Saudi Pharmaceutical Journal 28 (2020) 1197–1202

Contents lists available at ScienceDirect

Saudi Pharmaceutical Journal

journal homepage: www.sciencedirect.com

Original article

Evaluation of the chemical constituents and potential biological activities of *Cunninghamella blakesleeana*

Fatemah A. Alasmary [a,*], Amani S. Awaad [b], Safar M. Alqahtani [c], Reham M. El-Meligy [d], Dalal A. Abdullah [a], Saleh I. Alqasoumi [e]

[a] *Chemistry Department, Faculty of Sciences, King Saud University, Riyadh, Saudi Arabia*
[b] *Gateway to United Kingdom Education Ltd., Bradford, UK*
[c] *Pharmaceutical Chemistry Department, College of Pharmacy, Prince Sattam bin Abdulaziz University, Al-Kharj 11942, Saudi Arabia*
[d] *Department of Medicinal and Aromatic Plants, Desert Research Center, Cairo, Egypt*
[e] *Department of Pharmacognosy, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia*

ARTICLE INFO

Article history:
Received 14 June 2020
Accepted 11 August 2020
Available online 17 August 2020

Keywords:
Cunninghamella blakesleeana
Staphylococcus aureus
Antimicrobial activity
Liver and kidney function

ABSTRACT

The aim of this work is to evaluate the chemical constituents and potential biological activists of *Cunninghamella blakesleeana.* Three fatty acids were isolated using column chromatography and identified as palmitic acid (F1), oleic acid (F2) and stearic acid (F3) in addition to other two steroidal compounds; α-amyrin (A4), and β-sitosterol (A5). Using GC, ten fatty acids were detected the major fatty acid obtained was stearic acid (74.61%) while palmitic acid was the second high percentage (10.35%), and the least percentage obtained was arachidic acid (0.07%). *C. blakesleeana* extract showed *in-vitro* antimicrobial activities against some microorganisms. The highest activity of *C. blakesleeana* total extract was reported against *Staphylococcus aureus* (18.3 ± 0.03 mm.) followed by *Streptococcus pyogenes* (15.3 ± 0.05), while the lowest were for both *Candida albicans* & *Pseudomonas aeruginosa* (6.7 ± 0.06 and 5.9. 0 ± 0.9 mm. respectively). The three isolated compounds (F1-3) showed activities against *Staphylococcus* aureus, *Penicillium expansum,* and *Salmonella typhimurium* only. The highest activity was aganist Staphylococcus aureus (13.0 ± 0.1 mm.). The highest effect was obtained by compound F3 (stearic acid) (15.0 ± 0.5 mm.), and compound F1 (oleic acid) (13.0 ± 0.1 mm.) and F2 (palmitic acid) 11.0 ± 0.3 mm. The total ethanol extract of the investigated fungus was safe up to 5000 mg kg^{-1} and did not produce any significant change in liver and kidney functions after oral administration (400 mg kg^{-1}) for 14 consecutive days. The results reported the isolation of some fungal new driving compounds which has been not isolated before from *Cunninghamella* species in addition to their correlated new biological activities.

1. Introduction

The fungal kingdom includes many species which have been utilized by humans for a variety of purposes since ancient times such as; food preparation, fermentation of bread, production of soy sauce (Chaturvedi et al., 2018, Lange, 2017) in addition to many other medical purposes (Yang et al., 2019).

The fungal secondary metabolites have a great diverse of molecular structure (Gao et al., 2018). Many phytochemical groups were isolated and identified from fungi such as Alkaloids, Fatty acid, polyketides, Phenolic compound, Terpenes, and Carbohydrates (Metsämuuronen et al., 2018). Generally, at present more than 90,000 species of fungi have been described which indicates that only about 7% of the world's fungi have as yet been recognized (Salar, 2018), however, not all of them have been fully investigated for their potential chemical and biological activities (Pusztahelyi et al., 2015, Amare and Keller, 2014).

Cunninghamella is a genus of fungus belonging to family Cunninghamellaceae which can be found in soil, plant and animal material, cheese and Brazil nuts (Nguyen et al., 2017). Members of this genus possess cytochrome *P*-450 monooxygenase systems analogous to those in mammals (Chen et al., 2014) therefor they

* Corresponding author.
E-mail address: fasmari@ksu.edu.sa (F.A. Alasmary).
Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.jsps.2020.08.009
1319-0164/

are used in wide range of drugs in manners similar to mammalian enzyme systems (Kelly and Kelly, 2013). Accordingly this genus was used as biotransformation media due to its ability to convert molecules to valuable medicinally active substances (Palmer-Brown et al., 2019).

Cunninghamella elegans ATCC 9245, biotransformed doxepin, a tricyclic antidepressant drug, was obtained as 85: 15% mixture of other 8 derivatives (Joanna et al., 1999). Also *Cunninghamella echinulata* biotransform the drug loratadine to its active metabolite desloratadine which proved to have four times more potent at low dose and longer duration than desloratadine (Keerthana and Vidyavathi, 2018). Some researcher reported that there are a diverse pathways for *Cunninghamella blakesleana* AS 3. 153 to metabolize verapamil, which were similar to humans, so it may be used as a model of mammalian drug metabolism (Liu et al., 2002).

