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ANTI-CANDIDA EFFECT OF SAUDI PROPOLIS: GC/MS ANALYSIS, IN-SILICO STUDY AND NANO ENCAPSULATION

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Abstract

Owing to the high mortality and morbidity around the world due to resistant fungal infections, there is a growing interest in the potential role of natural products in curbing the fungal crawling. Considering this, our research targeted to investigate the efficacy of two Saudi propolis samples against two Candida Species (*C. albicans & C. krusei*). Saudi propolis samples were defatted firstly by petroleum ether yielding (P_1pt. & P_2pt.), then extracted with ethyl acetate yielding (P_1E & P_2E). The antifungal activity of the four fractions was estimated against *C. albicans*, the most popular infectious fungal pathogen and the multidrug resistant *C. krusei*. P_2E possessed the highest inhibitory activity for both *C. krusei* and *C. albicans* with inhibition zones of 37 ± 0.089 & 31 ± 0.073 mm and the MIC; 2.5& 4.0 mg/ml, respectively. Whilst P_1E showed a significant inhibition activity only for *C. krusei* with inhibition zone diameter of 30 ± 0.094 mm and MIC equal to 60 mg/ml. GC/MS investigation for (P_1E & P_2E) revealed the detection of 77 compounds from different chemical classes, caffeate esters were solely present in P_2E in a relatively high amount. In-silico study was conducted to predict the P_2E major bioactive components possible binding affinity mechanism to three potential enzymes for the growth and survival of candida species, results revealed that many of the examined compounds had a reasonable binding affinity which could explain the significant P_2E anti-candida activity. Eventually, P_2E was loaded in Soluplus-based self-nanoemulsifying tablets to overcome its low aqueous solubility and oral bioavailability, the selected formula showed a significant better active agents release compared to the plain fraction (100 % and 20 %, respectively), which proves the beneficial effect of the designed nano-system.

Keywords: Saudi propolis; *C. krusei*; *C. albicans;* in-silico study; nanoemulsifying tablets; GC/MS analysis.

1. Introduction

In recent decades, there are escalating in the population of immunocompromised patients (AIDS, cancer, organ and hematopoietic stem cell transplantation, preterm baby, etc.) combined with the emerge of opportunistic fungal infections [1]. These fungal infections have increased the morbidity and mortality rates worldwide to more than 1.35 million patient per year [2, 3]. Aspergillus and Candida species, Cryptococcus neoformans are the main causative agent of fungal infections. Candida genus is a commensal yeasts responsible for different opportunistic infections; from candidiasis to candidemia [4]. A major virulence attribute of C. albicans is its ability to form biofilm, a densely packed communities of cells adhered to a surface.

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These biofilms are resistant to classical antifungal drugs, the host immune system and environmental factors making biofilm associated infections a significant clinical challenge.[5] Nevertheless, in last decade non-albicans Candida (NAC) [6] species has been emerged as *C. auris, C. glabrata, C. krusei* and else [7, 8]. Some of the NAC species have intrinsic resistant and/or less susceptible to antifungal agents which is owing to the abuse of antimicrobial agents that leads to the recurrence of infection and emerging of highly resistant microbial strains, for example, the regular use of fluconazole (cheap, nontoxic, broad spectrum antifungal agent) as prophylaxis with immunocompromised patients has aroused fluconazole resistant fungal strains. Thus, this increases the demand for searching for a new safe, nontoxic antifungal agent [9-11].

C. krusei is the main causative agent of candidemia in immunocompromised patients specially those suffering from leukaemia, with high mortality rate than that of *C. albicans*, 49%-60% and 28%, respectively, furthermore, it is a rare cause of refractory vaginitis contributing to about 1% of cases [12, 13]. *C. krusei* is a multidrug resistant with intrinsic resistance to fluconazole, reduced susceptibility to Amphotericin B, caspofungin and flucytosine [14]. Although voriconazole is triazole (as fluconazole) it still effective aganist *C. krusei* which is related to the binding of this drug to the target enzyme (cytochrome P450 sterol 14-demethylase) [15, 16]. Incidence of yeast infection with *C. krusei* is very low compared by other yeast infections (2–5%), but it has a very high mortality rate with 90 days survival period, compared with other candida species and high drug resistance make it very interesting to explore new line in treatment for this life threatening yeast *C. krusei* [6, 17]. Candida species are characteristics mainly by consisting a highly drug endurance biofilms in the human host [18]which forced researchers to be back to the nature to explore out new bioactive nutraceuticals as alternatives for antifungal drug with less or almost free of adverse effects and have no resistance [19]. Bee propolis, is a resinous colloidal substance collected by bees from several buds exudate results in a great variation in its chemical composition as it depends mainly on the floral utilized by bees to gathering the propolis [20]. However, it consists mainly of polyphenolic compounds, as phenolic acids and their esters, flavonoids along with terpenes and many other various compounds. These chemical variations resulted directly in a spacious variation in the pharmacological activities [21]. Nevertheless, propolis is considered an inexhaustible source of bioactive metabolites which reported previously as anti-inflammatory, antimicrobial, immune-modulatory, anti-ulcer, and antitumor[22]. Different propolis types were previously reported to exhibit promising antimicrobial activity against different microorganisms[23] specifically as Candida species inhibitors[24]. Recently, the inspection of drug-protein interaction by the molecular docking using in silico studies has become a robust tool to depict a conceivable binding mechanism between the bioactive compounds and a given drug target protein[25]. Hence, three potential enzymes which are targets for anti-candida drug discovery; N-myristoyl transferase (NMT), thymidylate synthase (TS) and lanosterol 14 α-demethylase had been conducted into in-silico study to predict the binding affinity of the major bioactive components in the potent anti-candida fraction to these targets and accordingly conclude their possible anti-candida activity mechanism. Solid self-emulsifying drug delivery systems (SEDDS) is one of the formulation approaches for solving the problems of low solubility and oral bioavailability of active agents. SEDDS belong to lipid-based nano-formulations, they are isotropic mixtures composed of drug, oil/lipid and surfactant/ co-surfactant. They form fine emulsion nano-droplets on dilution with physiological fluid which facilitates the drug absorption from the gut [26]. Moreover, presence of the surfactant within the nano emulsion can avoid the toxicity of the free surfactant on the GIT. Conventional SEDDS are present in the form of liquids; however, they have some disadvantages like low stability and difficult portability. Solid SEDDS can combine the advantages of SEDDS together with those of solid dosage forms like low production cost, high stability and easy handling [27]. Thus it is expected that loading the P_2E in Soluplus-based self-nanoemulsifying could provide a great role in the P_2E fraction release rather than the plain fraction.Saudi propolis has been sparsely studied, hence, this study aimed to investigate the chemical composition of the Saudi propolis fractions which possess anti-candida activity against *C. krusei* and *C. albicans.*

