Seminaris de Recerca
Facultat de Farmàcia
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FACULTAT DE FARMÀCIA

Compostos d'origen vegetal amb activitat antifúngica

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Fungal infections

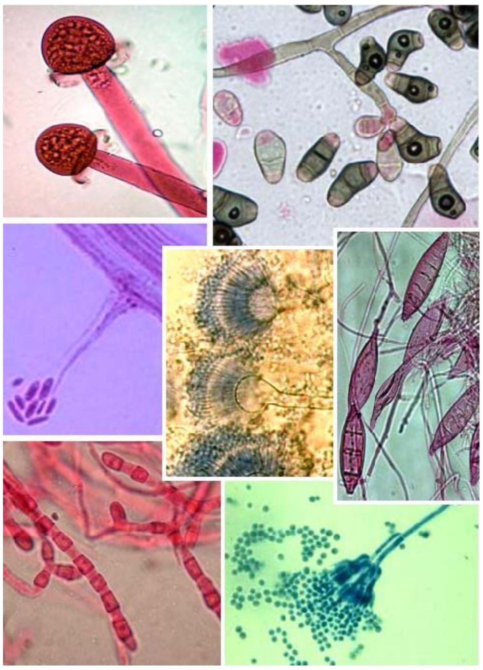
An increasing health problem

- ✓ Between 1979 and 2000 sepsis caused by fungi increased a 207% in USA.
- ✓ Deaths due to mycoses in USA increased from 1557 (1980) to 6534 (1997).
- ✓ Deaths were mainly related to infections by *Candida, Aspergillus and Cryptococcus* sp.

Pfaller and Diekema (2007) *Clin Microbiol Rev* 20(1):133-163

Fungal infections: Mycoses

Classification



Superficial

- ✓ Cutaneous
- ✓ Subcutaneous

Systemic

- ✓ Internal organs

Fungal infections

Main pathogenic fungi

Filamentous fungi

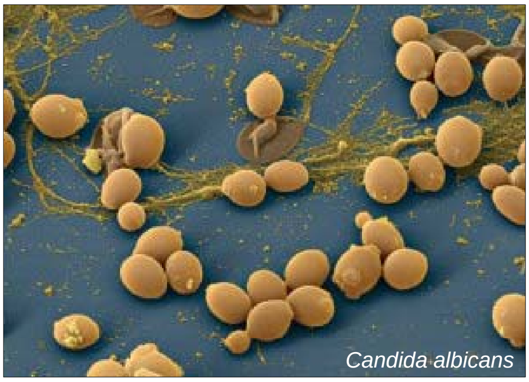
- ✓ *Aspergillus* sp.
- ✓ *Fusarium* sp.
- ✓ *Penicillium* sp.

Dermatophytes:

- ✓ *Tricophyton* sp.
- ✓ *Microsporum* sp.
- ✓ *Epidermophyton* sp.

Yeasts

- ✓ *Candida* sp.
- ✓ *Cryptococcus* sp.



Candida albicans

Fungal infections

An increasing health problem

Higher incidence related to:

- ✓ ↑ Immunocompromised population
- ✓ ↑ Population mobility, with higher exposure to endemic fungal pathogens

Effectiveness of antifungal drug limited by:

- ✓ Delayed diagnosis
- ✓ ↑ Antifungal drug resistance
- ✓ Drug toxicity
- ✓ Lack of oral and i.v. preparations

Pfaller and Diekema (2007) *Clin Microbiol Rev* 20(1):133-163
Mathew and Nath (2009) *Chem Med Chem* 4: 310-323

Research of antifungal drugs

There is a need for new antifungal agents

- ✓ New mechanisms of action
- ✓ Broad spectrum antifungal activity
- ✓ Fewer dose-limiting side effects
- ✓ Economic

Research of antifungal drugs

There is a need for new antifungal agents

Why plants?

- ✓ Biodiversity ⇨ Chemodiversity
- ✓ Traditional use
- ✓ Field partially explored

How to select the plant?

- ✓ Ethnopharmacological selection
- ✓ Chemotaxonomic selection
- ✓ At random

Drug discovery from plants

Why an ethnopharmacological approach?

- ✓ 80% of the people living in developing countries (64% of world population) are almost completely dependent on traditional medical practices.
- ✓ Higher plants are the main source of drug therapy in traditional medical systems.
- ✓ *Ca.* 74% of the drugs originated from higher plants were discovered in an ethnopharmacological context.
- ✓ Most of these plant-derived drugs are prototypes.

Ethnopharmacology in the development of new medicines

New analogues

Chemical or pharmacological leads

Used as drug in unmodified state

Bioactive molecules

Scientific basis for the use of the herbal drug or its preparations

Herbal medicinal products

Traditional use validation

Ethnomedical preparations

Ethnopharmacology and the discovery of new biologically active molecules

Data collection from indigenous population and data analysis

Collection of plant material and botanical identification

Literature search

Extraction

Biological / pharmacological testing

Bioguided isolation of active principles

Structure identification

Preparation of the extracts

With solvents of increasing polarity

- ✓ Dichloromethane
- ✓ MeOH 95%

Aqueous decoction

Distillation

- ✓ Essential oil

Screening of the antifungal activity

Assessment of the antifungal activity

Antifungigram

Growth inhibition diameters (mm)

Reference drugs:

- ✓ Nystatin
- ✓ Amphotericin B

Fungal strains

Af *Aspergillus fumigatus*
An *Aspergillus niger*
Ca *Candida albicans*
Cc *Cladosporium cladosporioides*
Cn *Cryptococcus neoformans*
Fo *Fusarium oxysporum* var. *pinaster*
Mg *Microsporum gypseum*
Nc *Neurospora crassa*
Pp *Penicillium purpurogenum*
Sc *Saccharomyces cerevisiae*
Tm *Tricophyton mentagrophytes*

