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Research Article

Chemical Composition and Evaluation of the Antimicrobial Activity of the Of *Illicium Verum* and the Derivations of Protocatechuic Acid

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ARTICLE INFO

Article history:
Received: 24 January, 2020
Accepted: 2 March, 2020
Published: 18 March, 2020

Keywords:
Illicium
antibacterial
protocatechic acid
gram positive
gram negative

ABSTRACT

In our present studies we identify the components of essential oil, crude extracts and fraction of *Illicium verum*, and evaluation of antibacterial activity. We isolated and identified as 3, 4-dihydroxybenzoic acid (protocatechuic acid) through spectroscopic studies and comparison with the data of the authentic sample [1]. The phase MEA itself was emerged as the active fraction comprising antibacterial, antioxidant and nematocidal constituents, including 3, 4-dihydroxybenzoic acid which also possessed antibacterial, Superb antioxidant and nematocidal activities [1].

Introduction

Extracts obtained from many plants have recently gained popularity and scientific interest. Plants have been used for different purposes, such as food, drugs and perfumery. Researchers have been interested in biologically active compounds isolated from plant species for the elimination of pathogenic microorganisms have built against antibiotics [1]. The plant family Illiciaceae has only one genus *Illicium.* This small genus has forty-two species, which are aromatic shrubs or small trees and are distributed in Atlantic North America, China and Indo China. The true star anise *Illicium verum* locally known as Badian is an evergreen tree. Star anise fruit has an agreeable, aromatic, sweet taste and a pleasant odor [2]. Star anise oil is used as a flavoring agent in confectionery, candy, chewing gum, tobacco, animal feeds, liqueurs and pharmaceutical preparations also used in perfumery and soaps. The oil is stimulant, stomachic, carminative, mildly expectorant and diuretic. It relieves colic and is an ingredient of cough lozenges [3].

However, to the best of our knowledge, there is no study regarding the antimicrobial activity against various microorganisms of that crude extracts and fractions [4]. This is the first report of the bioassay guided fractionation of methanolic extract of dried flower and their antimicrobial activity was studied in detail. Additionally, the analysis of the chemical composition and antimicrobial activity of essential oil by broth dilution method against several bacteria models.

Materials and Methods

I Plant Material

The dried fruits of Badian were purchased from local market in July of 2003 in Karachi.

II Essential Oil Isolation

Essential oil was extracted from the dried fruits of *I. verum* by the method of steam distillation, for this purpose passing dry steam to the plant material, whereby the steam volatile oil present in the plant material is volatilized and passed through the condenser along with steam to the receiver. This method gives better, cheaper and quicker, but not applicable to delicate flower.

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http://dx.doi.org/10.31487/j.GDT.2020.01.03

III Instrumentation

i Gas Chromatography

Gas chromatography using FID, was carried out on a shimadzu gas chromatograph GC-17A hooked with shimadzu class GC-10 software and equipped with a less polar capillary column (25 m x 0.53 mm ID x 0.50-micron fitter coating of carbowax-10 M). The analysis was performed with an initial temperature 50oC for 5 minutes, then temperature programmed 5°C/mm to a final temperature 22°C with final time 25 min (Program A). Injector with splitting ratio 1:30 was set at 260°C and FID at 280°C. Carrier and make up gas were nitrogen with a flow of 0.7 and 3 ml/min at a pressure of 0.5 Kg/cm2 and 4 Kg/cm2 respectively.

ii Gas Chromatography-Mass Spectrometry

For GC-EIMS experiments a Hewlett-Packard 5890 gas chromatography was combined with a JMS-HX 110 (Jeol Japan) was spectrometer operating in EI mode with ion source at 270ºC and electron energy at 70 eV. Injector was set at 270ºC with splitting ration 1:50. Analysis (program B) on an equivalent column SPB-5 (30m x 0.25 mm ID and 0.22-micron film thickness) carrier gas was helium. The column was kept initially at temperature 50ºC for 2 min, raised to a final temperature 260ºC at a rate of 5ºC/min with final holding time - minutes. Mass spectral survey was performed using MS-libraries (NIST Mass spectral Search Program, 1988; GC-MS library from Shimadzu, 1996) [5, 6].

