoids as a potential antimicrobial compounds (Nugroho et al., 2011).

Phytochemicals of the Leaf Extract of *Awar-Awar*

Phytochemical test of the methanolic leaf extract of *awar-awar* showed that the leaf extract of *awar-awar* containing compounds such as terpenoids, alkaloids, flavonoids, and phenols (Table 1). According to Baumgartner et al. (1990) the results of the methanol extract fractionation of *awar-awar* leaves contain active compounds in the form of 2 indolizidine alkaloid that is ficuseptine and antofine, the two compounds that have antifungal and antibacterial activity. Results of fractionation of ethanol and hexane leaf extract of *awar-awar* has potential as an anticancer compound, besides that of leaves, fruits and roots of *awar-awar* contain alkaloids, saponins and flavonoids that have the potential as antimicrobial compounds (Nugroho et al., 2013).

Table 1. Phytochemical test results of combined fraction.

Phytochemicaltest	Reaction result	Conclusion
Alkaloid	chocolatesediment	Alkaloid (+)
Triterpenoid	yellow to purple	Triterpenoid (+)
Phenolat	Blackish blue	Polyphenol (+)
Flavonoid	yellow	Flavonoid (+)
Saponin	Foamis not Constant	Saponin (-)
Tannin	No sediment is formed	Tannin (-)

Nduagu et al. (2008) reported the phytochemical content extracts of bark and root bark of five types of plants can inhibit the growth of *Colletotrichum capsici* causes anthracnose in pepper. The extract positive for chemical compounds such as alkaloids (*Citrus limon* and *Azadirachta indica*), tannins (*Vernonia amygdalina*, *Azadirachta indica* and *Ocimum gratissimum*), glycosides (*Vernonia amygdalina*, *Citrus limon*, *Azadirachta indica*, *Ocimum gratissimum* and *Anona senegalensis*), saponins (*Vernonia amygdalina* , *Citrus limon*, *Azadirachta indica*, *Ocimum gratissimum* and *Anona senegalensis*) and flavonoids (*Azadirachta indica*). Lawal et al. (2012) reported methanol and ethyl acetate extracts of the root bark of *Ficus exasperata* Vahl. at a concentration of 200 mg / ml can inhibit the growth of *Colletotrichum gloeosporioides* with inhibition zone diameter each by 19 mm and 13 mm, and after phytochemical test, the extract containing saponins and glycosides.

Antifungal Active Compounds Based on GC-MS

Eighteen fractions which showed the highest inhibition to the *Colletotrichum acutatum* were combined and then analyzed components contained therein by using GC-MS (GCMS-QP2010 Ultra SHIMADZU). Chromatogram of the fraction analysis results showed 15 peaks as shown in Fig. 4, so it is assumed that crude extract of *awar-awar* leaves may contain a maximum of 15 active compounds that are antifungal against *C. acutatum*. Each emerging peak was further identified by mass spectroscopy, so that each compound has a specific mass fragmentation pattern.