Ethnopharmacological selection

Paraguay

- *Acanthospermum australe*
- *Acanthospermum hispidum*
- *Baccharis articulata*
- *Borreria valens*
- *Calycophyllum spruceanum* var. *multiflorum*
- *Croton gracilipes*
- *Croton urucurana*
- *Equisetum giganteum*
- *Erythrina crista-galli*
- *Geophila repens*
- *Maytenus ilicifolia*
- *Senecio grisebachii*
- *Tabebuia avellanedae*
- *Vernonanthura tweedieana*

Portillo *et al* (2001) *J Ethnopharmacol 76: 93-98.*

Extracts selected

Paraguay

▶ *Acanthospermum australe*
Aerial parts
Dichloromethane extract

Calycophyllum spruceanum ◀
Bark
Dichloromethane extract

▶ *Geophila repens*
Aerial parts
Dichloromethane extract
Methanolic extract

Vernonanthura tweedieana ◀
Root
Dichloromethane extract

Portillo *et al* (2001) *J Ethnopharmacol 76: 93-98.*

Antifungals from plants

Latin-American species studied

Acalypha arvensis
Acanthosper
Acanthosper
Andira inerm
Andira surina
Baccharis ar
Bixa orellana
Blepharocaly
Borreria vale
Calycophyllu
Croton graci

Croton urucur

a
lia
thum
a
n
s
ropiperoides
achii
nedae
Vernonanthura tweedieana

33 Species:
Argentina
Mexico
Peru
Paraguay
Ecuador

***Screening* for antifungal activity**

Antifungals from plants

Bioguided isolation

Extract

Preparative Chromatography →
CC, CTLC, HSCCC

← **Bioautography**

↓

Active compounds

Preparative chromatography

Techniques

Solid stationary phase

✓ **Column chromatography:**
- Classical column chromatography
- Flash chromatography
- Medium pressure liquid chromatography

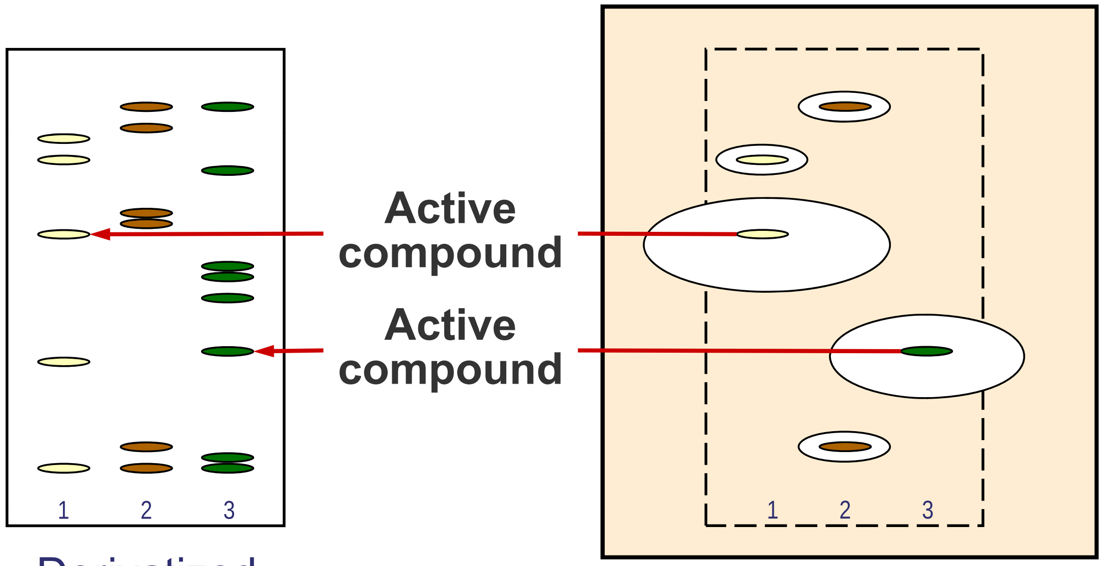
✓ **Planar Chromatography:**
- Thin-layer chromatography
- Centrifugal thin-layer chromatography

Liquid stationary phase

✓ High-speed counter current chromatography

Bioguided isolation

Bioautography



Derivatized Reference TLC

Bioautography

Antifungals from plants

Bioguided isolation

Extract

Preparative Chromatography →
CC, CTLC, HSCCC

← **Bioautography**

Structure elucidation →
Spectroscopic techniques:
MS, ^{1}H-NMR, ^{13}C-NMR, UV-Vis

← **Analysis of the composition**
GC-FID, GC-MS, ^{13}C-NMR

Active compounds

↑

Minimum inhibitory concentration (MIC)
Microdilution
Minimum fungicidal concentration (MFC)
Extension of the microdilution

Antifungals from plants

Classes of active compounds

- ✓ **Fatty acids**
- ✓ Peptides (defensins)
- ✓ Alkaloids
- ✓ **Simple phenols and phenolic acids**
- ✓ Coumarins
- ✓ **Flavonoids**
- ✓ Xanthones
- ✓ Quinones
- ✓ **Lignans**
- ✓ **Triterpenes**
- ✓ **Diterpenes**
- ✓ **Sesquiterpenes**
- ✓ **Monoterpenes**

Antifungal fatty acids

Undecylenic acid

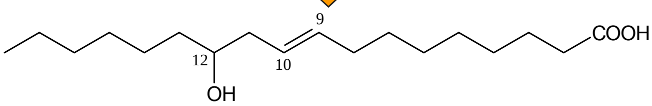
A semisynthetic compound

Ricinus communis

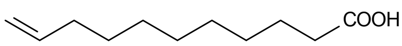
Seeds → Castor oil

$CH_2-OOC-(CH_2)_7-CH=CH-CH_2-CH_2OH-(CH_2)_5-CH_3$
$CH-OOC-(CH_2)_7-CH=CH-CH_2-CH_2OH-(CH_2)_5-CH_3$
$CH_2-OOC-(CH_2)_7-CH=CH-CH_2-CH_2OH-(CH_2)_5-CH_3$