Cunninghamella strains has ability to accumulate large quantities of unsaturated fatty acids, therefore is considered a promising strain for lipids particularly unsaturated fatty acids (Suleiman et al., 2018). Accordingly the aim of the present study is to isolate and identify the lipid contents of one species of this family (*Cunninghamella blakesleeana*) and evaluate its potential biological activities in order enrich its biotechnological application.

2. Material and methods

The current study was divided into two parts (chemical investigations and biological activities).

2.1. Fungal material

The investigated fungi, *Cunninghamella blakesleeana* (DSM 1906) was obtained from DSMZ (German Collection of Microorganisms and Cell Cultures**).** Sampling and isolation of fungal isolates were obtained using direct inoculation method (Kasper et al., 2018).

2.1.1. Fungal extraction

The mycelia mat (800 g.) of *Cunninghamella blakesleeana* (C. *blakesleeana*) was harvested, washed with distilled water, then extracted by refluxing in boiled ethanol 95% for two hours, filtered and the mate lifted was re-extracted using the same way for 3 extra times till exhaustion (Alkhulaifi et al., 2020). The total collected ethanol extracts of C. *blakesleeana* was concentrated under reduced pressure at low temperature to obtain residue of 60 g.

The alcohol dry extract (60 g.) of the fungi was suspended in water (350 ml) and extracted using chloroform till complete exhaustion. The collected chloroform extracts obtained was filtrated over anhydrous sodium sulphate to remove any excess of water, freed from chloroform using reduced pressure as before. 28 g. was obtained residue and kept for investigation.

2.2. Chemical investigations

2.2.1. Phytochemical screening

The alcohol extract *C. blakesleeana* subjected to phytochemical screening to investigate its different chemical constituents such as; carbohydrates and/or glycosides, flavonoides, tannins, sterols and/or triterpenes, proteins and/or amino acids, alkaloids and/or nitrogenous bases, saponins, anthraquinones, cardinolides and oxidase enzyme (Thilagavathi et al., 2015).

2.2.2. Isolation and purifications

For isolation of the fungal chemical components, the dry chloroform extract *C. blakesleeana* was saponified using the published method described by El-Kashef et al., (2014). From saponification, two types of matters were obtained (Saponifiable & nun-saponifiable matters) with total weight of 3 & 10 g.

Saponifiable matter was investigated using gas-liquid chromatography after methylation as described in published references (El-Kashef et al., 2014). On the other hand, un-saponifiable matter was subjected to isolation of its contents using column chromatography.

Un-saponifiable matters (8 g.) was fractionated using glass column (120 × 2 cm) packed with neutral alumina (200 g in each), eluted using benzene-ethyl acetate (86:14 v/v). Fractions of 60 ml. each were collected (100 fraction), concentrated, reduced to three sub-fraction (according to number, color and R_f of the spots) and symbolized as A, B& C with total weights of 2, 2.5 and 3 g. respectively. Each sub-fractions was subjected to multiple columns, eluted with different proportion of benzene gradually increased by different proportion of ether, from which four compounds A1 – A5 were isolated and identified using different instrumental analysis.

2.3. Biological studies

2.3.1. Antimicrobial activity

2.3.1.1. Preparation of the extracts. The alcohol extract of *C. blakesleeana* was used for testing its antimicrobial activity. The extract (0.5gm each) was dissolved in 10 ml water.

2.3.1.2. Test organisms. Representatives of Gram-negative bacteria; namely, *Klebsiella pneumoniae* (RCMB 0010093), *Salmonella typhimuium* (RCMB 0104), *Escherichia coli* (RCMB 0103), *Pseudomonas aeruginosa* (RCMB 0102) and Gram-positive bacteria; namely, *Bacillus subtilis* (RCMB 0107), *Staphylococcus aureus* (RCMB 0106), *Streptococcus pyogenes* (RCMB 0109), *Staphylococcus epidermidis* (RCMB 010024) and fungi; namely, *Candida albicans* (RCMB 5002), *Aspergillus fumigatus* (RCMB 2003), *Syncephalastrum racemosum* (RCMB 05922), and *Penicillium expansum* (RCMB 1006) were used as test organisms.

2.3.1.3. Antimicrobial screening. The disc diffusion and micro-dilution diffusion methods (Gupta et al., 2015, Boyanova et al., 2005) were used for the antimicrobial screening and determination of minimum inhibitory concentration (MIC), for the extract of *C. blakesleeana*.

The well diffusion method carried out using petri plates containing 20 ml of, nutrient (for bacteria) or malt extract (for fungi), agar medium were seeded with 1–3 day cultures of microbial inoculums. Wells (6 mm in diameter) were cut off from agar and 50 μl of fungal extract and the isolated compounds were tested in a concentration of 100 mg/ml and incubated at 37 °C for 24–48 h (bacterial strains) and for 3–5 days (fungal strains). The antibacterial and antifungal activities were determined by measurement of the diameter of the inhibition zone around the well (results were reported for the mean of three determinations).