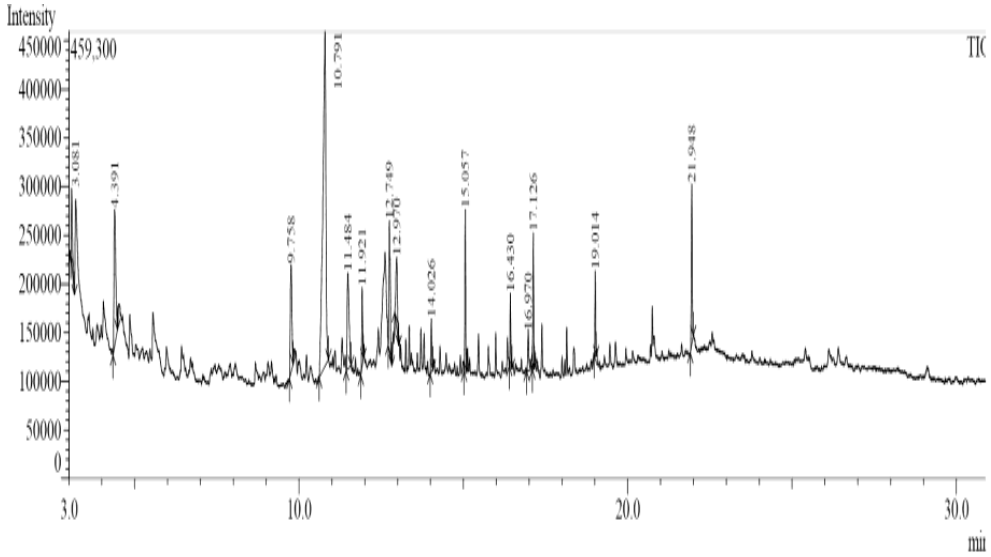

Figure 4. Chromatogram of GC-MS analysis of the active fractions capable of inhibiting the growth of *C. acutatum*.

The identification was done by comparing the mass spectrum of each peak in the mass spectrum of compounds that are already known to exist in the GC-MS library. Results of the analysis with GC-MS showed that the active fraction of the leaf extract of *awar-awar* contains 14 compounds namely dl-glyceraldehyde dimer, 2,3,5 trimethyl heptane, Sulfurous acid cyclohexylmethylhexadecyl ester, guanosine, D-Allose, dodecanoic acid methyl ester, 1,2-Benzenedicarboxylic acid diethyl ester, 3-Deoxy-d-mannonic acid, cyclohexane

tetraethyl 1,2,3,4, (Z) - 9-Tricosene, hexadecanoic acid methyl ester, octadecamethyl-cyclononasiloxane, 1-Heptacosanol and 1,2-Benzenedicarboxylic acid mono (2-etilhexyl) ester. Eight of them have been known as antifungal compounds. Those compounds are: 2,3,5 trimethyl heptane, Sulfurous acid cyclohexyl methylhexadecyl ester, dodecanoic acid methyl ester, 3-Deoxy-d-mannonic acid, hexadecanoic acid methyl ester, octadecamethyl-cyclononasiloxane, 1 -Heptacosanol and 1,2-Benzenedicar boxylic acid mono (2-etilhexyl) ester. The specification of each compound contained in the active fraction of the leaf extract of *awar-awar* is presented in Table 2.

Table 2. Active compounds that have the potential as a botanical fungicide identified in the leaf extract of *awar-awar* based on the analysis with GC-MS.

No.	Peak	MW (molecular weight)	MF (molecular formula)	Retention time	Active coumpound base on database GC-MS
1	Peak 1	180	$C_6H_{12}O_6$	3,079	dl-Glyceraldehyde dimer
2	Peak 2	142	$C_{10}H_{22}$	4,391	heptane 2,3,5 trimetil
3	Peak 3	402	$C_{23}H_{46}O_3S$	9,760	sulfurous acid cyclohexylmethyl hexadecyl ester
4	Peak 4	283	$C_{10}H_{13}N_5O_5$	10,791	guanosine
5	Peak 5	180	$C_6H_{12}O_6$	11,489	D-Allose
6	Peak 6	214	$C_{13}H_{26}O_2$	11,920	dodecanoic acid metil ester
7	Peak 7	222	$C_{12}H_{14}O_4$	12,750	1,2-Benzenedicarboxylic acid dietil ester
8	Peak 8	180	$C_6H_{12}O_6$	12,972	3-Deoxy-d-mannonic acid
9	Peak 9	196	$C_{14}H_{28}$	14,029	cyclohexane 1,2,3,4 tetraetil
10	Peak 10	322	$C_{23}H_{46}$	15,057	(Z)- 9-Tricosene
11	Peak 11	270	$C_{17}H_{34}O_2$	16,430	hexadecanoic acid metil ester
12	Peak 12	666	$C_{18}H_{54}O_9SI_9$	16,976	octadecamethyl cyclononasiloxane
13	Peak 13	396	$C_{27}H_{56}O$	17,125	1-Heptacosanol
14	Peak 14	396	$C_{27}H_{56}O$	19,015	1-Heptacosanol
15	Peak 15	278	$C_{16}H_{22}O_4$	21,947	1,2-Benzenedicarboxylic acid mono (2-etilhexyl)