Triricinoleine



Ricinoleic acid



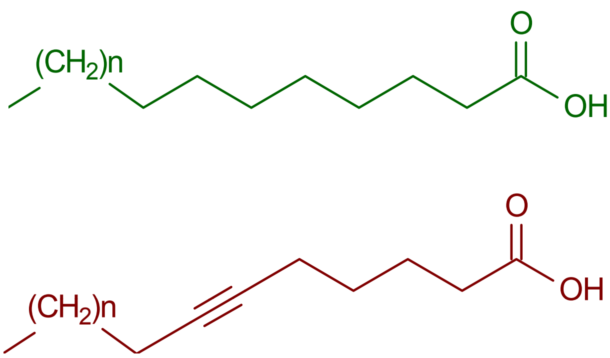
Undecylenic acid

Antifungal fatty acids

Calycophyllum spruceanum var. multiflorum (bark)

Paraguay

Mixture of fatty acids



Antifungal fatty acids

Calycophyllum spruceanum var. multiflorum (bark)

Paraguay

Dichloromethane extract

(13,80 g)

MPLC/Si60; Hexane:EtOAc:MeOH (1:0:0)-(0:1:0)-(0:0:1)

CM-I *CM*-II *CM*-III *CM*-IV *CM*-V *CM*-IX *CM*-X

CM-VI 0,94 g

CM-VII 2,44 g

CM-VIII 3,28 g

CC/LH-20; CH_2Cl_2:MeOH (50:50)

CM-VI1…*CM*-VI3

CM-VI-4 640 mg

CM-VI-5 27 mg

Antifungal fatty acids

Calycophyllum spruceanum var. multiflorum (bark)

Paraguay

***CM*-VI-4**
640 mg

2 x CC-Si60/LH20

CLC/Si60

↓

Mixture of compounds

Methylation (BF_3/CH_3OH)
GC-FID / GC-MS

↓

8 Fatty acids

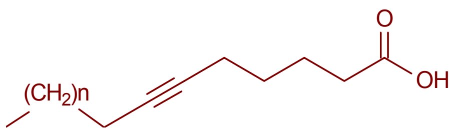
Antifungal fatty acids

Calycophyllum spruceanum var. multiflorum (bark)

Paraguay

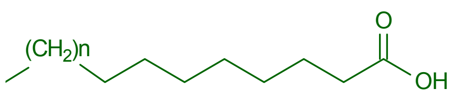
Structure of the fatty acids

Acetylenic fatty acids



n = 7 ➡ 6-Hexadecinoic
n = 8 ➡ 6-Heptadecinoic
n = 9 ➡ 6-Octadecinoic
n = 10 ➡ 6-Nonadecinoic
n = 11 ➡ 6-Eicosinoic

Saturated fatty acids



n = 7 ➡ Palmitic
n = 8 ➡ Heptadecanoic
n = 9 ➡ Estearic

Antifungal fatty acids

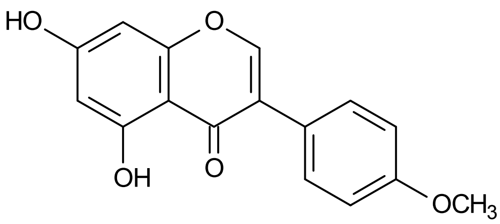
Calycophyllum spruceanum var. multiflorum (bark) Paraguay

MIC and MFC of the fatty acid mixture (*CM*-AG) (µg/ml)

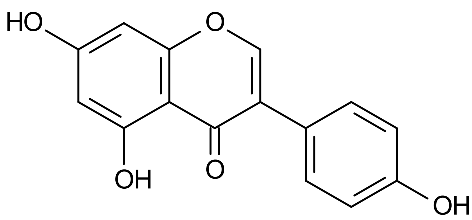
Fungi strains	*CM*-AG		Amphotericin B		Nystatin	
	MIC	MFC	MIC	MFC	MIC	MFC
Ca	-	-	0.16	0.63	1.25	2.50
Cn	-	-	0.16	0.31	0.31	0.63
Mg	0.25	0.25	0.31	0.31	2.50	2.50
Sc	-	-	0.16	0.31	0.31	0.63
Tm	0.25	0.25	0.63	0.63	1.25	1.25