Micro-dilution diffusion methods for MIC calculation were determined by dilution of concentrations from 0.0 to 100 mg/ml. Equal volume of each extract and nutrient broth were mixed in a test tube. Specifically 0.1 ml of standardized inoculum (1–2 × 107 cfu/ml) was added in each tube. The tubes were incubated at 37 °C for 24–48 h and/or 3–5 days. Two control tubes, containing the growth medium, saline and the inoculum were maintained for each test batch. The lowest concentration (highest dilution) of the algal extract that produced no visible microbial growth (no turbidity) when compared with the control tubes were regarded as MIC. These are the mean of three determinations.

2.3.2. Pharmacological activities

2.3.2.1. Preparation of fungal extract. The ethanol extracts of *C. blakesleeana* was suspended individually in sterile normal saline (0.9% NaCl) with the aid of few drops of tween 80 immediately before use. The concentration of the tested extract was 10%.

2.3.2.2. Animals. Swiss albino mice (20–25 g), male rats (160–180 g) and healthy guinea pigs (950–1200 g) were employed in the study. The animals were the animal house of Alazahar University, Cairo, Egypt. The animals were kept in standard polypropylene cages and maintained under standard conditions (Awaad et al. 2018).

Left for 2 weeks at room temperature to ensure stabilization before use. They were maintained on standard pellet diet and water *ad libitum* throughout the experiment.

2.3.2.3. Acute toxicity and median lethal dose (LD_{50}) test. The oral median lethal dose (LD_{50}) of the investigated alcohol extract determined at doses up to 4000 mg/kg (Alasmary et al., 2019).

2.3.2.4. Sub-chronic toxicity. Wister rats were casually separated into two groups each of ten Rats. The 1st group received the vehicle in a dose of 5 ml/kg and kept as normal control. On the other hand rats of the 2nd, group was given the total alcohol extracts of *C. blakesleeana,* (400 mg/kg each). The extract was administered orally day-to-day for 14 repeated days. Sera were separated to be used for the biochemical estimations of liver and kidney function (Soumen et al., 2013).

2.3.2.5. Statistical analysis. All values were expressed as mean ± S.D. Comparisons between means were carried out using a one-way ANOVA test followed by the Tukey HSD test using SPSS, version 14 (SPSS, Chicago, IL). Differences at p50.05 were considered statistically significant.

3. Results and discussions

3.1. Chemistry results

3.1.1. Phytochemical screening

Phytochemical Screening of *C. blakesleeana* reviled the presence of carbohydrates and /or glycosides, unsaturated sterols and/or triterpens, and traces of tannins, coumarins and saponins. The present investigation shows that significant variation in the contents which previously reported to have antioxidant activities. Its ability to produce phase I (oxidative) metabolites of drugs is associated with cytochrome P450 (CYP) activity which indicate the value of using this fungi as excellent media biotransformation (Palmer-Brown et al., 2019).

3.1.2. Fatty acid contents

Ten fatty acids (Table 1) were detected in *C. blakesleeana*. The major fatty acid obtained was stearic acid (74.61%) while Palmitic acid was the second high percentage (10.35%), and the least percentage obtained was Docosanoic acid (0.01%). The presence of higher amount of lipid can be used as essential oils, spice, oleoresins and natural food colors. In addition to its use in products that work with diverse requirements, as culinary, medicinal and cosmetics (Yadav and Tyagi, 2006).

3.1.3. Isolated compounds

Five Isolated compounds (Fig. 1) were identified using different instrumental analysis as following;

Compound F1: This compound obtained as yellowish residue (100 mg) with R_f 0.61 (n-hexane: ether 50:50 v/v), m.p. (13–14°). EI-MS *m/z* (% re. int): 282 (100) for M+, 253 (10%), 227 (5%). ^{1}H NMR ($CDCl_3$) δ: 0.87 (t, *J* = 6.9 Hz, 3H, CH_3-18), 1.28 (m, 20H, CH_2-[4 → 7] and CH_2-[12 → 17], 1.62 (quintet, *J* = 7.5 Hz, 2H, CH_2-3), 2.0 (q, *J* = 6.0 Hz, 4H, CH_2-11 and CH_2-8), 2.33 (t, *J* = 7.5 Hz, 2H, CH_2-2), 5.33 (td, *J* = 9.6, 5.4 Hz, 2H, CH-9 and CH-10), 11.0 (s, 1H, OH, exchangeable). ^{13}C NMR ($CDCl_3$) δ:180.54 (C-1), 130.14 (C-9),129.85 (C-10), 14 secondary carbons (CH_2) at 34.22 (C-2), 24.78 (C-3), 29.65 (C-4), 29.45 (C-5 and C-14), 29.80 (C-6), 29.20 (C-7), 27.31 (C-8 and C-11), 29.16 (C-12), 29.89 (C-13), 29.28 (C-15), 32.04 (C-16), 22.82 (C-17), 14.24 (CH3). By comparing the obtained spectroscopic data analysis (^{1}H NMR, ^{13}C NMR and DEPT-135COSY, HSQC and HMBC) with published one (Seebacher et al., 2003) this compound identified as; (9*Z*)-Octadec-9-enoic acid (Oleic acid).