Ultraviolet spectra were recorded in MeOH on Hitachi-µ-3200 and infra-red spectra were measured in $CHCl_3$ and KBr on VECTOR22 of BRUKER spectrophotometer. The High-resolution mass spectra were measured on JM-600 H spectrophotometer. ^{1}H-NMR were taken in deuteroacetone with trimethylsilane (TMS) as an internal standard using a Brucker AM 300 L instrument.

IV Identification of Constituents

Identification of the components of essential oil, crude extracts (IL-1-PE) and fractions (IL-1-EA-2) and IL-1EA-6 was based on (a) matches of their mass spectra with the National. Institute of Standards Technology (NIST 3.0) libraries provided with the computer controlling GCMS system, (b) further confirmation was obtained from Kovats retention index (RI) data generated against a series of n-alkanes.

V Extraction

The dried fruit of *I. verum* (5 Kg) purchased from local market were crushed in a food processor. A subsample of homogenate was extracted thrice with methanol at room temperature. The extracts were combined together, and solvent removed under reduced pressure to give a thick residue IL-1, (1287 g). The more left was extracted with methanol thrice giving methanolic extract, which on evaporation of solvent under reduced pressure give a residue IL-2 (105.86 g). The more ways than extracted with methanol, water (70:30) and the extract, on evaporation of the solvent afforded the residue IL-3 (50.02 g). the more left was extracted with 30% methanol, (30:70, MeOH: H2O) to give syrupy residue IL-4 (139.2598 g).

VI Bioassay Monitored Fractionation

The extract IL-1, (788 g) was partitioned between distilled water and petroleum ether to give petroleum ether and aqueous layers. The later phase was extracted successively with ethyl acetate and butanol and each layer were washed with water. The pet. ether and butanol phases on evaporation of the solvent afforded residues IL-1-PE (65.88 g), IL-1-But1 (139.46 g), IL-1-But2 (28.51 g) respectively. The combined EA phases were dried with anhydrous sodium sulphate and filtered, the filtrate on evaporation of solvent under reduced pressure furnished 32.74 g of IL-1-EA. The aqueous layer after freeze drying afforded the residue IL-1-Aq (220.62 g), which on treatment with methanol gave methanol soluble (IL-1-Aq M sol) and insoluble (IL-1-Aq M).

VII GC/GCEIMS Analysis

The EA (ethyl acetate) fraction (IL-1-EA, 32.57 g) was treated with petroleum ether affording petroleum ether soluble fraction (IL-1-EA-PEins, 30.00 g), the later portion was divided into ethyl acetate soluble (29.96 g) and insoluble fractions (0.31 g). The ethyl acetate soluble portion was concentrated under reduced pressure to a syrupy mass which was poured into large quantity of petroleum ether with shaking gently yielding petroleum ether-ethyl acetate soluble (IL-1-EA-EA:PE, 5.48 g) and petroleum ether-ethyl acetate) insoluble (IL-1-EA-EA:PE, 17.77 g) portions. The fractions IL-1-EA-PE, IL-1-EA-EA:PE solu. and essential oil (V-oil) which identified several compounds.

VIII Isolation and Preparations of the Compounds 1 and 2

i Isolation of 3, 4, 5-Trihydroxy-1-Cyclohoxene-1-Carboxylic Acid (Shikimic Acid)

Shikimic acid is a very important and well-known compound, as it is a precursor of a large number of natural products e.g. cinnamates, benzoic acids, flavonoids and alkaloids. When MeOH soluble portion of aqueous phase (IL-1-AqM solu.) was subjected to preparative thick layer chromatography (PTLC) affording five bands of which band 3 (IL-1-Aq-P3 Scheme 1) was found to be compound 1 through spectral studies compound 1 (Figure 1) obtained as a light brown powder easily soluble in methanol.

ii Isolation of 3, 4-Dihydroxybenzoic Acid (Protocatechuic Acid) 2

The fraction IL-1-EA-EA:PE In, 17.00 g (Scheme a) was subjected to vacuum liquid chromatography (VLC, silica gel, petroleum ether, ethyl acetate, methanol and water in order of increasing polarity) affording 38 fractions. Fractions 13F was separated into a number of fractions through classical solvent partition. Fraction 13G was divided into chloroform and chloroform methanol (1:1) soluble fractions, 13G-C and 13G-CM respectively. 13G-C on further solvent separation (chloroform and methanol twice) gave 13GC-C, 13GC-M1 and 13GC-M2 fractions. 13GC-M1 was subjected to PTLC affording four bands (Scheme 1) of which band four was found to be a known compound 3,4-dihydroxybenzoic acid (protocatechuic acid, 2, Figure 1). Further study on other fractions is going on.