Antifungal isoflavones

Andira surinamensis (bark) Ecuador



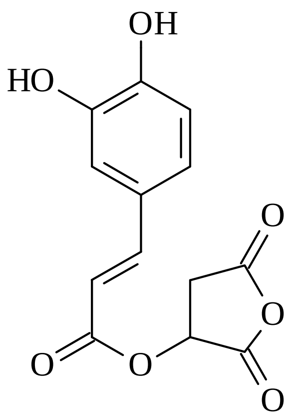
Biochanin A



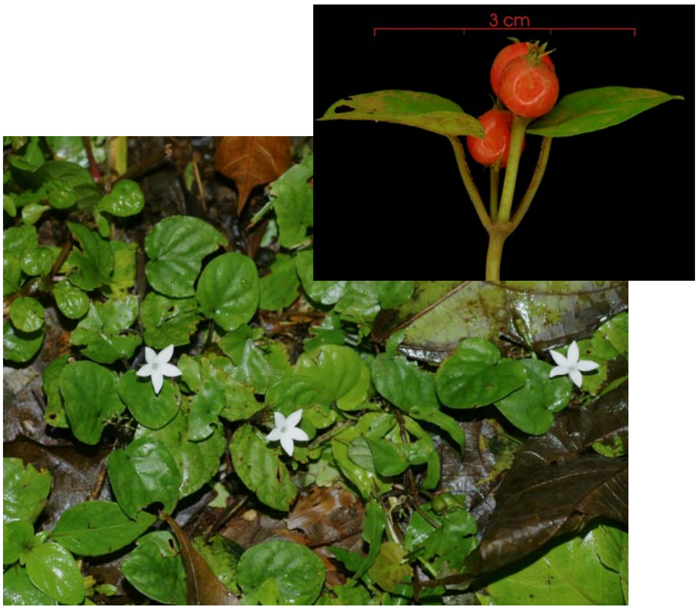
Genistein

Antifungal caffeic acid derivative

Geophila repens (aerial parts) Paraguay



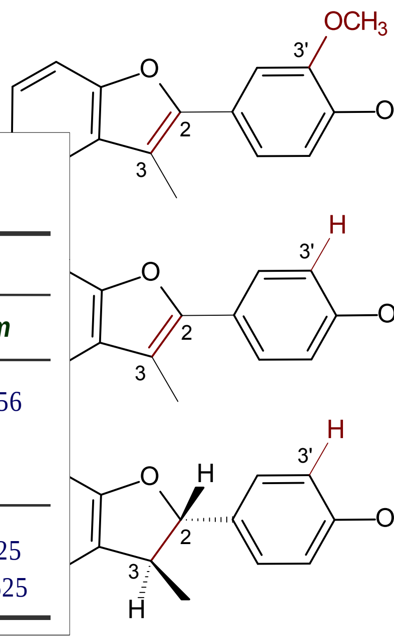
Malic anhydride caffeate



Portillo *et al.* (2004) 2nd Int. IOCD-CYTED, Sao Pedro (Brasil)

Antifungal lignans

Piper fulvescens (leaf) Paraguay


1
2
3

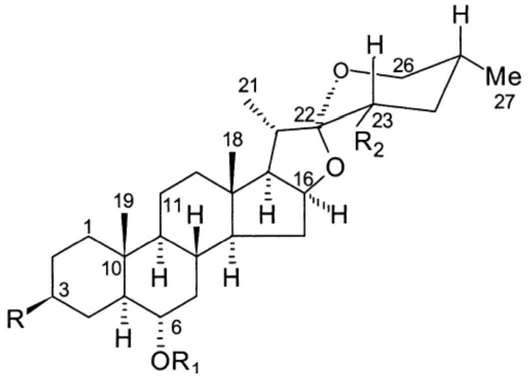
MIC (µg/mL)

Compound	Fungi strains				
	Ca	*Cn*	*Mg*	*Sc*	*Tm*
1	> 256	> 256	> 256	> 256	> 256
2	> 256	> 256	0,5	16	1
3	8	16	16	4	8
A	0,0625	0,125	0,25	0,0625	0,125
N	0,05	0,5	0,25	0,25	0,0625

Freixa *et al.* (2001) *Planta Med*, 67: 873-875.

Antifungal triterpenes

Saponins from *Solanum chrysotrichym*



	R	R_1	R_2
SC-2	OH	Xyl (1→3)-Qui	H
SC-3	OH	Xyl	H
SC-4	OH	Qui	H
SC-5	OH	Rha (1→3)-Qui	H
SC-6	OH	Rha (1→3)-Qui	OH

Leaves → MeOH extract →

Zamilpa et al (2002) J Nat Prod 65: 1815-1819

Antifungal triterpenes

Saponins from *Solanum chrysotrichym*

Double blind RCT on *Tinea pedis*

External application, 4 weeks, n= 101
Verum: Standardised extract of *S. chrysotrichym*
Control: ketoconazole (2%)

Clinical efficacy: 96.1% (*S. chrysotrichym)*
91.7% (Ketoconazole)

Mycological efficacy: 78.4% (*S. chrysotrichym)*
77.8% (Ketoconazole)

Tolerability: Both treatments were well tolerated in 100% of the patients

Herrera-Arellano *et al.* (2003) Planta Med 69: 390-395

Antifungal triterpenes

Saponins from *Solanum chrysotrichym*

Other clinical studies

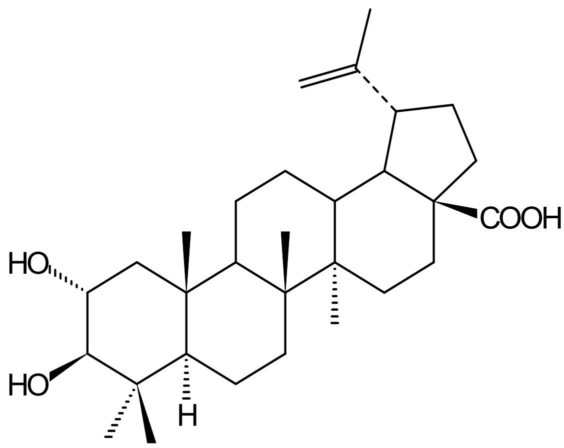
Double blind RCT on *Pityriasis capitis* (dandruff)
Efficacy: 92.2%
No differences to ketoconazole group

Exploratory study on *Candida* sp.-associated vaginal infection
Efficacy:
✓ Extract (125 mg/suppository): 57.1%
✓ Ketoconazole (400 mg/suppository): 72.5%

Herrera-Arellano *et al.* (2004) Planta Med 70: 483-488.
Herrera-Arellano *et al.* (2009) Planta Med 75: 466-471.