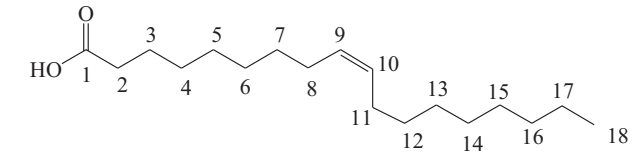
Compound F 2: Obtained as white residue (180 mg) with R_f 0.54 (n-hexane: ether 50:50 v/v), m.p. (62.10 °C) b.p. (351–352 °C). EI-MS *m/z* (% re. int): 60 (100%), 212(5%), 255 (3%)0.1-H NMR ($CDCl_3$) δ: 2.34 (2H, J = 7.60, H-2), 1.62 (2H, *J* = 7.39 Hz, H-3), 1.30 (24H (CH_2)12, H-4 → 15), 0.87 (3H, J = 7.05, H-16) for the terminal CH_3 group. 13-C NMR ($CDCl_3$) δ: 180.42 (C-1), 14.25 (C-16) and 14 CH_2 group, 34.20 (C-2), 32.06 (C-14), 24.80 (C-3), 29.19 (C-4), 29.37 (C-5), 29.56 (C-6), 29.80 (C-7,8,9,10,11), 29.72 (C-12), 29.50 (C-13), 22.83 (C-15). By comparing the obtained spectroscopic data analysis (1H NMR, ^{13}C NMR and DEPT-135, COSY, HSQC and HMBC) with published one (Martínez-Yusta et al., 2014) this compound identified as Palmitic acid.

Compound F 3: obtained as white needle crystals (250 mg), with R_f 0.51 (n-hexane: ether 50:50 v/v), (m.p. 69.6–72°). EI-MS *m/z* (% re. int): 60 (100%%), 212 (10%%), 255 (10), 281 (2.5%). ^{1}H NMR ($CDCl_3$) δ: 11.50 ppm (1H s, CO_2H), 2.34 (2H t, J = 7.60, H-2), 1.62 ppm (2H q, *J* = 7.56 Hz, H-3), 1.30 (28H m. (CH2)14, H-4 → 17), 0.87 (3H t, J = 7.05, H-18) CH_3. ^{13}C NMR ($CDCl_3$) δ: 180.27 (C-1), 14.25 (C-18), 34.18 (C-2), while 24.80 (C-3), 29.19 (C-4), 29.37 (C-5), 29.56 (C-6), 29.72 (C-14), 29.50 (C-15), 32.06 (C-16), 22.83 (C-17). By comparing the obtained spectroscopic data analysis (1H NMR.13C NMR and DEPT COSY, HSQC and HMBC)

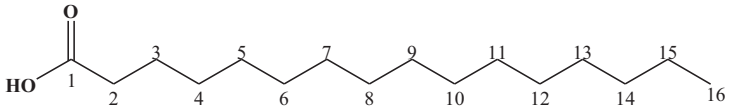
Table 1
GC analysis of fatty acids methyl esters for fungi.

Peak No	Fatty acids methyl ester	t_R (min)	tRR (min)	*C. blakesleeana*
1	**Tetradecanoic acid (Myristic acid)**	13.09	0.62	00.01
2	**(Pentadecylic acid)**	14.88	0.71	0.04
3	**Unknown**	16.33	0.78	0.15
4	**Hexadecanoic acid (Palmitic acid)**	17.31	8.93	10.35
5	***cis*-9-Octadecanoic acid (Oleic acid)**	19.52	0.93	6.62
6	**Octadecanoic acid (Stearic acid)**	20.98	1	74.61
7	**Unknown**	21.97	1.05	0.15
8	**cis,*cis*-9,12-Octadecadienoic acid (Linoleic acid)**	22.60	1.08	0.13
9	**Eicosanoic acid (Arachidic acid)**	24.44	1.15	0.07
10	**Docosanoic acid (Behenic acid)**	29.32	1.40	0.01

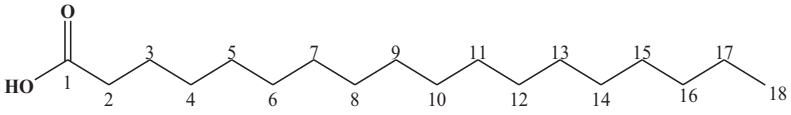
t_R: retention time in minutes; t_{RR} is relative retention time to stearic acid.