iii Preparation of Acetyl Derivatives of Protocatechuic Acid 3 and 4

Protocatechuic and acid 2 (20 mg) was subjected to acetylation with acetic anhydride (10 ml) was add in pyridine (15 ml) and the reaction mixture was kept at room temperature under hood for 24 hours. After 24 hours the reaction mixture showing single spot on TLC was evaporated at room temperature under hood, and identified as 3,4-diacetylbenzoic acid (3, HBA-1, Figure 1). It showed instability as after two days and converted into mono acetyl derivative, 3-acetyl-4-hydroxybenzoic acid (4, HBA-2). Employing the same acetylation process for the direct formation of monodactyl derivative, 3, 4-dihydroxybenzoic acid (0.20 g) with was acetic anhydride (0.19 ml) in the presence of pyridine (0.20 ml) was kept at room temperature under fuming hood. After half an hour TLC showed the 3-acetyl-4-hydroxybenzoic acid (4, HBA-2).

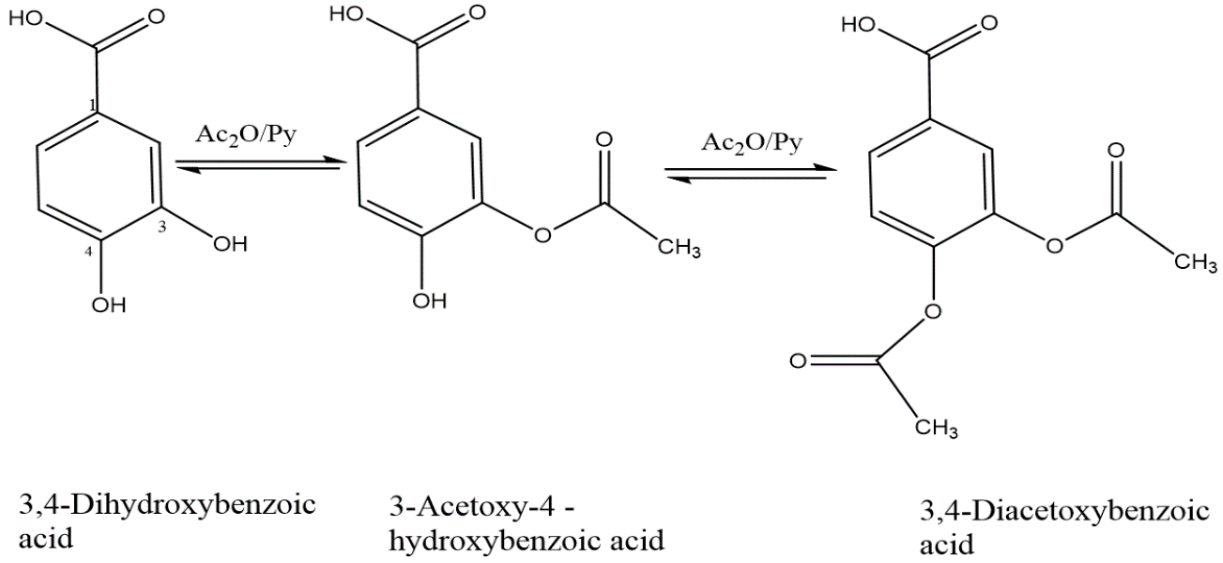

Figure 1: Acetyl derivatives of 3, 4-dihydroxybenzoic acid.

IX Characterization of Compound 3(HBA-1)

UV/VIS (MeOH): (max (log () In the range of 250 nm-260 nm no band observed. IR (KBr): (max (cm 1) 3069, 2923, 1686, 1600 and 1499, 1296.

^{1}H NMR (C3D6O; 400 MHz); δ 7.87 (1H, d, J = 1.5 Hz, H-2); 7.38 (1H, d, J = 8.4 Hz, H-5); 7.94 (1H, dd, J = 1.5 Hz, 8.4 Hz, H-6); 2.26 (6Hs, s, H-). EI-MS (eV): m/z .