Antifungal triterpenes

Bixa orellana (leaf) Ecuador

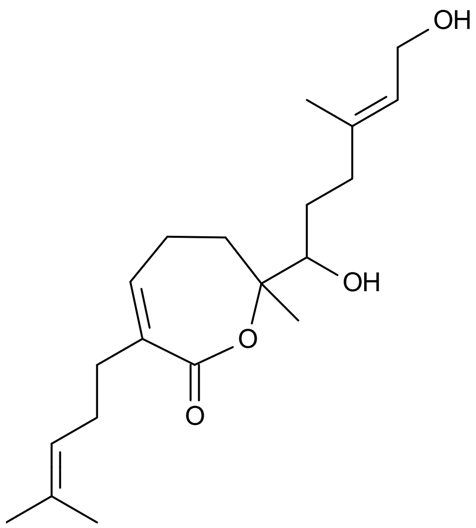


2α,3ß-alphytolic acid

Freixa *et al.* (2002) 50th GA Congress. Barcelona (Spain).

Antifungal diterpenes

Acanthospermum australe **(leaf)** Paraguay



Acanthoaustralide

Portillo *et al.* (2003) WOCMAP III, Chieng Mai (Thailand).

Antifungal diterpenes

Acanthospermum australe **(leaf)** Paraguay

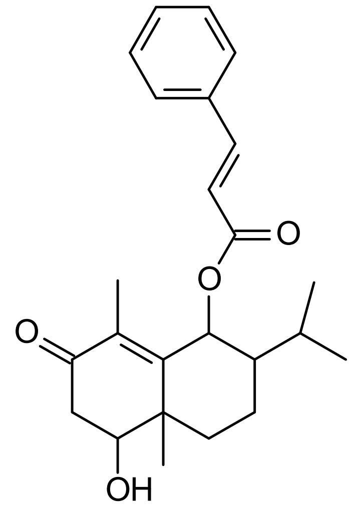
MIC and MFC (µg/mL) of acanthoaustralide (*AA*-B)

Fungi strains	*AA*-B		Amphotericin B		Nystatin	
	MIC	MFC	MIC	MFC	MIC	MFC
Ca	8	16	0.16	0.63	1.25	2.50
Cn	2	4	0.16	0.31	0.31	0.63
Mg	8	8	0.31	0.31	2.50	2.50
Sc	4	8	0.16	0.31	0.31	0.63
Tm	**2**	**2**	0.63	0.63	1.25	1.25

Portillo *et al.* (2003) WOCMAP III, Chieng Mai (Thailand).

Antifungal sesquiterpene derivatives

Vernonanthura tweedieana (leaf) Paraguay



6-Cinnamoyl-1-hidroxy-eudesm-4-en-3-one

Portillo *et al.* (2005) *J Ethnopharmacol* 97: 49-52.

Antifungal sesquiterpene derivatives

Vernonanthura tweedieana (leaf) Paraguay

MIC and MFC (µg/mL) of 6-cinnamoyl-1-hydroxi-eudesm-4-en-3-one (*VT*-E)

Fungi strains	*VT*-E		Amphotericin B		Nystatin	
	MIC	MFC	MIC	MFC	MIC	MFC
Ca	16	32	0.16	0.63	1.25	2.50
Cn	8	8	0.16	0.31	0.31	0.63
Mg	8	8	0.31	0.31	2.50	2.50
Sc	8	16	0.16	0.31	0.31	0.63
Tm	**4**	**4**	0.63	0.63	1.25	1.25

Portillo *et al.* (2005) *J Ethnopharmacol* 97: 49-52.

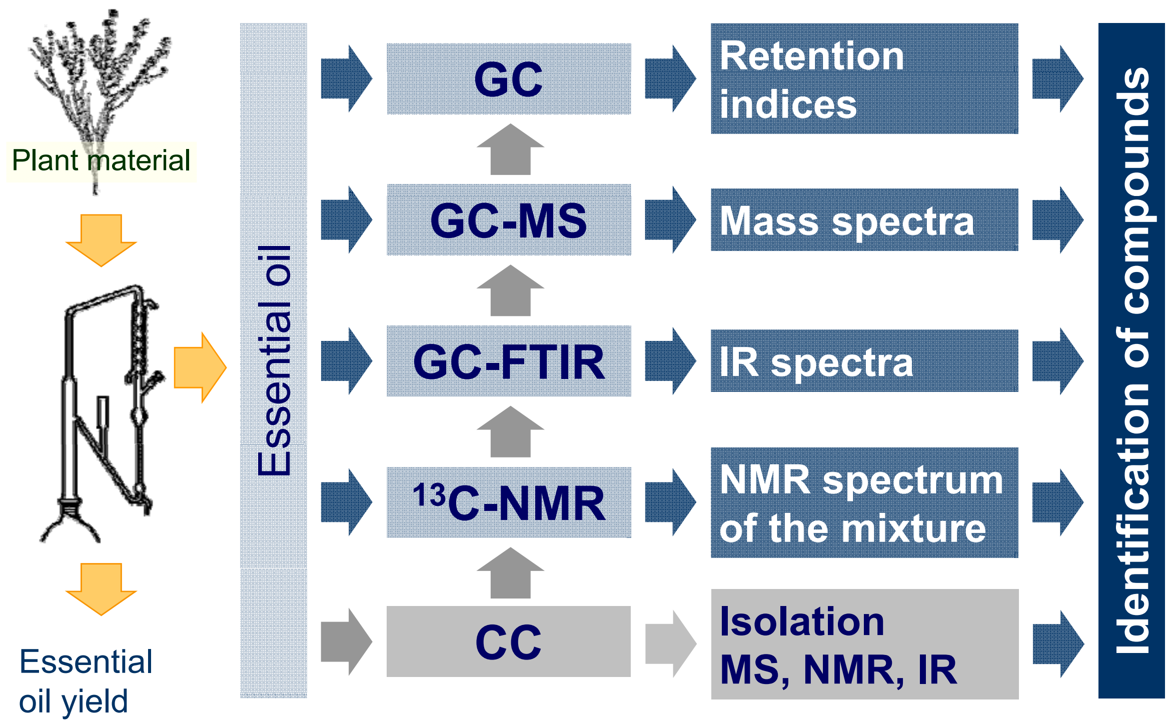
Essential oils

Chemodiverse mixtures

- ✓ (Hemiterpenes)
- ✓ Monoterpenes
- ✓ Sesquiterpenes
- ✓ (Diterpenes)
- ✓ Fenilpropanoids
- ✓ C6-C1 aromatic compounds
- ✓ Aliphatic com-pounds
- ✓ Etc…