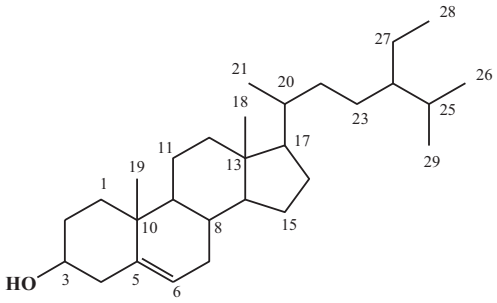
Oleic acid



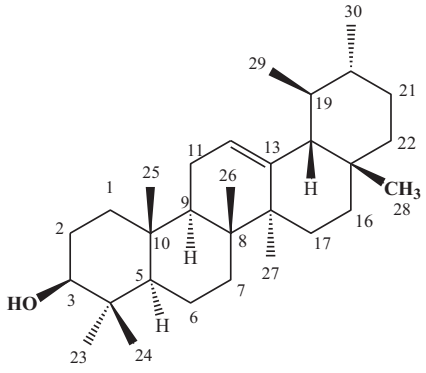
Palmitic acid



Stearic acid



β–sitosterol



α-amyrin

Fig. 1. Isolated compounds from *C. blakesleeana.*

Table 2
Antibacterial activity of *L C. blakesleeana* and some of its isolated compounds against some microorganisms.

Microorganism	Sample									
	C. blakesleeana		Oleic acid		Stearic acid		Palmitic acid		Standard Antibiotic	
	Inhibition zone (mm)	MIC (μg/ml)	Inhibition zone (mm)	MIC (μg/ml)	Inhibition zone (mm)	MIC (μg/ml)	Inhibition zone mm)	MIC (μg/ml)	Inhibition zone (mm)	MIC (μg/ml)
Gram negative									**Gentamycin**	
Escherichia coli (RCMB 010056)	14.5 ± 0.03	00.0	00.0	00.0	00.0	00.0	00.0	00.0	20.30 ± 0.85	03.90
Pseudomonas aeruginosa (RCMB 0100243-5)	5.9.0 ± 0.9	750	00.0	00.0	00.0	00.0	00.0	00.0	20.60 ± 1.50	01.95
Salmonella typhimurium RCMB 006 (1) ATCC 14,028	11.5 ± 0.03	480	6.5.0 ± 0.3	700	5.9.0 ± 0.9	750	8.0 ± 0.8	690	22.60 ± 1.50	01.95
Gram Positive									**Ampicillin**	
Staphylococcus aureus (RCMB 010027)	18.3 ± 0.03	250	13.0 ± 0.1	250	15.0 ± 0.5	200	11.0 ± 0.3	360	22.00 ± 1.00	01.95
Streptococcus pyogenes (RCMB 0100174-2)	15.3 ± 0.05	380	00.0	00.0	00.0	00.0	00.0	00.0	22.70 ± 0.58	00.98
Bacillus substilis (RCMB 0100169-3)	13.7 ± 0.03	520	00.0	00.0	00.0	00.0	00.0	00.0	22.30 ± 0.63	01.95
Fungi									**(Amphotericin B)**	
Candida albicans RCMB 005,003 (1) ATCC 10,231	6.7 ± 0.06	750	00.0	00.0	00.0	00.0	00.0	00.0	21.30 ± 1.50	01.95
Aspergillus fumigatus RCMB 002,008	14.3 ± 0.04	400	00.0	00.0	00.0	00.0	00.0	00.0	25.70 ± 1.50	0.49
Penicillium expansum RCMB 001,001 (2)	13.4 ± 0.01	525	8.5.0 ± 0.6	500	6.3.0 ± 0.9	550	7.0 ± 0.5	660	21.70 ± 2.00	01.95

These are the mean of three determinations.
ND, not determined. MIC, minimum inhibitory concentration.

with published one (Seebacher et al., 2003) this compound identified as; stearic acid.

Compound A 4: Compound obtained as white needle crystals from methanol (200 mg), Rf (Benzene –Ethyl acetate 86:14) was 0.62, (m.p 185–187°). EI-MS *m/z* (% re. int): 412 (100%), 221 (78%), 194 (37%), 208 (17%), 426 (3%). ^{1}H NMR ($CDCl_3$) δ: 1.61 (H-2), 4.48 (dd H-3), 0.81(H-5), 1.53 (Hb-6), 1.30 (Ha-6), 1.54(H-9), 1.84(H-11), 5.11 t (3.6) H-12, 18(H-1.29), 19(H-1.38 m), 20 (H-1.98), 23 (H-0.85 s), 24 (H-0.84 s), 25 (H-0.96 s), 26 (H-0.98 s), 27 (H-1.04 s), 28 (H-0.78 s), 29 (H-0.77 d), 30 (H-0.83 d). ^{13}C NMR ($CDCl_3$) δ:142.07 (C-13), 122.13 (C-12), also one carbon at δ 76.48 (C-3) next to –OH group, at 60.14(C-18), 51.84 (C-9) hithermost of double bound, at δ 50.33 (C-5), at δ 44.36(C-16),at δ 42.96 (C-4), at 40.96 (C-8), at δ39.42(C-14), at δ38.72(C-10), at 35.52 (C-11), at δ34.93(C-17), at 34.25(C-2), at 29.19 (C-20), CH_3 group at δ 30.92(C-26), four CH_2 group at δ 29.20 (C-1), 29.09 (C-6), at δ 28.44 (C-7) and 27.89 (C-19), three CH_3 group position see in 25.61 (C-25), at 23.06 (C-23) and 22.08 (C-24), at 24.16 (C-15), at 20.03 (C-22), at 18.18 (C-21), also CH3 group position at 17.99 (C-27), 16.20 (C-28), δ 15.87 (C-29), and 15.12 (C-30). Accordingly, this compound identified as α-amyrin. By comparing the obtained spectroscopic data analysis (1H NMR.13C NMR and DEPT COSY, HSQC and HMBC) with published one (Nnamonu et al., 2016) this compound identified as; α-amyrin.