X Characterization of Compound 4 (HBA-2)

UV/VIS (MeOH): (max (log () 251 nm. IR (KBr): (max (cm 1) 3370, 3075, 2924, 1678, 1612 and 1444, 1267. 1H NMR (C3D6O; 400 MHz); δ 7.71 (1H, d, J = 1.5 Hz, H-2); 7.04 (1H, d, J = 8.4 Hz, H-5); 7.80 (1H, dd, J = 1.5 Hz, 8.4 Hz, H-6); 2.28 (3Hs, s, H-); hump show on δ 9.50. EI-MS (eV): m/z .

XI Identification of Compounds 1-4

Identification of the compounds 1 and 2 have been made through spectral (UV, IR, Mass, 1H) studies and completion of reaction, at this stage reaction mixture was cease, with distilled cold water and readily filtered, was characterized as 3-acetyl-4-comparison of the data with the reported spectral values [7, 8]. As the spectral data of compounds 2 and 3 of synthesized compounds is not present in literature so, the spectral values are being given in the paper.

XII Sample Preparation for Antibacterial Assay

Primary screening: The disc diffusion technique of (Bauer *et al*. 1966) was used to determine the antibacterial activity of all the compounds. Sterile disc (6 mm) containing 200 µg of each compound / disc were prepared from a 10 mg /ml stock in DMSO (as the compound were insoluble in water). The Iso-sensitest agar (oxoid) plates were seeded with 24 h old culture grown in trypticase soya broth (TSB; oxoid) containing 107 ml (CFU: colony forming units). The prepared discs were placed on to the surface at different positions and plates were incubated at 37°C for 24 hours. The results were recorded by measuring the zones of inhibition against each derivative. DMSO was used as a negative control. The test samples showing zones of inhibition of 10 mm diameter were considered significant, which those giving 8 mm or 9 mm diameter zones were considered less active. Antibacterial activities of all the samples were also compared with the Gentamycin at a concentration of 30 µg/ml by disc diffusion method (Table 1).

XIII Evaluation of Antifungal Activity

All the fungal cultures were grown on Sabouraud's dextrose medium at 30°C. The activity against fungal cultures (except *Candida*) was determined by using compound incorporation method. Sabouraud's dextrose agar slants (3 ml) incorporated with the different test samples were prepared in concentrations of 100 µg/ml and 200 µg/ml. Terbinafine, an antifungal antibiotic was used as positive control, at a concentration of 10 µg/ml. Slants of each concentration of the samples in triplicate along with plain Sabouraud's dextrose agar were inoculated with the fungal culture and incubated at 30-32°C for 7-9 days according

to growth characteristics of the culture. The slants were then examined for growth inhibition of the inoculated accordingly.

XIV Evaluation of Anticandidal Activity

This was performed by using disc diffusion method (as described for the determination of antibacterial activity) on Sabouraud's dextrose agar medium (Table 2)

Table 1: Antimicrobial activity of the crude extracts, fractions and pure compounds of *I. verum*.

Gram Positive Bacteria	**IL-1**	**IL-2**	**IL-3**	**IL-4**	**IL-1-PE**	**IL-1-EA**	**IL-1-Butl**	**IL-1-But2**	**IL-1-Aq**	**IL-1-AqMsol**	**IL-1-AqMinsol**
Bacillus cereus	14	8	-	-	10	9	-	-	17	11	8
B. stearothermophillus NCTC 10003	-	-	-	-	-	-	-	-	-	-	-
B. subtilis	10	10	9	-	10	8	-	7	10	11	10
B. thuringiensis	-	8	-	-	-	-	-	-	-	-	-
Corynebacterium hoffmanii	-	-	-	-	-	-	-	-	-	-	-
C. xerosis	10	-	-	-	10	7	-	-	10	-	8
Micrococccus luteus	-	8	-	-	10	12	10	-	-	10	8
Staphylococcus aureus	12	8	-	-	10	8	8	9	7	12	9
S. aureus AB 188	11	8	-	-	8	10	-	7	7	8	7
S. epidermidis	9	8	-	8	7	-	-	-	-	8	-
S. saprophyticus	-	8	-	8	-	-	-	-	-	-	-
Streptococcus faecalis	-	-	-	-	13	8	-	9	-	-	-
S. pyogenes	-	-	-	-	9	7	-	-	-	-	7
Escherichia coli	7	-	-	-	7	8	-	8	8	10	9
Klebsiella pneumoniae	10	-	-	-	8	7	9	8	9	9	9
Proteus mirabilis	8	-	-	-	8	9	-	-		-	7
P. vulgaris	10	-	-	-	-	10	8	12	15	10	7
Pseudomonas aeruginosa PAO 286	-	-	-	-	-	10	-	10	9	-	8
Salmonella typhi	8	-	-	-	8	-	-	-	-	8	-
S. paratyphi A	-	-	-	-	-	9	-	9	8	10	-
S. paratyphi B	-	-	-	-	7	8	-	8	11	9	7
Shigella flexneri	-	-	-	-	9	8	-	7	-	-	-
Fungi											
Aspergillus niger	10	-	-	-	25	15	-	-	-	-	14
Penicillium	18	-	-	-	20	-	-	-	-	-	11
Candida albicans	10	-	-	-	9	-	-	-	-	-	-