2. Materials and method:

2.1. Materials:

Tween 80 (T80), Isopropyl myristate (IPM), Isopropyl palmitate (IPP) and Methyl laurate (ML) were purchased from Sigma Chemical Company, St. Louis, USA. Soluplus® was kindly gifted from BASF, Germany. BSTFA was purchased from Sigma-Aldrich, Germany. C. krusei isolate was provided by researcher, Mona M. H. Soliman, Department of microbiology and immunology, National Research Centre, Cairo, Egypt. C. albicans is (NRRL-Y 477), positive control; miconazole from (Miconaz, MUP, Egypt) and voriconazole from

(Vfend, Pfizer, Egypt), from the Egyptian market. All other chemicals used were of analytical grade.

2.2. Methods:

2.2.1. Collection of propolis samples:

Two Saudi propolis samples were collected from different districts; Abhaa (P1) and Qassium (P2), during March-April 2019 and were stored in freezer until processed. The samples were frozen, ground and homogenized prior to extraction.

2.2.2. Sample extraction:

Twenty grams of each propolis sample were cut into small pieces and defatted by petroleum ether yielding; P1pt. & P2pt. (3 & 4.2 g/ dry weight, respectively).Then the residue of each sample was further extracted with ethyl acetate yielding; P1E &P2E (3.5 & 3 g/ dry weight, respectively).

2.2.3. Antifungal activity:

C. krusei and C. albicans were maintained on potato dextrose agar (PDA) medium. Antifungal activity of ethyl acetate and petroleum ether fractions of the two propolis samples was estimated as previously described by [28, 29] using the disc diffusion method. Fungi were sub-cultured onto fresh potato dextrose agar slants and incubated for 3 days at 37 °C. Fungal spore suspension of optical density 0.5 was prepared and the sterilized medium was inoculated by 80µl of spore suspension, mixed well, poured into a sterile petri dish and allow to solidify at room temperature. Wells of diameter 1cm were punched in each plate. 100 µl of different fractions and positive control solution was used (concentration 100 mg /ml), in addition to, a negative control (methanol and petroleum ether). The positive control used are miconazole and voriconazole. The plates were kept in the refrigerator for two hours then incubated at 37 °C for 3-5 days. The growth was daily observed and the diameters of the inhibition zones were measured in mm. MIC of the fractions with higher activity were evaluated using different concentrations of the sample. The MIC recorded as the lowest concentration of the fraction that completely inhibits growth.

2.2.4. GC/MS analysis

2.2.4.1. Preparation of propolis samples:

Sample preparation for GC/MS analysis was carried out by derivatization of 2.5 mg of each dried sample for 30 min. at 85 °C with 20µl pyridine + 30µl N,O,bis-(trimethylsilyl) trifluoroacetamide (BSTFA) [30].

2.2.4.2. Mass spectrometer:

A Finnigan MAT SSQ 7000 mass spectrometer was coupled with a Varian 3400 gas chromatograph. DB-5 column, 30 m x 0.32 mm (internal diameter), was employed with helium as carrier gas (He pressure, 20 Mpa/cm2), injector temperature, 310ºC; GC temperature program, 85 – 310 ºC at 3 ºC/ min (10 min. intial hold). The mass spectra were recorded in electron ionization (EI) mode at 70 eV. The scan repetition rate was 0.5 s over a mass range of 39 – 650 atomic mass units (amu). [30]

2.2.4.3. Compounds Identification:

The identification was accomplished using computer search user-generated reference libraries, incorporating mass spectra. Peaks were examined by single-ion chromatographic reconstruction to confirm their homogeneity. In some cases, when identical spectra have not been found, only the structural type of the corresponding component was proposed on the bases of its mass spectral fragmentation. Reference compounds were co-chromatographed when possible to confirm GC retention times.

2.2.5. In-silico study:

The x-ray crystal structure of candida N-myristoyl-transferase (PDB code 1IYL) and lanosterol 14 α-demethylase (PDB code 5FSA) were downloaded from Protien Data Bank while the homology model of candidal Thymidylate synthase prepared by Perez-Castillo *et al*., using *Cryptococcus neoformans* thymidylate synthase crystal structure (PDB code 2AAZ) as template was downloaded from SWISS-MODEL repository using Uniprot ID P12461, [31]. All ligand structures were drawn using ChemDraw software and energy minimization was performed using the MMFF94X force field at the gradient 0.05. The protein structures have been prepared by using the Structure Preparation module in MOE 2019.01 (Molecular Operating Environment, Version 2019.01, Chemical Computing Group Inc., Montreal, Canada) after all water molecules were removed. Docking calculations were performed using the induced fit method, where the co-crystallized ligands were considered as the centre of the docking site while other docking parameters were kept as default [32] One hundred docking poses were calculated for each compound; then the generated docking poses were visualized using MOE 2019.01.The generated docking poses were ranked according to their docking scores, and the best energy pose was selected. To validate our docking method, the native ligands were redocked and RMSD (Root Mean Square Deviation) values were determined.

2.2.6. Preparation of SEDDS:

Various SEDDS were prepared using surfactant, co-surfactant and oil combination (S/CoS/oil) so that

the ratio was equal to 45:45:10 w/w. Tween 80 (T80) and ethanol were used as the surfactant and CoS, respectively. Different oils were used: Isopropyl myristate (IPM), Isopropyl palmitate (IPP) and Methyl laurate (ML). The mixture was stirred using vortex mixing (JULABO Labortechnik, Germany) and left for equilibrium at room temperature till 24 h to form a homogenous isotropic mixture [27].