- ✓ Saturated
- ✓ Unsaturated
- ✓ Aromatic
- ✓ Monocyclic
- ✓ Bicyclic
- ✓ Tricyclic
- ✓ Alcohols
- ✓ Phenols
- ✓ Esters
- ✓ Etc…

Essential oil analysis

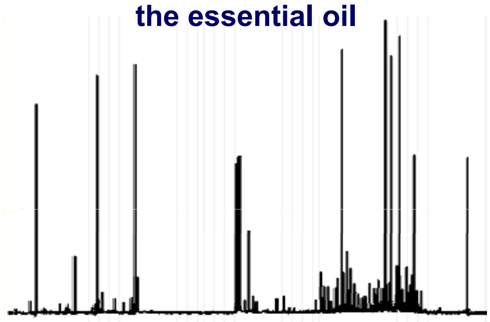


Essential oil analysis by ^{13}C-NMR

Library of ^{13}C-NMR spectra

Essential oil

^{13}C-NMR spectrum of the essential oil



Computer processing

Identification of compounds

- ✓ Number of signals observed
- ✓ $\Delta\delta$ between signal in oil and reference
- ✓ Number of overlapped signals

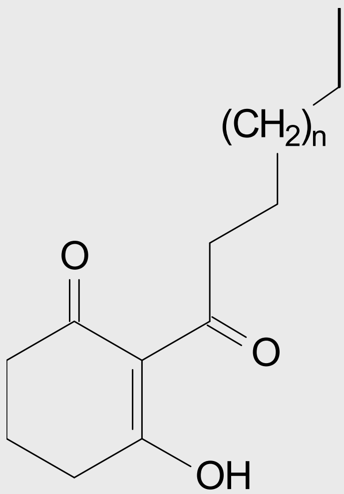
Antifungal essential oils

Piper amalago (spike, leaf, stem) Panama

Identified: > 95%

40 Constituents

Major constituents



2-acyl-3-hydroxycy-clohex-2-enones

1: n=1   3: n=5
2: n=3   4: n=7

Vila *et al.* (2002) 50th GA Congress, Barcelona (Spain).

Antifungal essential oils

Piper amalago (spike, leaf, stem) Panama

Compounds	Fungi strains *Ca*	*Cl*	*Sc*
1	64	32	64
2	256	16	256
3	>256	128	>256
4	>256	>256	>256
A	0.25	0.25	0.5
N	2	1	1

MIC (µg/ml) of compounds **1**, **2**, **3** and **4**.

Ca: *Candida albicans*
Cl: *Candida lactis-condensi*
Sc: *Saccharomyces cerevisiae*
A: Amphotericin B
N: Nystatin

2-Hexanoyl-3-hydroxycyclohex-2-enone (**1**)
2-Octanoyl-3-hydroxycyclohex-2-enone (**2**)
2-Decanoyl-3-hydroxycyclohex-2-enone (**3**)
2-Dodecanoyl-3-hydroxycyclohex-2-enone (**4**)

Freixa *et al.* (2005) 53th GA Congress, Florence (Italy).

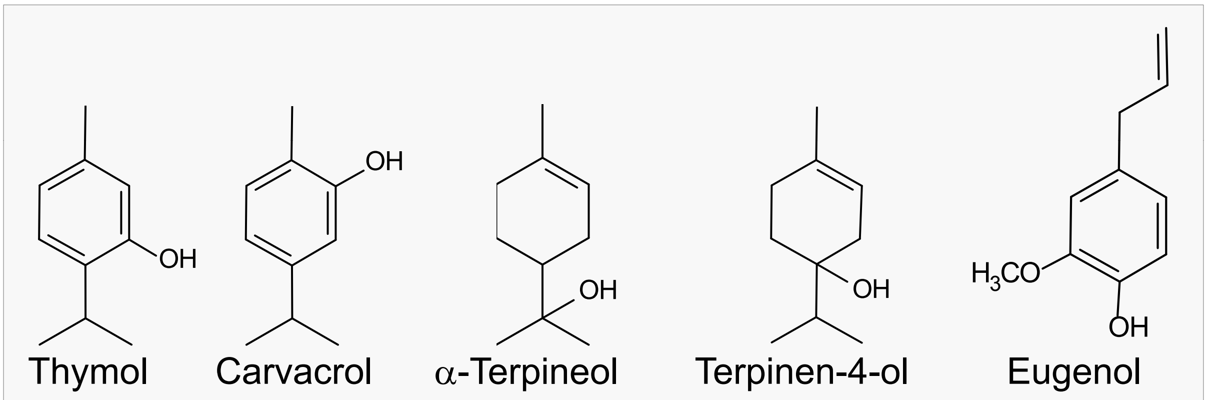
Essential oils with antimicrobial activity

Applications in the infections of skin and mucous membrane

- ✓ Acne
- ✓ Onychomycosis
- ✓ Vaginal infections
- ✓ Gingivitis
- ✓ Teeth and gums health care

Antifungal activity

Essential oil constituents

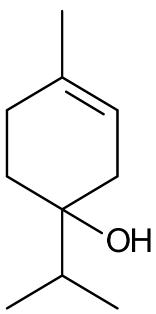


Tea tree oil

Melaleuca alternifolia Cheel leaf

Essential oil content in the fresh herbal drug: 1-2%

Essential oil



Terpinen-4-ol (30-48%)

1,8-Cineol (≤ 15%)