Note: Results are not shown for extracts or fractions that were inactive against all organisms.

Table 2: The MIC in µg/disc of crude extract /fractions against bacteria.

Gram Positive Bacteria	**IL-1**	**IL-1-PE**	**IL-1-EA**	**IL-1-Aq In**
Bacillus cereus	125	125	1000	1000
B. subtilis	250	125	1000	1000
Corynebacterium xerosis	1000	1000	-	-
Micrococccus luteus	-	250	250	1000
Staphylococcus aureus	125	125	500	500
S. aureus AB 188	500	1000	125	1000
S. epidermidis	500	1000	-	-
Sreplococcus faecalis	-	125	1000	-
S. pyogenes	500	500	1000	1000
Gram negative Bacteria				
Escherichia coli	-	-	-	1000
Klebsiella pneumoniae	1000	1000	-	1000
Proteus mirabilis	-	1000	1000	-
P. vulgaris	-	1000	-	-
Pseudomonas aeruginosa	-	-	250	1000
Salmonella typhi	1000	1000	-	-
S. paratyphi A	-	-	1000	-

S. paratyphi B	-	-	1000	-
Shigella flexneri	1000	-	1000	-
Fungi				
Aspergillus niger	125	250	500	1000
Penecillium	500	125	-	1000
Candida albicans	-	62.5	125	-

- = No zone of inhibition.

XV Determination of Minimum Inhibitory Concentration (MIC)

Compounds showing antimicrobial activities were further tested for MIC in tryptone soya both (oxoid). Briefly, broth was prepared containing varying concentrations of thest compound ranging from 7.8125-500 µg/ml 200 µg/ml of each concentration and control in triplicate was transferred into sterile 96 well plates. Cultures containing 102-103 CFU were incubated in each well and the plates incubated at 37°C for 24 hours. Growth was measured and viability of culture was confirmed by inoculation of broth on tryptone soya agar plates (oxoid). Higher dilution showing no growth (MIC) was recorded. MIC against Gram +ve and –ve bacteria were also compound with the standard antibiotics Ampicillin and Gentamicin while, MIC against fungi compared with the Amphotericin B.

XVI Essential Oil (V-Oil) Analysis by Broth Dilution Method

i Preparation of Broth Medium

Oil sample was mixed in broth to achieve final concentration of 20 µg/ml were dispensed into screw cap tubes. Muller Hinton broth was used from all test cultures except for streptococci that were cultured in BHI. Media without oil was also prepared as control.

ii Transfer of Culture

Fresh suspensions of all cultures were prepared and mixed by vortexing, 100 µl was transferred to all tubes with and without oil. All tubes were incubated at 37ºC for 3 hours in shaking water bath at 150 rpm.

Plant media was adjusted as blank difference between O.D. of cultures without oil and with oil is taken as inhibition. Present inhibition was calculated, and it was considered as an indicative of antimicrobial activity. Determination of minimum inhibitory Concentration (MIC).

