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COMPARATIVE STUDIES OF THE ANTIMICROBIAL ACTIVITY OF CRUDE EXTRACTS AND FRACTIONS FROM *EUGENIA CARYOPHYLLUS* AGAINST *CANDIDA ALBICANS* ISOLATE FROM CHRONIC DISEASE AFFECTED PATIENTS

Panagal Mani[1], M. Boominathan[2]*

1. Department of Biotechnology, School of Life Sciences, Vels University (VISTAS), Chennai, Tamil Nadu, India

2. Department of Biotechnology, Marudhu Pandiyar College, Thanjavur-613 403, Tamil Nadu, India.

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For Correspondence:

M. Boominathan

Department of Biotechnology, Marudhu Pandiyar College, Thanjavur-613 403, Tamil Nadu, India

E-mail:

master.maniji@gmail.com

ABSTRACT

This study was carried out to evaluate the antimicrobial activity of water, ethanol, methanol, acetone, hexane and butanol crude extract and fractions of *Eugenia caryophyllus*. The antimicrobial activity was determined by the disc diffusion method and significant inhibitory concentration against fungal strain. The combining ethanol with methanol at 2:18 concentration moderately inhibits (6 mm inhibition zone) *Candida albicans* than all individual extract and fractions. In the GC-MS analysis 65 bioactive phytochemical compounds were identified in the ethanolic fraction of *Eugenia caryophyllus*. The results indicated that fractions of *Eugenia caryophyllus* were highly potent as antibacterial agent.

INTRODUCTION

Oral candidosis is the most common fungal infection encountered in general dental practice[1] caused by *Candida albicans.* It is an opportunistic pathogen present in about 50-60% of the healthy human population, and becomes pathogenic when the host immune defense is undermined such as in HIV infection[2]. Liu et al.[3] reported 90% of AIDS patients affect oral and/or or opharyngeal candidiasis in various stages. However, synthetic drug treatments against *Candida albicans* can cause various side effects in chronic disease affected patients (i.e. HIV/AIDS, Cancer, Diabetes etc). hence, the search for more effective agents with low side effect from plant source.

Eugenia caryophyllus (clove), belonging to the family Myrtaceae, an ever-green plant of ten-to-twenty centimeters in height with spear-shaped leaves and racemiferous yellowish flowers[4]. Species of this family are often used for several medical purposes. In particular, *Eugenia caryophyllus* has pronounced anti-fungal[5], anti- bacterial[6], antioxidant[7] and hepatoprotective[8] properties. The objective of the present study was to examine effects of *Eugenia caryophyllus* extract and different solvent against human pathogenic fungus *Candida albicans.*

MATERIALS AND METHODS

Plant material

Eugenia caryophyllus fresh leaves were collected from various area of Tamil Nadu and analysis was carried out at Vels University, Chennai, Tamil Nadu, India.

Extraction

The shade dried plant material was chopped into small pieces and finally pulverized into fine powder. 500g of powdered plant material was soaked and then extracted successively water, ethanol, methanol, acetone, hexane and butanol solvent in separate Soxhlet extractor for 48h. The extract was concentration to dryness in rotary vacuum evaporator and stored -30˚C until further use.

Fractionation

Column was packed with ethanol with silica gel, sample was loaded as slurry of silica gel and the column was eluted with increasing concentration of water, ethanol, methanol, acetone, hexane and butanol solvent to increase polarity. After, active fraction was stored in a refrigerator until used for further usage.

Micro organisms

Clinical pathogenic fungal organisms *Candida albicans* was used for this study. These organisms were clinical isolates of patients isolated from clinical patients at dental clinics in and around Thanjavur and Chennai, Tamil Nadu, India.

Determination of antimicrobial activity

Culture supernatants with fractions of extract were used in the disc-diffusion method separately. *Candida albicans* swabbed on the surface of the sabouraud agar plates and discs (Whatman No.1 filter paper with 9 mm diameter) impregnated with the 50 µl of each plant sample was place on the surface individually. To compare the antifungal activities, Nystatin (20 µg/disc) used as standard antibiotic and negative control, a blank disc impregnated with solvent followed by drying was used. The plates (triplicates) were incubated 28°C for 72 h. The antimicrobial potency of the test samples was measured by determining the diameter of the zones of inhibition in millimeter.