Tea tree oil

Indications and preparations

Topical use	Preparations
• Infections of skin and mucous membranes: ✓ Acne ✓ Onychomycosis ✓ Furunculosis ✓ Vulvovaginitis • Wounds • Burns	✓ Unguents ✓ Creams ✓ Gels ✓ Shampoos ✓ Liquid soap for intimae hygiene

Tea tree oil

Clinical trial in candidiasic vaginitis

Topical treatment of women with candidiasic vulvovaginitis

Design

- ⇨ Number of patients: 28
- ⇨ Duration: 90 days
- ⇨ 200 mg/day essential oil

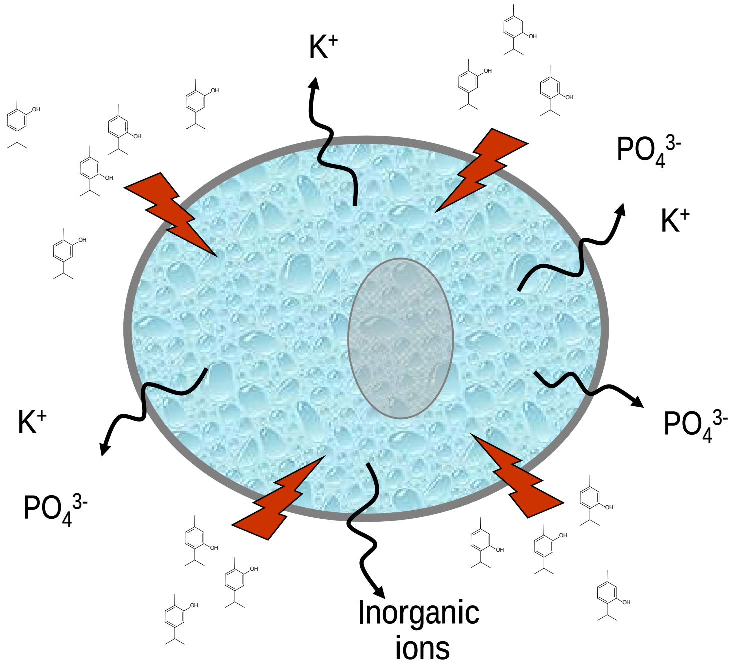
Result

- 85% patients symptom free
- 77% patients free of *C. albicans*

Belaiche 1988

Thymol and carvacrol

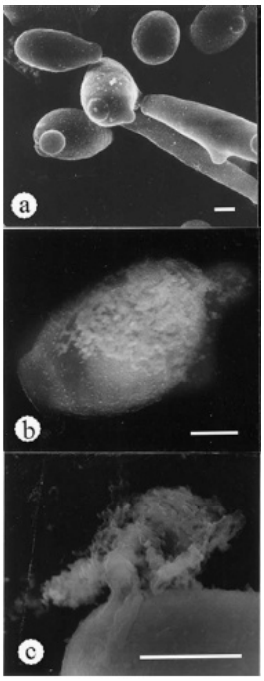
Antibacterial mechanism



- Damage to the membrane integrity:
  - ✓ ↑ Permeability
  - ✓ pH alteration
  - ✓ Loss of inorganic ions
- ↓ Intracellular ATP

Antifungal essential oils

Mechanisms



⇨Degradation of cell wall

⇨Damage to the cytoplasmic membrane

⇨Inhibition of germ tube formation

⇨↓ Ergosterol biosynthesis

Candida albicans untreated (a) and treated (b and c) with the essential oil of *Ocimum gratissimum* (main constituent: eugenol)

Pinto *et al.* (2009) *J Med Microbiol* 58: 1454-1462.
Carlson *et al.* (2006) *Clin Microbiol Rev* 19(1): 50-62.
Nakamura *et al.* (2004) *Res Microbiol* 155: 579-586.

Antifungal essential oils

Synergism among constituents

Table 4 Checkerboard assay of combinations of the four major components of *Thymus* (thymol, 1,8-cineole, *p*-cymene and carvacrol)

	Fractional inhibitory index (FIX)		
	C. albicans M1	*C. krusei* H9	Outcome
Thymol/Cineole	0.125	0.125	Synergy
Thymol/*p*-Cymene	0.125	0.125	Synergy
Thymol/Carvacrol	0.500	0.500	Indifferent
p-Cymene/Carvacrol	0.500	0.500	Indifferent
Carvacrol/Cineole	0.250	0.250	Synergy
p-Cymene/Cineole	0.250	0.250	Synergy

MIC was determined by the macrodilution technique.

Pina-Vaz *et al.* (2004) JEADV 18: 73-78.