2.2.6.1. Assessment of self-emulsification:

The first step to formulate SEDDS is to determine the capability of being self-emulsified upon dilution. The evaluation of their self-emulsifying properties is performed by adding one gram of the prepared formulation dropwise into a beaker containing 20 ml of distilled water stirred using at100 rpm at 37 ± 0.5 ^{o}C. The self-emulsification was visually assessed according to the following grading [33]: A: Rapid formation of a clear nano emulsion. B: Formation of a translucent nano emulsion. C: Formation of an emulsion with a bluish white appearance. D: Formation of a milky white emulsion. E: Separation of the emulsion and formation of large oil droplets. Droplet size determination: The average droplet size of the selected preparation was measured using a Zetasizer (Malvern Instruments, UK) after suitable dilution with distilled water.

2.2.6.2. Preparation of S3-loaded Soluplus-based self-emulsifying tablets:

Preparation was done following the method previously described by Attama et al [34]. The selected SEDDS was mixed with Soluplus in a ratio equal to 1:2 to form a homogenous mixture, then was poured into a plastic mold and allowed to set at room temperature. Then tablets were stored in a cool place until use after removed from the mold. Each tablet (0.8 g) contained 20 mg extract.

2.2.6.2.1. Physical evaluation:

2.2.6.2.1.1. Weight uniformity:

The average weight was calculated by weighing 5 tablets individually and the percentage deviation from the mean was determined.

2.2.6.2.1.2. Disintegration time:

The disintegration (liquification) time of S3-loaded Soluplus-based self-emulsifying tablets was determined by placing the tablet in the round bottom flask of the dissolution tester (Hanson Research Corporation, United States) [34]. The experiment was done in 250 ml of methanolic phosphate buffer pH 6.8 (same media used for release testing), maintained at 37 ± 1 ◦C. The tablet was observed carefully, and the melt time was recorded.

2.2.6.2.1.3. In-vitro release studies:

In-vitro drug release properties were investigated using USP Apparatus I (rotating basket) (HansonSR8plus, USA) in 500 ml phosphate buffer pH 6.8 containing 15% methyl alcohol to maintain sink condition at a speed of 100 rpm and the temperature was maintained at 37 ± 0.5 ^{o}C. At predetermined time intervals (0.25, 0.50, 1, 2, 3, 4, 5, 6 and 24 h); aliquots of 5 ml were withdrawn and replaced by fresh solution in order to maintain sink condition throughout the experiment. The withdrawn samples were filtered through a 0.2 um Millipore membrane for spectrophotometric analysis of the released active moieties. Calibration curve was assessed from the absorbance values of a serial dilution containing different concentrations of the extract in phosphate buffer pH 6.8 /15% methyl alcohol; the maximum wavelength was at 293 nm [35]. Release profiles were plotted and release efficiency was calculated. The experiments were carried out in triplicate and data were expressed as mean value ±S.D.

3. Results and Discussion:

3.1. Antifungal activity:

The propolis four fractions exhibited variable inhibition activity against *C. krusei* with different inhibition zone as illustrated in Table 1. The ethyl acetate fractions of both propolis samples P_1E and P_2E showed higher inhibition with a zone of 30 ± 0.094 &37 ± 0.089 mm respectively, compared to voricanzole 70 ± 0.069 mm , whilst P_1pt., P_2pt. showed lower inhibition (22 ± 0.06 mm &18 ± 0.092 mm respectively). Miconazole was inactive, Table 1. On contrary, only P_2E manifested significant inhibition activity towards *C. albicans* with inhibition zone of 31 ± 0.073 mm compared to Miconazole 60 ± 0.043 mm. Moreover, the MIC of the highly active fractions, P_2E and P_1E was evaluated, on one hand, P_2E showed the least MIC against *C. krusei* (2.5mg/ml), on the other hand, P_2E MIC was (4.0 mg/ml) for *C. albicans*, while P_1E MIC against *C. krusei* was (60 mg/ml).

3.2. GC/MS analyses of propolis samples:

GC/MS analyses revealed the identification of 77 compounds; many compounds are common in both P_1E & P_2E, whereas other compounds are solely present in each sample. 49 compounds were detected in P_1E and 45 compounds in P_2E from different chemical classes, aliphatic acids/esters, phenolic compounds, phenolic acids/esters, flavonoids and sugars (Fig.1, Table 2).

Table 1. Zone of inhibition (mm) for propolis four fractions

Fractions	Zone of inhibition (mm)	
	C. krusei	*C. albicans.*
P_1pt	22 ± 0.060	-ve
P_1E	30 ± 0.094	-ve
P_2pt.	18 ± 0.092	-ve
P_2E	37 ± 0.089	31 ± 0.073
Miconazole	-ve	60 ± 0.043
Voriconazole	70 ± 0.069	ND
ND: Not Done		

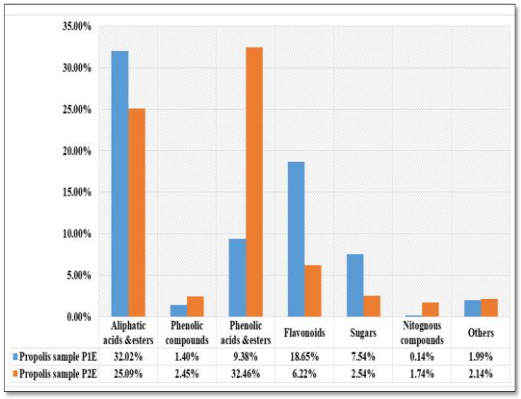

Fig. 1: Total percentage of different chemical classes detected by GC/Ms in (P1E & P2E).

3.2.1. Aliphatic acids &esters:

Only 16 aliphatic compounds are identified in P_1E with total concentration of 32.02 % while 17 aliphatic compounds are present in P_2E with total concentration of 25.09%, both P_1E & P_2E have a relatively high concentration of hexadecanoic acid (palmitic acid) (12.06% & 10.91% respectively) and octadecanoic acid (stearic acid) (8.77% & 6.16 % respectively). (Table 2).

3.2.2. Phenolic compounds:

phenolic compounds are detected in P_1E, all of them have a relative low concentration ranged from 0.14% to 0.7%, five phenolic compounds identified in P_2E, benzyl methyl ketone has the highest concentration 1.35%. (Table2).