GC-MS analysis

30 g powdered sample of *Eugenia caryophyllus* were soaked and dissolved in 75 ml of methanol for 24 h. Then the filtrates were collected by evaporated under liquid nitrogen. The GC-MS analysis was carried out using a Clarus 500 Perkin- Elmer (Auto System XL) Gas Chromatograph equipped and coupled to a mass detector Turbo mass gold – Perking Elmer Turbomas 5.2 spectrometer with an Elite-1 (100% Dimethyl ply siloxane), 300 m x 0.25 mm x 1 µm df capillary column. The instrument was set to an initial temperature of 110°C, and maintained at this temperature for 2 min. At the end of this period, the oven temperature was raised upto 280°C, at the rate of an increase of 5°C/min, and maintained for 9 min. Injection port temperature was ensured as 250°C and Helium flow rate as 1 ml/min. The ionization voltage was 70 eV. The samples were injected in split mode as 10:1. Mass Spectral scan range was set at 45-450 (mhz). The chemical constituents were identified by GC-MS. The fragmentation patterns of mass spectra were compared with those stored in the spectrometer database using National Institute of Standards and Technology Mass Spectral database (NIST-MS). The percentage of each component was calculated from relative peak area of each component in the chromatogram.

Table 1: Antimicrobial activity of individual extract and fraction of *Eugenia caryophyllus* tested against *Candida albicans* by disk diffusion method.

Plant sample / Solvent	Zone of inhibition (mm)					
	Water	**Ethanol**	**Methanol**	**Acetone**	**Hexane**	**Butanol**
***E. caryophyllus* extract**	1	3	4	2	0.5	1
***E. caryophyllus* fraction**	3	5	4	0.5	1	2

Table 2: Antimicrobial activity of ethanol and methanol combined fractions of *Eugenia caryophyllus* tested against *Candida albicans* by disk diffusion method.

Plant sample/ Fraction concentration	Zone of inhibition (mm)								
	18:2 (E/M)	**16:4 (E/M)**	**14:6 (E/M)**	**12:8 (E/M)**	**10:10 (E/M)**	**8:12 (E/M)**	**6:14 (E/M)**	**4:16 (E/M)**	**2:18 (E/M)**
Eugenia caryophyllus	0.5	1	1	-	3	1	1	-	6

Table 3: The main compounds identified by GC-MS in the extracts of *Eugenia caryophyllus*

S.No.	Peak Name	Retention time	Peak Area	%Peak Area
1.	Name: Methyl acetoxyacetate Formula: $C_5H_8O_4$ MW: 132	4.08	1015507	0.1008
2.	Name: 2-Cyclopentene-1,4-dione Formula: $C_5H_4O_2$ MW: 96	5.76	14442367	1.4337
3.	Name: N,N-Dinitropiperazine Formula: $C_4H_8N_4O_4$ MW: 176	6.33	1033862	0.1026
4.	Name: 2,5-Furandione, dihydro-3-methylene- Formula: $C_5H_4O_3$ MW: 112	6.88	1250462	0.1241
5.	Name: Cycloheptanone Formula: $C_7H_{12}O$ MW: 112	8.50	1316550	0.1307
6.	Name: 4-Amino-5-imidazolecarboxamide hydrochloride Formula: $C_4H_6N_4O$ MW: 126	9.71	7997190	0.7939
7.	Name: 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- Formula: $C_6H_8O_4$ MW: 144	10.98	8897269	0.8833
8.	Name: Leucinocaine Formula: $C_{17}H_{28}N_2O_2$ MW: 292	11.81	1549334	0.1538
9.	Name: 5-Methyl-2-(2-methyl-2-tetrahydrofuryl)tetrahydrofuran Formula: $C_{10}H_{18}O_2$ MW: 170	11.99	397154	0.0394
10.	Name: Benzofuran, 2,3-dihydro- Formula: C_8H_8O MW: 120	12.51	3212366	0.3189
11.	Name: 1-Ethyl-2-hydroxymethylimidazole Formula: $C_6H_{10}N_2O$ MW: 126	12.60	6201052	0.6156
12.	Name: Phenol, 4-(2-propenyl)-	13.05	20639580	2.0489