Antifungal essential oils

Synergism between estragole and ketoconazole

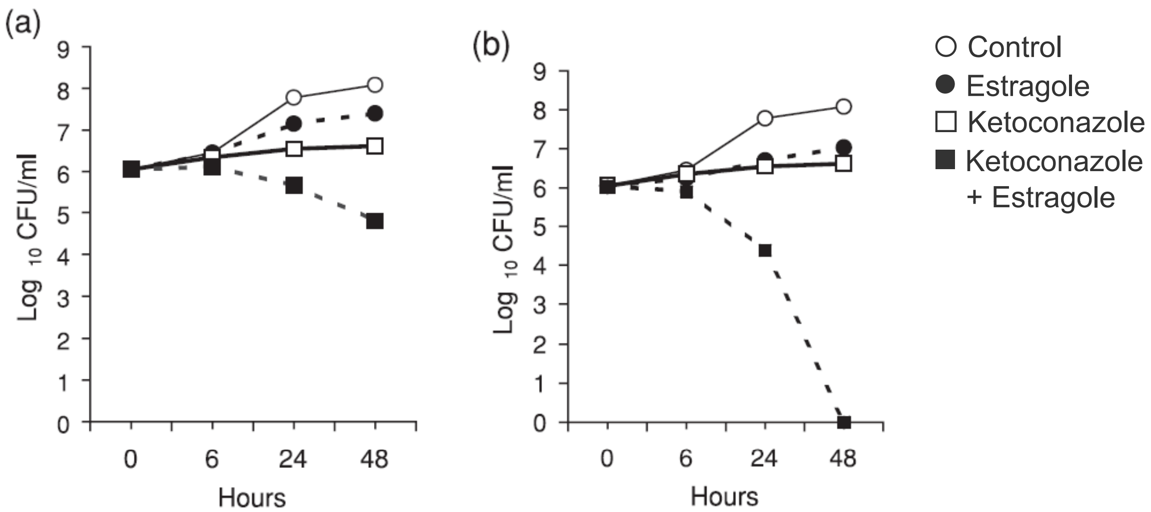


Figure 1. Time-kill curves of ketoconazole (MIC) in combination with estragole in concentration of 1/2 MIC (a) and MIC (b) against *C. albicans*. The fungal suspensions were cultured with estragole alone (●), ketoconazole alone (□), and ketoconazole plus estragole (■),with 30 ul of tween 80 in all cultures. Data were compared with control (○).

Shin and Pyun (2004) Phytother Res 18: 827-830.

Antifungal essential oils

Plinia cerrocampanensis Leaf

Vila *et al.* (2010) Bioresour Technol, 101: 2510-2514

Zuccagnia punctata Aerial part

Alvarez *et al.* (2012) Phytochem Lett 5: 194-199

Ferula hermonis Root

Al-Ja'fari *et al.* (2011) Phytochemistry, 72: 1406-1413

Antifungal essential oils

***Ferula hermonis* (root)** Lebanon/Syria

Shirsh-el-Zallouh

- ✓ Used in the Middle East to improve sexual behaviour in the treatment of frigidity and impotence.
- ✓ Several *Ferula* sp. are traditionally used for the treatment of skin infections.

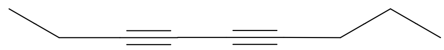
Al-Ja'fari *et al.* (2011) Phytochemistry, 72: 1406–1413

Antifungal essential oils

Ferula hermonis (root) Lebanon/Syria

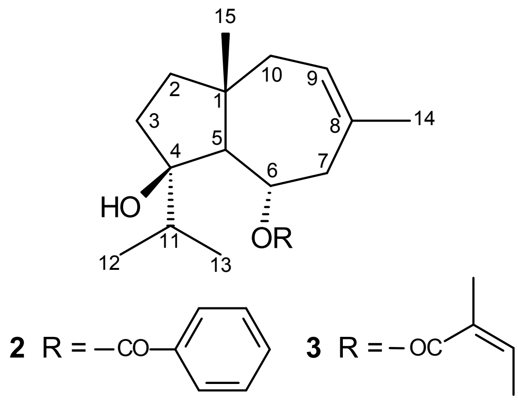
Constituents of the essential oil

Identified: > 90% (79 constituents)



1

Major constituents:
α-Pinene (43.3 %)
α-Bisabolol (11.1 %)
3,5-Nonadiyne (4.4 %) (**1**)



Other constituents:
Jaeschkeanadiol derivatives:
Jaeschkeanadiol (1.9 %)
Jaeschkeanadiol benzoate (1.9 %) (**2**)
Jaeschkeanadiol angelate (0.1 %) (**3**)

Al-Ja'fari *et al.* (2011) Phytochemistry, 72: 1406–1413

Antifungal essential oils

Ferula hermonis (root) Lebanon/Syria

MIC and MFC (µg/ml) of the active constituents and fractions

Substance	*M. gypseum* MIC	*M. gypseum* MFC	*T. mentagrophytes* MIC	*T. mentagrophytes* MFC
α-Pinene	32	32	64	64
α-Bisabolol	16	16	16	32
Nonadiyne	**8**	**16**	**8**	**8**
trans-Verbenol	32	32	32	32
α-Bisabolol oxide	64	128	64	64
Jaeschkeanadiol benzoate 73%	64	128	**0.25**	**0.25**
Jaeschkeanadiol angelate	32	64	32	32
Spathulenol 50%	32	32	32	32
Amphotericin B	1	1	0.5	0.5
Nystatin	4	8	2	2
Ketokonazole	8	>8	0.25	0.25
Clotrimazol	0.125	0.25	0.0625	0.0625

Al-Ja'fari *et al.* (2011) Phytochemistry, 72: 1406–1413

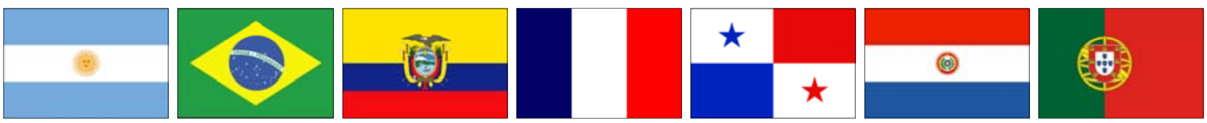
Antifungal compounds from plants

Conclusions

- Fungal infections are still an unsolved health problem.
- There is a need of new antifungal agents.
- Plants offer a good opportunity for new develop-ments.
- Unsaturated fatty acids, triterpene derivatives and essential oils have been shown as promising groups.
- There is a limited knowledge on mechanisms, pharmacokinetics and clinical profile.

International cooperation on antifungals

Country	Institution	Researcher
ARGENTINA	Universidad de Rosario	S. Zacchino
BRASIL	Universidade Bandeirante de São Paulo	S. Mendonca
ECUADOR	Fundación Hábitat Siglo XXI	F. Ghia
FRANCE	Université de Corse/CNRS	J. Casanova, F. Tomi
PANAMA	Universidad de Panamá	M.P. Gupta
PARAGUAY	Universidad Nacional de Asunción	E. Ferro
PORTUGAL	Universidade de Coimbra	L. Salgueiro



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