Results and Discussion

Antimicrobial Activity

The antibacterial activity of bio-guided crude extracts of dried flowers of Badian IL-1, IL-2, IL-3 and IL-4 and their fractions IL-1PE (petroleum ether fraction of IL-1), IL-1-EA (ethyl acetate fraction), IL-1-But 1 and 2 (butanol fractions), IL-1-Aq (aqueous phase), IL-1-Msol (methanol soluble portion of aqueous phase), and IL-1-AqM In. (methanol insoluble portion of aqueous phase) were tested against a range of Gram positive (nine strains) and Gram negative (nine strains). Bio-guided crude extract IL-1 being very good antibacterial and antifungal active than the IL-2, which is IL-2 antimicrobial less active while IL-3 and IL-4 was inactive against Gram positive (thirteen strains) and Gram negative (twelve strains) including four different Gram positive bacteria namely *Corynebacterium hofmannii*, *Staphylococcus saprophyticus*, *Bacillus thuringiensis*, *Bacillus* and three Gram negative bacteria *Shigella boydii*, *Shigella dysenteriae* and *Enterobacter* (same Gram positive and negative bacteria for IL-2). IL-1 showed activity against Gram positive bacteria *Staphylococcus aureus* and *Bacillus cereus* and significant antifungal activity against *Penicillium* and Aspergillus niger, resulting from them, the antifungal activity of IL-1 against *Penicillium* and *Aspergillus niger* went to IL-1-PE showed very promising activity.

Some activity view in IL-1-Aq M In showed antifungal activity against both fungi *Penecillium* and *Aspergillus niger*. IL-1-Aq phase being more antibacterial active than the IL-1-But 1 and 2, showed good activity against Gram positive bacteria *Bacillus cereus* and Gram-negative bacteria *Proteus vulgaris* (Table 2). The antibacterial activity of essential oil against a range of Gram positive (thirteen strains) and Gram negative (twelve strains). Obtained results revealed that essential oil exhibited Gram negative bacteria seemed to be more sensitive than the Gram positive bacteria, such are *Salmonella typhi*, *Salmonella typhi* para A and *Salmonella typhi* para B, percentage inhibition 98.8%, 86.3%, 91.4% respectively, while other than bacteria *Shigella flexneri*, *Shigella dysenteriae* and *Shigella boydii* showed percentage inhibition 95.6%, 81.6% and 89.4% as well as *Proteus vulgaris* 86.3% inhibit. Especially considerable is that the highest sensitivity of star-aniseed oil was observed activity against Gram positive bacteria Bacillus subtilis 99.7% inhibition, as well oil showed good antimicrobial activity against other than Gram positive bacteria such as *Streptococcus faecalis* and *Corynebacterium xerosis* 98.01%, 87% inhibit respectively.

Table 3: Antibacterial activity of V-Oil by Broth dilution method.

Gram Positive Bacteria	**Percentage Inhibition**
Bacillus cereus	55.8%
B. subtilis	99.7%
B. thuringiensis	70.8%
Corynebacterium hofmannii	50.1%
C. xerosis	87%
Micrococcus luteus	1.06%
Staphylococcus aureus	51%
S. aureus AB 188	55.2%
S. epidermidis	49.5%
S. saprophyticus	53.9%
Streptococcus faecalis	89.1%
S. pyogenes	75.1%
Gram negative Bacteria	
Enterobacter	68.7%
Escherichia coli	53.1%
Klebsiella pneumoniae	60.3%
Proteus mirabilis	46.2%
P. vulgaris	86.3%
Pseudomonas aeruginosa PAO 286	53.8%

Salmonella typhi	98.8%
S. paratyphi A	86.3%
S. paratyphi B	91.4%
Shigella boydii	89.4%
S. dysenteriae	81.6%
S. flexneri	95.6%

The MIC (µg/disc) of active crude extract IL-1 and its fractions IL-1-PE, IL-1-EA and IL-1-Aq M In were tested against Gram positive (nine strains) and Gram negative (nine strains) bacteria revealed that IL-1-PE was more active than IL-1 and IL-1-EA, while IL-1 Aq M In was found to be inactive. Fraction IL-1-PE displayed good activity against Gram positive bacteria *Stayphylococcus aureus*, *Staphylococcus fecalis* and *Bacillus subtilis*. Crud extract IL-1 exhibited the activity against *Staphylococcus aureus* and *Bacillus cereus*, while fraction IL-1-EA active against only *Staphylococcus aureus* AB 188. MIC of antifungal activity of above cited extract and fractions were tested against three fungi. IL-1 active against *Aspergillus niger*, while IL-1-PE showed activity against *Penicillium* and *Candida albicans* with MIC 125 and 62.5 mm respectively and IL-1-EA displayed activity against *Candida albicans* with MIC 123 mm (Table 3).

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