According to Appuaka et al. (2013) the utility of the benzene compound is the most important thing as a solvent and as a raw material for making other aromatic compounds which are derivatives of benzene. These compounds act as antioxidants, antifungal and antimicrobial. Haptane a class of organic compounds alkanes used for petroleum fuels, solvents, lubricants and industrial raw materials. Akpuaka et al. (2013) reported the heptane has biological activity as antifungal and antibacterial compounds. Sulfurous acid is an organic compound containing groups sulfur, are corrosive and hazardous in high concentrations can cause death. Mazid et al. (2011) reported the Sulfurous acid in higher plants function as an antifungal compound. Dodecanoic acid is a fatty acid is generally known as lauric acid. Nakatsuji et al. (2009) reported the lauric acid has biological activity as antimicrobial compounds. Carolina et al. (2011) reported the dodecanoic acid had biological activity as an antifungal. 3-Deoxy-D-mannonic acid ester is a compound known as uronat acid. According to Martinez et al. (2009) uronat acid having biological activity as an antifungal. Hexadecanoic acid ester compound belonging that is often called sour palmintat. Hexadecanoic acid compounds have activity as an antioxidant, nemasida, pesticides, (Murugesan et al., 2013; Elezabeth and Arumugam, 2014). According to Akpuaka et al. (2013) hexadecanoic acid has activity as antifungal and antibacterial compounds. Octadecamethyl cyclononasiloxane are compounds that are volatile. Ojekale et al. (2013) reported the compound octadecamethyl cyclononasiloxane predominantly found in leaf

extracts of *Thaumatococcus Danielli* (Benn.) Benth potential as antioxidants. Dubal et al. (2013) reported extracts of rhizomes *Tectaria coadunata* contains octadecamethyl cyclononasiloxane potentially be used as antioksidaan and antimicrobial. Heptacosanol an alcoholic, Raman et al. (2012) reported the 1-heptacosanol have biological activity as nemasida, anticancer, antioxidant and antimicrobial. Benzenedicarboxylic acid ethyl ester is a compound that is commonly known as monoetilheksil phthalate. Raman et al. (2012) reported the 1, 2-Benzenedicarboxylic acid had biological activity as antimicrobial, antioxidant and anticancer. According to Akpuaka et al. (2013) the 1, 2-benzenedicarboxylic acid compounds have biological activity as antifungal, antibacterial, antiviral and antioxidant.

CONCLUSION

The leaf extract of *awar-awar* contains several phytochemicals groups of compounds such as terpenoids, alkaloids, flavonoids, and phenols. The active fraction of the leaf extract of *awar-awar* contains 14 compounds namely dl-glyceraldehyde dimer, 2,3,5 trimethyl heptane, Sulfurous acid cyclohexylmethylhexadecyl ester, guanosine, D-Allose, dodecanoic acid methyl ester, 1,2-Benzenedicarboxylic acid diethyl ester, 3-Deoxy-d-mannonic acid, cyclohexane tetraethyl 1, 2, 3, 4, (Z) - 9- Tricosene, hexadecanoic acid methyl ester, octadecamethylcyclononasiloxane, 1-Heptacosanol and 1,2-Benzenedicarboxylic acid mono (2-etilhexyl) ester. Eight of them have been known as antifungal compounds. Those compounds are: 2,3,5 trimethyl heptane, Sulfurous acid cyclohexyl methylhexadecyl ester, dodecanoic acid methyl ester, 3-Deoxy-d-mannonic acid, hexadecanoic acid methyl ester, octadecamethylcyclononasiloxane, 1 -Heptacosanol and 1, 2-Benzenedicar boxylic acid mono (2-etilhexyl) ester.

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