	Formula: $C_9H_{10}O$ MW: 134			
13.	Name: Benzene, 1-methoxy-4-(1-propenyl)- Formula: $C_{10}H_{12}O$ MW: 148	13.46	450396	0.0447
14.	Name: 1,3-Benzodioxole, 5-(2-propenyl)- Formula: $C_{10}H_{10}O_2$ MW: 162	13.57	544346	0.0540
15.	Name: Butanoic acid, 3-oxo-, 1-methylpropyl ester Formula: $C_8H_{14}O_3$ MW: 158	13.76	3157539	0.3135
16.	Name: 2-Methoxy-4-vinylphenol Formula: $C_9H_{10}O_2$ MW: 150	13.99	4206333	0.4176
17.	Name: Eugenol Formula: $C_{10}H_{12}O_2$ MW: 164	14.82	11265536	1.1184
18.	Name: Phenol, 4,4'-(1-methylethylidene)bis[2-methyl- Formula: $C_{17}H_{20}O_2$ MW: 256	15.40	4607156	0.4574
19.	Name: Vanillin Formula: $C_8H_8O_3$ MW: 152	15.56	3982290	0.3953
20.	Name: 1,2,3-Benzenetriol Formula: $C_6H_6O_3$ MW: 126	15.67	49543320	4.9183
21.	Name: Caryophyllene Formula: $C_{15}H_{24}$ MW: 204	15.84	97118320	9.6411
22.	Name: Phenol, 2-methoxy-4-(1-propenyl)- Formula: $C_{10}H_{12}O_2$ MW: 164	16.29	6814702	0.6765
23.	Name: à-Caryophyllene Formula: $C_{15}H_{24}$ MW: 204	16.43	11333587	1.1251
24.	Name: Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-, (1à,4aà,8aà)- Formula: $C_{15}H_{24}$ MW: 204	16.65	2484150	0.2466

25.	Name: à-Farnesene Formula: $C_{15}H_{24}$ MW: 204	16.90	3695149	0.3668
26.	Name: 2-(3-Isopropyl-4-methyl-pent-3-en-1-ynyl)-2-methyl-cyclobutanone Formula: $C_{14}H_{20}O$ MW: 204	16.97	5231360	0.5193
27.	Name: Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene- Formula: $C_{15}H_{24}$ MW: 204	17.06	4164709	0.4134
28.	Name: Phenol, 2-methoxy-4-(2-propenyl)-, acetate Formula: $C_{12}H_{14}O_3$ MW: 206	17.25	409168320	40.6190
29.	Name: Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)- Formula: $C_{15}H_{24}$ MW: 204	17.32	20927576	2.0775
30.	Name: Naphthalene, 1,2,3,4-tetrahydro-1,6-dimethyl-4-(1-methylethyl)-, (1S-cis)- Formula: $C_{15}H_{22}$ MW: 202	17.42	3769918	0.3742
31.	Name: Naphthalene, 1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl)- Formula: $C_{15}H_{24}$ MW: 204	17.59	13419531	1.3322
32.	Name: à-Calacorene Formula: $C_{15}H_{20}$ MW: 200	17.77	904913	0.0898
33.	Name: 1H-Indene, 1-ethylideneoctahydro-7a-methyl-, (1E,3aà,7aá)- Formula: $C_{12}H_{20}$ MW: 164	18.05	2340252	0.2323
34.	Name: N-Acetyltyramine Formula: $C_{10}H_{13}NO_2$ MW: 179	18.17	1164902	0.1156
35.	Name: Caryophyllene oxide Formula: $C_{15}H_{24}O$ MW: 220	18.51	35726160	3.5466
36.	Name: Phenol, 2-methoxy-4-(2-	18.65	514664	0.0511