Compound A5: It is white needle crystals (150 mg), R_f (Benzine: ethyl acetate 86/14) was 0.54, (m.p. 137°–139°). EI-MS *m/z* (% re. int): 415 *m/z* (100%) for M^{+1}, and other peaks at: 149 *m/z* (52%) for $C_{10}H_{13}O^+$, 214 *m/z* (31%) for $[C_{16}H_{22}]^{+\cdot}$ 273 *m/z* (31%) for $C_{19}H_{29}O^+$, 161 *m/z* (30%) for $C_{12}H_{17}^+$, 385 *m/z* (24%) for M-C_2H_5 $[C_{27}H_{45}O^+]$, 95 *m/z* (24%) for $C_6H_7O^+$, 397 *m/z* (21%) for M-OH $[C_{10}H_{13}O^+]$, 230 *m/z* (9%) for $[C_{16}H_{22}O]^{+\cdot}$, 200 *m/z* (9%) for $C_{15}H_{20}^+$, 315 *m/z* (6%) for $C_{22}H_{35}O^+$. 1-H NMR ($CDCl_3$) δ: Triplet at δ 5.34 ppm (1H d, J = 2.2 Hz, H-6), singlet at δ 3.51 ppm (1H, s, –OH), at δ 2.26 ppm (2H q, J = 4.5 Hz, H-3) nearest from –OH, triplet at δ 1.98 ppm (2H,t, J = 3.1 Hz, H-5 & H-8), at δ 1.83 ppm (3H, t, H-28), sextet at 1.63 ppm (1H,s, H-18), singlet at 1.57 ppm (8H,s,H-1,H-2,H-15 & H-16), at 1.33 ppm(5H, m, H-9,H-11, & H-12), multiplet at 1.14 ppm(6H, m, H-4,24,21,17, & 22), at 1.12 ppm(6H, d, H-29 & H-30), at 0.91 ppm(4H d, J = 6.4 Hz,H-19 &H-20),singlet at 0.81 ppm(9H, s, H-24, H-25 &H-26), singlet at 0.66 ppm (3H,s, H-23). ^{13}C NMR in $CDCl_3$ showed 30 carbons, and DEPT-135 revealed the presence of three quaternary carbon C-6 at δ 140.87 ppm, δ42.43 (C-10), at δ 36.62 (C-13). From previous data this compound was identified as β-sitosterol (Fig. 1). By comparing the obtained spectroscopic data analysis (1H NMR.13C NMR and DEPT COSY, HSQC and HMBC) with published one (Kamboj and Saluja, 2011) this compound identified as; as β–sitosterol.

3.2. Biological activities

3.2.1. Antimicrobial activities

Antimicrobial activities against representatives of Gram positive, Gram negative bacteria, and fungi of *C. blakesleeana* extract and some isolated compounds revealed that there was a significance activity against some of the microorganisms (Table 2).

The highest activity of C. *blakesleeana* total extract was reported against *Staphylococcus aureus* (18.3 ± 0.03 mm.) followed by *Streptococcus pyogenes* (15.3 ± 0.05), while the lowest were for both *Candida albicans* &*Pseudomonas aeruginosa* (6.7 ± 0.06 and 5.9.0 ± 0. 9 mm. respectively).

The isolated compounds F1-F3 showed activities against *Staphylococcus aureus*, *Penicillium expansum*, and *Salmonella typhimurium* only. The highest activity was aganist *Staphylococcus aureus* (13.0 ± 0.1 mm.). The highest effect was obtained by compound F_3 (stearic acid) (15.0 ± 0.5 mm.), and compound F_1(Oleic acid) (13.0 ± 0.1 mm.) and F_2 (Palmitic acid) 11.0 ± 0.3 mm. There are other minor effects were reported on *Salmonella typhimurium* and *Penicillium expansum*. The MIC of total extract was varied according to the microorganism the best was on *Staphylococcus aureus* (250 μg/ml).

3.2.2. Pharmacology

3.2.2.1. Acute toxicity and median lethal dose (LD_{50}) test. All the animals that received the three tested extracts in doses up to 5000 mg Kg^{-1} survived beyond the 24 h of observation. On administration of

Table 3
Effect of prolonged oral administration of the alcoholic extract of *C. blakesleeana,* in a dose of 200 mg Kg^{-1} for 2 weeks Liver and kidney parameters (n = 6).