3.2.3. Phenolic acids & their esters:

Eight compounds of 9.38 % total concentration have been detected in P_1E fraction. Meanwhile, ten compounds represented by total concentration 32.46% have been detected in P_2E fraction. Two phenolic acids are identified in both P_1E & P_2E, benzoic Acid (1.01% & 2.17% respectively) and isoferulic acid (2.69% & 0.33% respectively). *trans*-Caffeic and vanillic acids are detected only in P_2E in adequate concentration (4.26% & 1.37% respectively) (Table 2).

3.2.4. Three caffeic acid esters are solely present in P_2E in a relative high concentration, 3-methyl-2-butenyl-*trans*-caffeate (11.61%), 3-methyl-3-butenyl-*trans*-caffeate (9.48%) and phenyl ethyl *trans*-caffeate 2.35%, and only one isoferulate ester is detected in P_1E, 3-methyl-3-butenyl isoferulate (1.12%) (Table 2).

3.2.5. Flavonoids: Nine flavonoids are detected in P_1E with a total concentration of 18.65%, chrysin has the highest concentration of 5.37%, then galangin, naringenin & pinocembrin (4.85%, 1.68 %& 1.41% respectively). The other three flavonoids have very low concentrations ranged from 0.17% to 0.69%. Only 2 flavonoids are detected in P_2E, naringenin has the highest concentration 2.2% and galangin 0.63% (Table 2).

3.2.6. One chalcone, 2',4',6'-trihydroxy-chalcone, presents in adequate amount in both P_1E& P_2E (3.3% & 3.39% respectively) (Table 2).

3.2.7. Many sugars, nitrogenous compounds & other compounds were identified in both P_1E & P_2E (Table 2).

Results of GC/MS analysis demonstrated that propolis samples P_1E & P_2E contain a varied amount of bioactive compounds. In consequence, many previous studies proved that propolis samples from different provenances have different compounds or may be the same compounds but with varied concentration which considered a pronounced evidence on the influence of climate and geographic flora on propolis chemical content as well as, the solvent and the extraction method have an obvious impact [36]. The two ethyl acetate fractions contain adequate amounts of polyphenolic compounds like flavonoids and phenolic acids and their esters such as isoferulic acid, benzoic acid and caffeate esters which have many significant biological activities.

Sugars were identified in both P_1E & P_2E, however, the inquiry about the sugar origin in propolis has not been answered yet. Many studies suggested nectar and honey to be the sources of glucose, fructose and sucrose, whilst others referred that they originate in propolis from flavonoid glycosides hydrolysis as it is rare to find glycosides in propolis; only aglygons[37]. Moreover, Crane and his group listed plant mucilages as a potential propolis sugar sources[38].

Propolis anti-candida activity was previously reported; where the alcoholic extract of propolis samples from Poland, Iran and Brazil showed variable potent activities against *C. krusei*, [39-41]. Additionally many others researchers proved that the ethanolic extracts of Brazilian, Iranian and Polish propolis had a significant inhibition activity against *C. albicans* [39, 42, 43].

On the contrary, Monzote and his group reported the inactivity of 20 samples of Cuban propolis [44]. This deviation in the activity could be attributed to the diversity in the phytochemical constituents of different propolis samples which consequently affect their biological activity [45].

Besides, many identified compounds in both P_1E & P_2E have been proved in several previous studies as Candida inhibitors.

Various fatty acids such as conjugated linoleic acid (CLA), inhibited the yeast-to-hypha transition by inhibiting the hyphal growth through two main axis, affecting the cellular localization of Ras1p and obstructing the raise in RAS1 mRNA and protein levels [46]. Other studies tackled the inhibition activities of flavonoids to the resistant *C. krusei*, for instant Cushnie and Lamb stated that many flavonoids as galangin possess antifungal activity [47].

Table 2: Detected Compounds by GC/MS in P_1E and P_2E

Peak	RT	Compound	P_1E Area%	P_2E Area%
Aliphatic acids &esters				
1.	5.7	D-lactic acid	0.37	ND
2.	6.2	Acetic acid	0.34	ND
3.	15.34	Octanoic acid (Caprylic acid)	ND	0.1
4.	32.63	Dodecanoic acid (Lauric acid)	0.12	0.18
5.	38.0	Azelaic acid	0.48	ND
6.	41.47	9-Tetradecenoic acid	0.35	0.47
7.	42.86	Pentadecanoic acid(Pentadecylic acid)	ND	0.28
8.	46.4	Hexadecanoic acid (Palmitic Acid)	12.06	10.91
9.	51.0	9-Octadecenoic acid (oleic acid)	5.01	ND
10.	52.30	Octadecanoic acid (Stearic acid)	8.77	6.16
11.	53.50	9,12-Octadecadienoic acid(Linoleic acid)	0.49	0.57
12.	54.0	13-Octadecenoic acid	0.84	ND
13.	57.26	Arachidic acid	0.37	0.96
14.	57.84	Glycidyl oleate	ND	0.24
15.	61.2	1-Monopalmitin	0.92	ND
16.	61.44	13-Docosenoic acid	ND	0.43
17.	62.07	Behenic acid	0.19	0.42
18.	63.70	Glycerol monostearate	ND	0.33
19.	65.21	Mono-oleoyl- glycerol	ND	0.49
20.	66.7	Tetracosanoic acid	0.86	ND
21.	66.85	Lignoceric acid	ND	2.74
22.	67.3	Hexadecanedioic acid	0.63	ND
23.	71.04	Hexacosanoic acid	0.22	0.57
24.	80.67	9,12,15-Octadecatrienoic acid, 2,3-dihydroxy-propyl ester	ND	0.11
25.	88.08	1,3-Dipalmitin	ND	0.13
Total			**32.02**	**25.09**
Phenolic Compounds				
26.	8.10	Benzyl methyl ketone	ND	1.35