	propenyl)-, acetate Formula: $C_{12}H_{14}O_3$ MW: 206			
37.	Name: Ledol Formula: $C_{15}H_{26}O$ MW: 222	18.84	2135293	0.2120
38.	Name: 12-Oxabicyclo[9.1.0]dodeca-3,7-diene, 1,5,5,8-tetramethyl-, [1R-(1R*,3E,7E,11R*)]- Formula: $C_{15}H_{24}O$ MW: 220	18.93	6585502	0.6538
39.	Name: Cubenol Formula: $C_{15}H_{26}O$ MW: 222	19.11	13030455	1.2936
40.	Name: Tetracyclo[6.3.2.0(2,5).0(1,8)]tridecan-9-ol, 4,4-dimethyl- Formula: $C_{15}H_{24}O$ MW: 220	19.34	37401368	3.7129
41.	Name: Methyl steviol Formula: $C_{21}H_{32}O_3$ MW: 332	19.57	14958485	1.4850
42.	Name: Kauran-18-al, 17-(acetyloxy)-, (4á)- Formula: $C_{22}H_{34}O_3$ MW: 346	19.78	16116079	1.5999
43.	Name: 2',3',4' Trimethoxyacetophenone Formula: $C_{11}H_{14}O_4$ MW: 210	19.95	69654616	6.9148
44.	Name: Benzeneacetic acid, 4-hydroxy-3-methoxy-, methyl ester Formula: $C_{10}H_{12}O_4$ MW: 196	20.59	3264975	0.3241
45.	Name: 4-Hydroxy-2-methoxycinnamaldehyde Formula: $C_{10}H_{10}O_3$ MW: 178	20.77	8510876	0.8449
46.	Name: Cyclohexanol, 1,3,3-trimethyl-2-(3-methyl-2-methylene-3-butenylidene)-, (E)- Formula: $C_{15}H_{24}O$ MW: 220	21.08	6965316	0.6915
47.	Name: Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-	21.31	1462924	0.1452

	methylethyl)-, (1S-cis)- Formula: $C_{15}H_{24}$ MW: 204			
48.	Name: Menthol, 1'-(butyn-3-one-1-yl)-, (1S,2S,5R)- Formula: $C_{14}H_{22}O_2$ MW: 222	21.53	1257368	0.1248
49.	Name: 2,6,10-Dodecatrien-1-ol, 3,7,11-trimethyl-, acetate, (E,E)- Formula: $C_{17}H_{28}O_2$ MW: 264	21.66	7888633	0.7831
50.	Name: Spiro[4.5]decan-7-one, 1,8-dimethyl-8,9-epoxy-4-isopropyl- Formula: $C_{15}H_{24}O_2$ MW: 236	21.97	5478447	0.5439
51.	Name: 6-Benzyloxy-2,6-dimethyl-octa-2,7-dien-1-ol Formula: $C_{17}H_{24}O_2$ MW: 260	22.70	1599378	0.1588
52.	Name: 4,4,8-Trimethyltricyclo[6.3.1.0(1,5)]dodecane-2,9-diol Formula: $C_{15}H_{26}O_2$ MW: 238	23.15	5597798	0.5557
53.	Name: 5,6,7,7-Tetramethyl-octa-3,5-dien-2-one Formula: $C_{12}H_{20}O$ MW: 180	23.53	2984011	0.2962
54.	Name: Phenol, 4-[3-(acetyloxy)-1-propenyl]-2-methoxy-, acetate Formula: $C_{14}H_{16}O_5$ MW: 264	24.33	1576108	0.1565
55.	Name: 9,12-Octadecadienoic acid, methyl ester Formula: $C_{19}H_{34}O_2$ MW: 294	26.64	1473753	0.1463
56.	Name: 4-Cyclopentene-1,3-dione, 4-(3-methyl-2-butenyl)- Formula: $C_{10}H_{12}O_2$ MW: 164	27.16	1027403	0.1020
57.	Name: Cyclopropane, 1-(1,2-dimethylpropyl)-1-methyl-2-nonyl- Formula: $C_{18}H_{36}$ MW: 252	28.91	1906665	0.1893
58.	Name: Methanone, 1H-imidazol-4-yl(octahydro-4a(2H)-naphthalenyl)-	29.19	2428757	0.2411