Groups	Control	*C. blakesleeana*
Uric acid (mg dl^{-1})	2.18 ± 0.21	2.25 ± 0.19
Urea (mg dl^{-1})	45.4 ± 2.54	44.2 ± 2.84
Creatinine (mg dl^{-1})	0.66 ± 0.05	0.62 ± 0.05
Total bilirubin (mg dl^{-1})	0.28 ± 0.02	0.29 ± 0.02
Total proteins (g dl^{-1})	6.44 ± 0.28	6.46 ± 0.28
Albumin (g dl^{-1})	4.13 ± 0.20	4.20 ± 0.21
Globulin (g dl^{-1})	2.31 ± 0.16	2.26 ± 0.18
AST (U L^{-1})	80.6 ± 5.22	87.0 ± 5.46
ALT (U L^{-1})	46.2 ± 2.74	47.2 ± 3.44
ALP (U L^{-1})	88.5 ± 5.20	95.7 ± 5.63

Values represent the mean ± S.E. No significant difference from control (LSD).
ALP, Alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

any of the three extracts, no immediate behavioral changes were noted. There were no changes in the nature of stool, urine and eye color in any of the animals. All mice move and fed normally. They did not exhibit any abnormal symptoms during the experimental period. The median acute toxicity value (LD_{50}) of the alcoholic extract of *C. blakesleeana* determined to be greater than 5000 mg kg^{-1} body weight which considered to be save for human use (Awaad et al., 2018).

3.2.2.2. Sub-chronic toxicity. None of the tested extracts were induced any deleterious effect on the liver and kidney functions of rats after 2 weeks of oral administration (Table 3). Oral administration of the alcoholic extract of *C. blakesleeana,* in a dose of 400 mg kg^{-1} b. wt to rats for 2 weeks did not disturb the serum activity of AST, ALT and ALP as compared to the normal control animals. No significant changes in the mean values of total bilirubin, total protein, albumin and globulin were found in serum of rats following 2 weeks of extracts administration when compared with the control. The obtained results showed insignificant effect on serum levels of urea, uric acid, and creatinine in the all medicated groups when compared to the control group.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgment

This research project was supported by a grant from the "Research Center of the Female Scientific and Medical Colleges", Deanship of Scientific Research, King Saud University.

References

Alasmary, Fatemah A., Assirey, Eman A., El-Meligy, Reham M., Awaad, Amani S, El-sawaf, Lara A., Allahe, Menatallah M., Alqasoumi, Saleh I., 2019. Analysis of Alpina Officinarum Hance, chemically and biologically. Saudi Pharm. J. 27 (8), 1107–1112.

Alkhulaifi Manal M. , Amani S. Awaad , Hind A. AL-Mudhayyif , Monerah R. Alothman, Saleh I. Alqasoumi, Sarah M. Zain, 2019. Evaluation of antimicrobial activity of secondary metabolites of fungi isolated from Sultanate Oman soil. Saudi Pharm. J. 27(3), 401–405.

Amare, M.G., Keller, N.P., 2014. Molecular mechanisms of Aspergillus flavus secondary metabolism and development. Fungal Genet. Biol. 66, 11–18.

Awaad, Amani A., Alkanhal, Haya F., El-Meligy, Reham M., Zain, Gh.ada.M., Desikan, Vidya, Alqasoumi, Saleh I., 2018. Anti-ulcerative colitis activity of *Calotropis Procera* Linn. Saudi Pharm. J. 26 (1), 75–78.

Lyudmila, Boyanova, Gergova, Galina, Nikolov, Rossen, Derejian, Sirigan, Lazarova, Elena, Katsarov, Nikolai, Mitov, Ivan, Krastev, Zacharii, 2005. Activity of Bulgarian propolis against 94 Helicobacter pylori strains in vitro by agar-well diffusion, agar dilution and disc diffusion methods. J. Med. Microbiol. 54, 481–483.

Chaturvedi V, Bouchara JP, Hagen F, Alastruey-Izquierdo A, Badali H, Bocca AL, Cano-Lira JF, Cao C, Chaturvedi S, Chotirmall SH, et al., 2018. Eighty years of mycopathologia: a retrospective analysis of progress made in understanding human and animal fungal pathogens. Mycopathologia 183(6), 859-877.

Chen, Wanping, Lee, Mi-Kyung, Jefcoate, Colin, Kim, Sun-Chang, Chen, Fusheng, Yu, Jae-Hyuk, 2014. Fungal cytochrome P450 monooxygenases: their distribution, structure, functions, family expansion, and evolutionary origin. Genome Biol. Evolut. 6 (7), 1620–1634.

El-Kashef, Dina F., Hamed, Ashraf N.E., Khalil, Hany E., Kame, Mohamed S., 2014. Investigation of the unsaponifiable and saponifiable matters of pachypodium lamerei drake leaves and stems by GC/MS. J. Pharm. Phytochem. 3 (1), 128–132.

Han, Gao, Li, Gang, Lou, Hong-Xiang, 2018. Structural diversity and biological activities of novel secondary metabolites from endophytes. Molecules 23 (3), 646.