27.	9.1	Benzyl alcohol	0.19	ND
28.	18.1	1,2-Benzenediol	0.7	ND
29.	18.81	2-phenylpropenol	ND	0.23
30.	19.83	Benzyl isobutyl ketone	ND	0.13
31.	23.1	Cinnamyl alcohol	0.14	ND
32.	32.91	1,3,5-Benzentriol	ND	0.53
33.	34.25	2,5-Dihydroxyacetophenone	ND	0.21
34.	73.4	5,7-Dihydroxy-3-{4-hydroxy-phenyl}-4H-chromen-4-one	0.37	ND
Total			**1.4**	**2.45**
Phenolic acids & esters				
35.	14.15	Benzoic Acid	1.01	2.17
36.	22.6	Benzenepropanoic acid	0.27	ND
37.	27.22	p-Anisic acid	ND	0.24
38.	28.2	Cinnamic acid	1.73	ND
39.	36.96	Vanillic Acid	ND	1.37
40.	38.8	4-Methoxycinnamic acid	0.4	ND
41.	39.04	Protocatechoic acid(3,4-dihydroxy-benzoic acid)	ND	0.54
42.	44.37	*cis*-Caffeic acid	ND	0.11
43.	45.4	3,4-Dimethoxycinnamic acid	2.02	ND
44.	47.19	Isoferulic acid	2.69	0.33
45.	49.23	*trans*-Caffeic acid	ND	4.26
46.	53.2	3-Methyl-3-butenyl isoferulate	1.12	ND
47.	55.47	3-Methyl-3-butenyl- *trans* -Caffeate	ND	9.48
48.	56.78	3-Methyl-2-butenyl- *trans*-Caffeate	ND	11.61
49.	57.4	3,4,5-Trihydroxybenzoic acid	0.14	ND
50.	65.92	Phenyl ethyl-*trans*-caffeate (CAPE)	ND	2.35
Total			**9.38**	**32.46**
Flavonoids				
51.	35.5	6,7-Dihydroxycoumarin	0.33	ND
52.	58.8	Pinostorbin	0.69	ND
53.	59.6	Pinocembrin	1.41	ND
54.	59.66	2',4',6'-trihydroxy-chalcone	3.3	3.39
55.	61.24	Naringenin	1.68	2.2
56.	64.5	Chrysin	5.37	ND
57.	64.96	Galangin	4.85	0.63
58.	72.4	Kaempferol	0.17	ND
59.	77.19	Apigenin	0.37	ND
60.	75.0	Isorhamnetin (monomethoxyflavone)	0.48	ND
Total			**18.65**	**6.22**
Sugars				
61.	35.2	Levoglucosan	0.24	ND
62.	39.35	D-Fructofuranose	ND	2.54
63.	40.1	D-Pinitol	4.47	ND
64.	40.4	Glucofuranoside	1.18	ND
65.	46.3	alpha.-D-Glucopyranoside	0.16	ND
66.	74.2	D-Pinitol(isomer)	1.49	ND
Total			**7.54**	**2.54**
Nitrogenous compounds				

67.	55.60	N-[.alpha.-Cyano(4-methoxybenzyl)]-N-benzyl-2-azido-5-chlorobenzamide	ND	0.66
68.	67.90	4,6,2',6'-Tetramethyl-biphenyl-2,4'-diamine.	ND	0.59
69.	73.72	4,6,2',6'-Tetramethyl-biphenyl-2,4'-diamine isomer	ND	0.49
70.	74.6	2,4-Imidazolidinedione-5-[3,4-dihydroxy-phenyl]-3-methyl-5-phenyl	0.14	ND
Total			**0.14**	**1.74**
		Others		
71.	15.0	Cyclohexane	0.27	ND
72.	16.57	Phosphoric acid	0.29	0.1
73.	16.77	Glycerol	ND	0.78
74.	18.12	Acetin	0.7	0.70
75.	19.45	Diacetin	ND	0.41
76.	35.73	2-[4-methyl-6-(2,6,6-trimethylcyclohex-1-enyl)hexa-1,3,5-trienyl]cyclohex-1-en-1-carboxaldehyde	ND	0.15
77.	41.3	beta.-Eudesmol, [sesquiterpene]	0.73	ND
Total			**1.99**	**2.14**

ND: Not Detected

In addition, Candiracci and his team proved that chrysin and galangin had the highest anti-candida activity and contributed this to the low hydroxyl groups they have so they are much less polar than the corresponding flavone (apigenin) and flavonols (quercetin and kaempferol) [48]. This finding supports the assumption that anti-candida activity depends on their relative lipophilic characters, suggesting that they may reach a possible intracellular site of action without compromising membrane-associated functions. Understanding how natural flavonoids inhibit the growth of microorganisms can help finding new technologies for the development of food products with particular nutritional functionalities [49], our results are in agreement with these studies.

In consequence, De Vita and his team illustrated that caffeic acid phenethyl ester (CAPE) possess anti-candida activity through hindering both the biofilm formation and Candida filamentation, they attributed this to the conjugation of the ester function with the unsaturated system of the aromatic ring [50]. As well, Sun and his group elaborated that caffeic acid and its esters inhibit Candida growth through many effective mechanisms as damaging the cell wall, inhibit the dimorphism and disrupting plasma membrane, along with suppressing the Isocitrate Lyase enzyme activity. Not only this but caffeic acid and its esters exhibited curbing activity to biofilm formation. Thus they have a drastic inhibition influence on Candida by hindering almost all its main virulence factors [51]. These evidences relevant for the powerful inhibition activity of P_2E against both *C. krusei and C. albicans* as many caffeic acid esters have been detected solely in a relatively high concentration as illustrated in Table 2.

Some bioactive major compounds in P_2E fraction (Fig.2), which had the highest antifungal activity were further conducted into in-silico study to estimate their possible binding affinity to three pivotal enzymes for candida species survival, N-myristoyl transferase (NMT), thymidylate synthase (TDS) and lanosterol 14 α-demethylase(LDM), where N-myristoyl transferase is responsible for transfer the myristate moiety from myristoyl-CoA to the N-terminal glycine residue of a variety of cellular proteins as an essential process for vegetative growth.