	, trans- Formula: $C_{14}H_{20}N_2O$ MW: 232			
59.	Name: 1,6,10,14,18,22-Tetracosahexaen-3-ol, 2,6,10,15,19,23-hexamethyl-, (all-E)- Formula: $C_{30}H_{50}O$ MW: 426	30.68	1361649	0.1352
60.	Name: Pentadecanoic acid, ethyl ester Formula: $C_{17}H_{34}O_2$ MW: 270	33.11	4642035	0.4608
61.	Name: Phenol, 2-methoxy-4-(1-propenyl)-, (Z)- Formula: $C_{10}H_{12}O_2$ MW: 164	33.30	3316504	0.3292
62.	Name: Phenol, 2-methoxy-4-(2-propenyl)-, acetate Formula: $C_{12}H_{14}O_3$ MW: 206	34.06	1115013	0.1107
63.	Name: Estragole Formula: $C_{10}H_{12}O$ MW: 148	34.24	15350748	1.5239
64.	Name: Phenol, 4-[2,3-dihydro-7-methoxy-3-methyl-5-(1-propenyl)-2-benzofuranyl]-2-methoxy- Formula: $C_{20}H_{22}O_4$ MW: 326	34.59	1355893	0.1346
65.	Name: o-Anisic acid, 2-adamantyl ester Formula: $C_{18}H_{22}O_3$ MW: 286	36.24	2399016	0.2382

RESULTS AND DISCUSSION

Eugenia caryophyllus all soul fractions and crude extracts showed different degree of antifungal activities against candidiasis causing fungi (Table 1). The substantial activity against *Candida albicans* was pointed out in ethanol and methanol solvent fraction, therefore those fractions were combining at various combinations to treat newly plated *Candida albicans* strain (Table 2). The favorably antimycotic activity of 6 mm zone of inhibition was exposed 2:18 combinations, which were corresponding to that of standard antibiotics such as Nystatin. Teke et al.[9] reported that the fraction had the highest activity against both bacterial

and fungal isolates. However, the individual crude extract of ethanol, methanol, acetone, hexane, water, and butanol showed lower antifungal activity with the average zone.

In addition, GC-MS analysis, totally 65 compounds identified from the methanol fraction of the *Eugenia caryophyllus* is presented in Table 3. The plant samples revealed the synthesis of Methyl acetoxyacetate; 2-Cyclopentene-1,4-dione; N,N-Dinitropiperazine; 2,5-Furandione, dihydro-3-methylene-; Cycloheptanone; 4-Amino-5-imidazolecarboxamide hydrochloride; 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-; Leucinocaine; 5-Methyl-2-(2-methyl-2-tetrahydrofuryl)tetrahydrofuran; Benzofuran, 2,3-dihydro-; 1-Ethyl-2-hydroxymethylimidazole; Phenol, 4-(2-propenyl)-; Benzene, 1-methoxy-4-(1-propenyl)-; 1,3-Benzodioxole, 5-(2-propenyl)-; Butanoic acid, 3-oxo-, 1-methylpropyl ester; 2-Methoxy-4-vinylphenol; Eugenol; Phenol, 4,4'-(1-methylethylidene)bis[2-methyl-; Vanillin; 1,2,3-Benzenetriol; Caryophyllene; Phenol, 2-methoxy-4-(1-propenyl)-; à-Caryophyllene; Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-, (1à,4aà,8aà)-; à-Farnesene; 2-(3-Isopropyl-4-methyl-pent-3-en-1-ynyl)-2-methyl-cyclobutanone; Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-; Phenol, 2-methoxy-4-(2-propenyl)-, acetate;. All these compounds are of pharmacological importance as they possess the properties such as analgesic, anti-diabetic, antibacterial, and antifungal. In conclusion, the present study was resulted to develop newer lead for better and safer antimicrobial agents from *Eugenia caryophyllus* fraction. Isolation and structure determination of these active metabolites are in process. Further studies are needed to identify the pure component and establish the exact mechanism of action for antibacterial action of the plant fractions.

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