Gupta Prashant, Vineeta Khare, Deepak Kumar, Abrar Ahmad, Gopa Banerjee,d Mastan Singh, 2015. Comparative evaluation of disc diffusion and E-test with broth micro-dilution in susceptibility testing of Amphotericin B, voriconazole and caspofungin against clinical Aspergillus isolates. J. Clin. Diagn. Res. 9(1), DC04–DC07.

Joanna D. Moody, James P. Freeman, Carl E. Cerniglia, 1999. Biotransformation of doxepin by cunninghamella elegans. Drug Metabolism Disposit. 27(10), 1157–1164.

Kamboj, A., Saluja, A.K., 2011. Isolation of Stigmasterol and B-Sitosterol from Petrolium Ether of Aerial Parts of Ageratum Conyzoides (Asteraceae). Inter. J. Phar. Pharm. Sci. 3 (1), 94–96.

Kasper Nørskov Kragh, Maria Alhede, Morten Rybtke, Camilla Stavnsberg,Peter. Jensen, Tim Tolker-Nielsen, Marvin Whiteley, Thomas Bjarnsholta, 2018. The inoculation method could impact the outcome of microbiological experiments 84(5), e02264-17.

Keerthana, M., Vidyavathi, M., 2018. Screening and evaluation of fungal resources for loratadine metabolites. J. Biosci. 43 (5), 823–833.

Kelly, S.L., Kelly, D.E., 2013. Microbial cytochromes P450: biodiversity and biotechnology. Where do cytochromes P450 come from, what do they do and what can they do for us?. Philos. Trans. R Soc. B 368. 20120476.

Lene, Lange, 2014. Fungal enzymes and yeasts for conversion of plant biomass to bioenergy and high-value products. Microbiol. Spectr. 5 (1), 1–19.

Liu, L., Huang, H.H., Sun, L., Zhong, D.F., 2002. Capability of Cunninghamella blakesleana to transform verapamil. Chinese J. Pharm. Toxicol. 16 (4), 292–298.

Metsämuuronen, Sari, Sirén, Heli, 2018. bioactive phenolic compounds, metabolism and properties: a review on valuable chemical compounds in Scots pine and Norway spruce. Phytochem. Rev. 18, 623–664.

Nguyen, T.T., Choi, Young-Joon, Lee, Hyang Burm, 2017. Isolation and characterization of three unrecorded Zygomycete Fungi in Korea: *Cunninghamella bertholletiae*, *Cunninghamella echinulata*, and *Cunninghamella elegans*. Microbiology 45 (4), 318–326.

Lami, Nnamonu, Tor-Anyiin, T.A., Ugbenyo, Nicholas, Anyam, John, 2016. Isolation and characterization of α–Amyrin from stem bark of ficus exasperata (Vahl). Biotechnol. J. Int. 16 (4), 1–7.

Martínez-Yusta, A., Goicoechea, E., Guillén, M.D., 2014. A review of thermo-oxidative degradation of food lipids studied by 1H NMR spectroscopy: Influence of degradative conditions and food lipid nature. Compr. Rev. Food Sci. Food Saf. 13, 838–859.

Palmer-Brown, W., Miranda-CasoLuengo, R., Wolfe, K.H., et al., 2019. The CYPome of the model xenobiotic-biotransforming fungus *Cunninghamella elegans*. Sci. Rep. 9, 9240.

Pusztahelyi, Tünde, Holb, Imre J., Pócsi, István, 2015. Secondary metabolites in fungus-plant interactions. Front. Plant Sci. 6, 573.

Salar, Raj Kumar, 2018. Fungi: Basic Concepts and Biotechnological Applications. CRC Press, Taylor and Francis group, p. 334.

Werner, Seebacher, Simic, Nebojsa, Weis, Robert, Saf, Robert, Kunert, Olaf, 2003. Complete assignments of 1H and 13C NMR resonances of oleanolic acid, 18α-oleanolic acid, ursolic acid and their 11-oxo derivatives. Magn. Reson. Chem. 41 (8), 636–638.

Soumen, S., Dhar, T.N., Sengupta, C., Ghosh, P., 2013. Biological activities of essential oils and methanol extracts of five ocimum species against pathogenic bacteria. Czech J. Food Sci. 31 (2), 194–202.

Suleiman, W.B., El-Sheikh, H.H., Abu-Elreesh, G., Hashem, A.H., 2018. Recruitment of *Cunninghamella echinulata* as an Egyptian isolate to produce unsaturated fatty acids. RJPBCS 9 (1), 765–1674.

Thilagavathi, T., Arvindganth, R., Vidhya, D., Dhivya, R., 2015. Preliminary phytochemical screening of different solvent mediated medicinal plant extracts evaluated. Thilagavathi. T et al. Int. Res. J. Pharm. 6(4).

Yadav, P.R., Tyagi, R., 2006. In: Lipid biotechnology. Discovery Publishing House, New Delhi, p. 89.

Yang Dandan, Zijing Zhou, Lijuan Zhang, 2019. Chapter Eight - An overview of fungal glycanbased therapeutics. Prog. Mol. Biol. Trans. Sci. 163, 135-163.