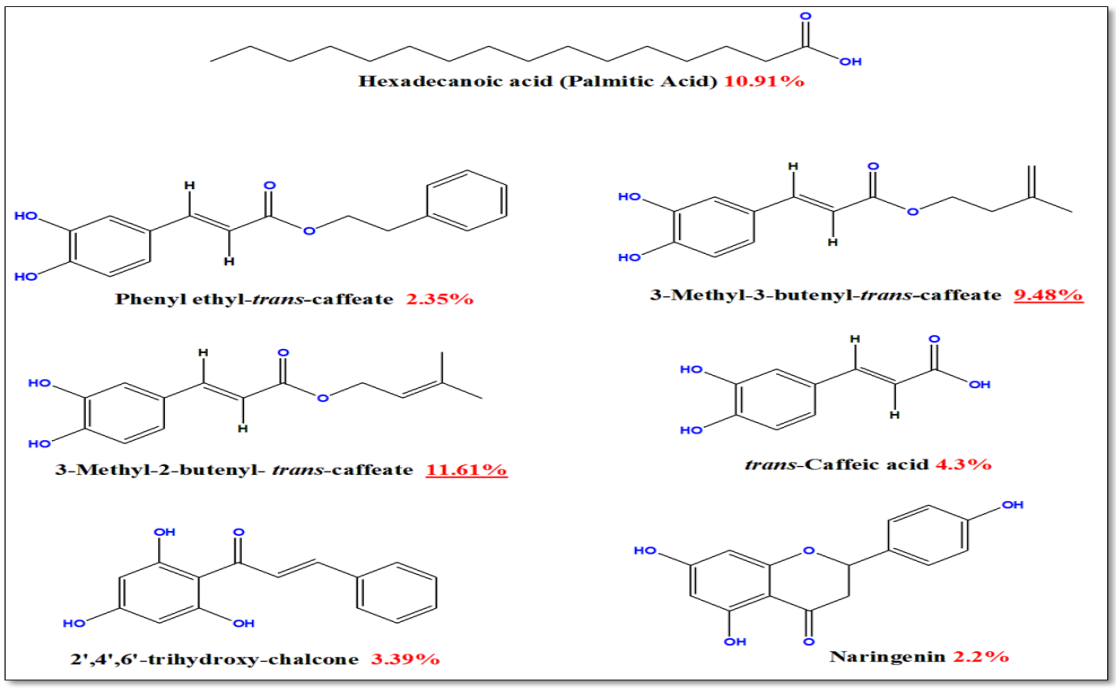

Fig.2: Chemical structure of some major compounds detected by GC/MS analysis in P2E fraction

Table 3. The docking score of major compounds in P2E with NMT, TDS and LDM

Ligand	Binding affinity ΔG (kcal/mol)		
	N-myristoyl-transferase	**Thymidylate synthase**	**lanosterol 14 α-demethylase**
Co-crystallized inhibitor (RMSD)	-9.090 (0.193)	-9.455 (0.244)	-16.511 (1.356)
Palmitic Acid (C16:0)	-8.025	-7.75	-7.967
Phenyl ethyl-trans-caffeate	-7.055	-7.003	-8.041
3-methyl-3-butenyl caffeate	-6.51	-6.97	-6.45
3-methyl-2-butenyl caffeate	-6.746	-6.798	-7.348
trans-Caffeic acid	-5.332	-5.449	-5.203
2`,4`,6`-trihydroxy-chalcone	-6.801	-6.608	-6.5

Naringenin	-7.013	-6.425	-6.72

In addition, thymidylate synthase is essential for thymidine synthesis and hence DNA synthesis while inhibition of lanosterol 14 α-demethylase which participates in ergosterol biosynthesis result in accumulation of intermediates such as 14a-methylsterol and subsequent fungal cell death [52], [53]&[54] these three enzymes conducted into our in silico study are potential targets for anti-candida drug discovery, thus compounds possessing certain binding affinity towards the three targets likely have a potential anti-candida activity. Redocking of native ligands resulted in an excellent superposition between the native and docked pose with RMSD values between 0.193Å and 1.356Å, revealing that docking protocol is valid for the study (Fig.3).

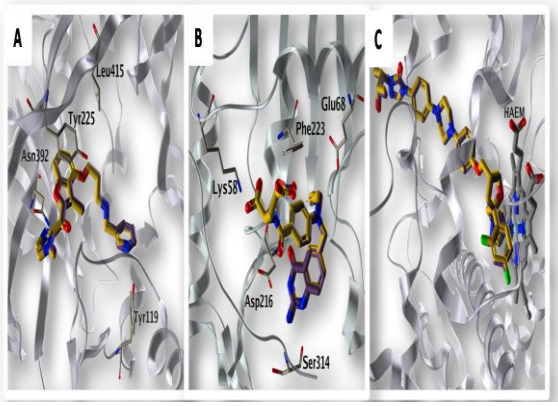

Fig.3: Superposition of native (magenta sticks) and (yellow sticks) poses for native ligand : (A) N-myristoyltransferase. (B) Thymidylate synthase. (C) Lanosterol 14-alpha demethylase.

Docking study revealed that some of the identified major compounds in P_2E had a plausible binding affinity to the three target candida enzymes as expressed as binding free energy (Table 3). Palmitic acid has demonstrated the highest biding affinity toward N-myristoyl-transferase (-8.025 kcal/mol) and thymidylate synthase (-7.750 kcal/mol) while phenyl ethyl-trans-caffeate showed the best binding to lanosterol 14 α-demethylase (-8.041 kcal/mol). 3-methyl-2-butenyl-*trans*-caffeate and 3-methyl-3-butenyl-*trans*-caffeate esters have demonstrated a good binding affinity toward the three enzymes ranging from -6.450 to -7.348 kcal/mol (that is the first time to investigate the anti-candida activity of these caffeate prenyl esters). Additionally, free caffeic acid exhibited the weakest binding affinity toward the three enzymes among all docked compounds, as shown in (Table 3), palmitic acid is stabilized in the hydrophobic pocket of N-myristoyl-transferase through interaction with the pocket hydrophobic residues in proximity (Fig.4).

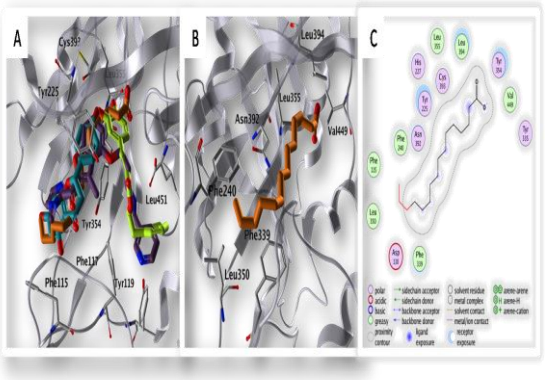

Fig.4: The predicted docking poses of P2E major compounds and the x-ray crystal structure of candida N-myristoyltransferase (PDB code 1IYL) (A) Superposition of best docking pose of the three top ranking major compounds in P2E, Palmitic Acid (orang sticks), Phenyl ethyl-trans-caffeate (yellow sticks), Naringenin (cyan sticks) and cocrystalized inhibitor (magenta sticks). (B) Detailed binding mode of palmetic acid (orange sticks) with the pocket amino acid residues (gray sticks). (C) 2D representation of palmetic acid binding interactions with the pocket amino acid residues.

Regarding the interaction with the thymidylate synthase active site, palmitic acid carboxylic group formed hydrogen bonds with Arg 29 and Arg 213 while the tail formed arene-H interaction with Phe 223 (Fig.5).

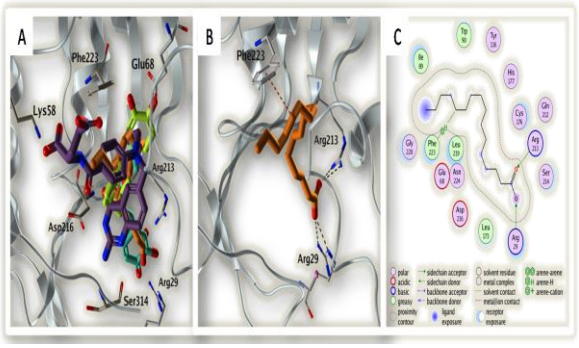

Fig.5: The predicted docking poses of P2E major compounds and the homology model of candida Thymidylate synthase (A) Superposition of best docking pose of the three top ranking major compounds in P2E, Palmitic Acid (orang sticks), Phenyl ethyl-trans-caffeate (yellow sticks), 3-methyl-3-butenyl caffeate (cyan sticks)

and cocrystalized inhibitor (magenta sticks). (B) Detailed binding mode of palmetic acid (orange sticks) with the pocket amino acid residues (gray sticks) hydrogen bonds (black dashed line) and π-interaction (red dashed line). (C) 2D representation of palmetic acid binding interactions with the pocket amino acid residues.

Moreover, Phenyl ethyl-*trans*-caffeate was stabilized in the active site of lanosterol 14 α-demethylase by ionic interaction with haem group (Fig.6).

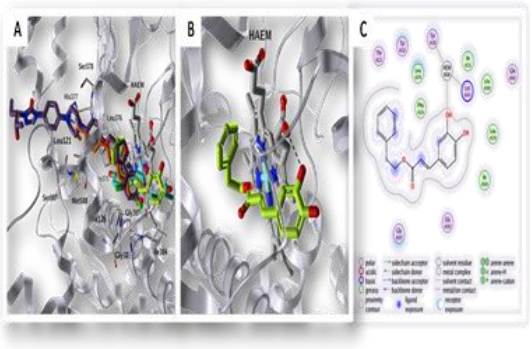

Fig.6: The predicted docking poses of P2E major compounds and the x-ray crystal structure of candida Lanosterol 14-alpha demethylase (PDB code 5FSA) (A) Superposition of best docking pose of the three top ranking major compounds in P2E, Palmitic Acid (orang sticks), Phenyl ethyl-trans-caffeate (yellow sticks), 3-methyl-3-butenyl caffeate (cyan sticks) and cocrystalized inhibitor (magenta sticks). (B) Detailed binding mode of Phenyl ethyl-trans-caffeate (Yellow sticks) with the Haem molecule (gray sticks) hydrogen bonds (black dashed line). (C) 2D representation of Phenyl ethyl-trans-caffeate binding interactions with the pocket amino acid residues.

The aforementioned results are in agreement with previously reported literature. Prasath et al. has related the significant anti-candida activity of Palmitic acid to the reduction in mature biofilm formation and ergosterol biosynthesis with down regulation of lanosterol 14 α-demethylase [55].

Moreover, caffeic acid esters especially phenethyl ester were reported as potent inhibitor for candida species compared to the weak activity exhibited by free caffeic acid [56, 57]. Although 3-methyl-2-butenyl-*trans*-caffeate and 3-methyl-3-butenyl-*trans*-caffeate esters have not achieved the best binding score against the candida enzymes but they illustrated a reasonable binding affinity range which could contribute to the P2E anti-candida activity owing to their high concentration in this fraction.

According to the chemical investigation along with docking results, inhibition of N-myristoyl-transferase, thymidylate synthase and/or lanosterol 14 α-demethylase by the major components of P2E could be the underlying mechanism for its significant anti-candida activity.

3.3. Assessment of self-emulsification:

Different SEDDS were prepared using different oils, and then their self-emulsifying capability was tested visually. The designed systems should form clear and monophasic liquid when diluted with aqueous medium to allow for the active moieties to be present in a solubilized form. Table 4 is showing the composition of the prepared formulae and the visual grading of the self-emulsification process upon dilution; it is clear that only S3 presented a successful nanoemulsifying system. This may be explained by the difference in fatty acid length chain of the different oils, where laurate, myristate and palmitate were C12, C14 and C16, respectively. In this case, the shortest fatty acid chain length has the highest self-emulsification power which may be due to its highest hydrophilicity. Therefore, S3 was selected to characterize and prepare the Soluplus-based self-emulsifying tablets [58].

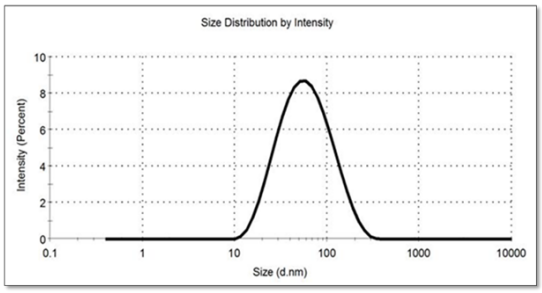

Fig. 7: Particle size analysis of the selected SEDDS (S3).

3.3.1. Droplet size determination:

The average droplet size of S3 was found to be 68.67±9.51, the size distribution is shown in (Fig. 7). Such a small particle size is an indicator of the stability and rapid emulsification of the nanosystem [27, 59]. Previous studies have reported that nanosystems with oils of short chain fatty acids attained small droplet size [27]. It was concluded that the chain length of the oil plays a crucial role in nanoemulsion stability and droplet size as well as easiness of emulsification.

3.3.2. Assessment of physical properties of the formulated tablets:

The average weight of the tablets was found to be 0.8 ± 0.05 g showing uniformity of prepared tablets, and the disintegration time was 11 ± 2 min. which is a reasonable time for such a step after oral administration.

3.3.3. In-vitro release study:

The standard curve for the spectrophotometric determination of the active moiety in methanolic buffer at 293 nm is shown in (Fig.8). Soluplus ® is a polyvinyl caprolactam–polyvinyl acetate–polyethylene glycol graft copolymer having amphiphilic properties which can be used as a solubility enhancer of poorly soluble drugs [60, 61].

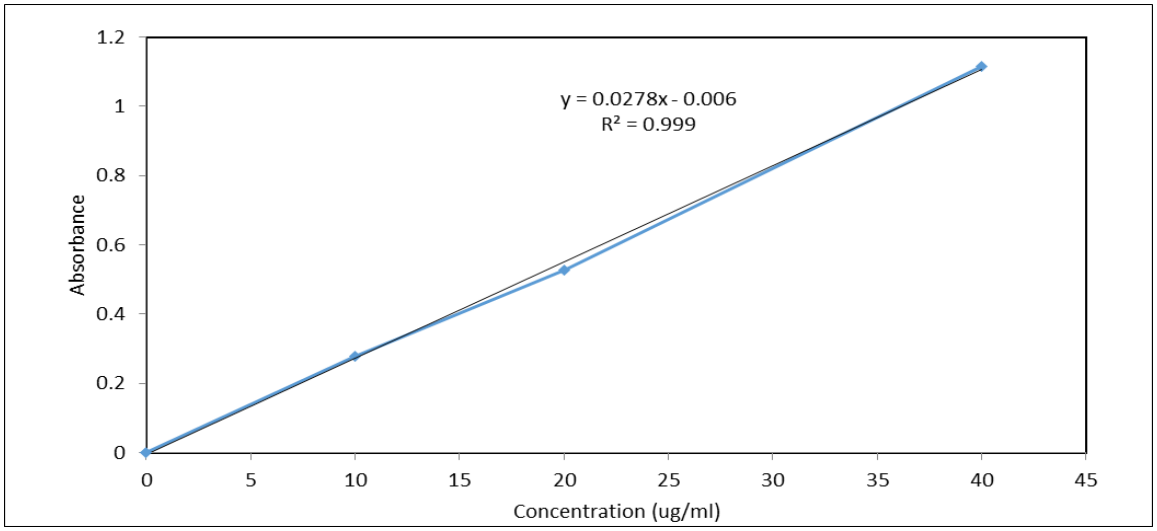

Fig. 8: Standard curve for the spectrophotometric determination of the active moiety in methanolic buffer at 293 nm.

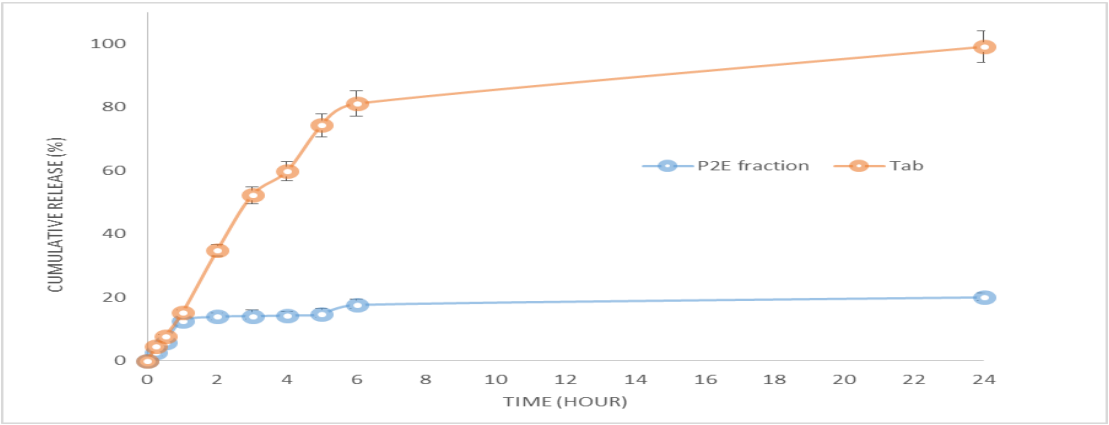

Fig.9: Release profile of the bioactive moieties from S3-loaded Soluplus-based self-emulsifying tablets in comparison with P_2E fraction (n=3).

Soluplus® has a dual functional being a matrix polymer for solid solutions as well as an active solubilizer through micelle formation in water [62]; therefore, as it was expected, this formula provided a great role in the enhancement of the active agent release as seen in (Fig.9), S3-loaded Soluplus-based self-emulsifying tablet attained a far better release than the plain extract; the cumulative release of the active agent was 100 % and 20 %, respectively, at 24 hr. This was expected as it is well known that SEDDS ameliorate the bioavailability of drugs with low aqueous solubility by increasing their solubility; after oral administration the drug is kept in oil nano-

droplets all over the gastrointestinal tract [27, 63]. These results also confirm the role of Soluplus which was used as a carrier for the SEDDS to form a liquisolid preparation, and a solubilizer as well.

4. Conclusion:

Having manifested all the above it could be concluded that the examined Saudi propolis is considered an attractive and promising source for curbing both the most prevalent infectious fungal pathogen *C. albicans* and the multi-drug resistant *C. krusei*.

Docking study confirmed that all of the identified major compounds in P_2E had a plausible binding affinity to the three target candida enzymes, which could explain the significant P_2E anti-candida activity. As well, we can state that Soluplus-based self-nanoemulsifying tablets represents a promising strategy for the administration of the propolis extract and for the improvement of its bioavailability ,so it is highly recommended to carry out further in-vivo studies to evaluate the safety and clinical utility of these active ingredients in patients.

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6. Conflict of interest:

The authors declare that they have no conflict of interest.

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