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Research Article

Novel Nano-Propolis Emulsion using Aqueous Solution Preparation Method without Ethanol

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ABSTRACT

Synthesized Propolis Nano-Emulsion (PNE) have been prepared by our team with some novel effective modifications, and newly compound with specific physical and chemical properties was obtained. Novel preparation method of propolis nano-emulsion depended mainly on an aqueous solution composite without any ethanol extraction as initial step. The obtained PNE with diameters of 32 nm have been prepared by a long time stirring process in organic surfactant; with temperature adjustment. Its average dynamic nano-size by zeta sizer reach to 16 nm. The prepared propolis -nano particles showed a good stability condition of nano-solution as the polydispersity index (pdi) is lower than 0.5. Its antimicrobial activity against the A. fumigatus isolates is proved by the developed inhibitory zones on PDA cultured media. The present work promotes to develop new compounds from the natural ones using nano- formulation techniques to increase their level of efficacy.

INTRODUCTION

Propolis, addressed from ancient times, owns antimicrobial, antioxidant, anti-inflammatory, anaesthetic, hepatoprotective, immunostimulating and cytostatic activities [1]. Propolis known also bee glue is a brownish waxy product collected by the honeybee from plant buds, leaves, and exudates. The propolis is noticed as a better source of natural antioxidants and antibacterial. Propolis contains variable of chemical compunds as coumarins, steroids, polyphenols, sesquiterpene, quinines, and inorganic components. Phenolic compounds are the most important compounds contained in propolis, especially flavonoids and phenolic acids derivatives [2]. Recently, nanotechnology has a clear role of increasing the efficacy and safety in therapeutic and diagnostic agents, synthesized Propolis Nano-Emulsion (PNE) have been prepared by our team with some novel effective modifications. Our invention idea relates to a propolis nano-emulsion solution and a preparation method is based on producing nano-emulsions using low-energy means due to the fact that expensive specialized equipment. The emulsions formed using Spontaneous Emulsification SE described by McClements [3], but our methodology has some additions. It is speculated that when PNE size decreases, the surface/volume ratio of particles become very large [4,5]. This feature makes nanoparticles highly reactive and they can overcome some of the limitations which exist in pure propolis [6,7]. Thus, the present work objected to develop a new form of nano propolis from the crude

compound using simple, novel, cheap and safer nano-formulation techniques to enhance the level of efficacy and safety in therapeutic and diagnostic purposes.

MATERIAL AND METHODS

Chemicals

The pure propolis samples were collected during (January-December 2020) from an apiary hive bee's located in Assiut Governorate by scraping the wall sand frames of the hives. Aqueous propolis extraction was prepared and lyophilized according to Crane [8]. Tween-80 was purchased from (Thermo Fisher Scientific USA).

Pure propolis (PP) extract preparation

The aqueous propolis extract was prepared by adding 10gm of propolis powder to 100 ml deionized water and left for 5 hrs at room temperature, the resulted extract was filtered by What man No. 1 filter paper and stored at - 4˚C until used [9].

Propolis nano- emulsion (PNE) preparation

We have used the Spontaneous Emulsification (SE) based technique with low-energy formation (thermal method) of nano-emulsions which utilized with food-grade ingredients [3]. Novel modifications were added to this basic manufactured methodology by our team. A fine powder of pure propolis were weighted 200mg and added to 100 ml double deionized distilled water W/V ratio (2 mg¬/ml). This oil-water phase was containing a hydrophilic surfactant Tween 80 in (3%) concentration was kept on a magnetic stirrer at 3000rpm for 7hrs at 40˚C. Then, the solution was sonicated for 10 minutes and filtered with 200nm nano-filter.

Characterization of propolis nano- emulsion: After sonication for 10min, it was analyzed by dynamic light scattering (DLS), TEM examination and FTIR analysis.

Particle size and zeta potential: The determination of average diameter and polydispersion index of the synthesized PNE nano-emulsion were made using Dynamic Light Scattering (DLS). The suspensions were fourfold diluted in Milli-Q water and analyzed in Zetasizer apparatus, model Nano-ZS from Malvern (Malvern analytical, a spectra's company, UK) at Nanotechnology Unit, Faculty of Pharmacy, Alazhar University, Assiut branch. The results were determined by the average of two cycles of 20 scans. The zeta potential of the nano- emulsion was obtained by the electrophoretic mobility technique in the zetasizer apparatus in which the samples were diluted fourfold with Milli-Q water and results were expressed in millivolts (mV) from the average of two cycles of 20 scans.

TEM analysis: Transmission electron microscopy images were analyzed to confirm the morphology and approximate size of nanoparticles. The compositions of synthesized propolis nano-emulsion were fixed on stubs with double carbon tape and covered by a gold film during the metallization process with 10 mA for 7 min. TEM micrographics have been taken from Joel (Japan) microscope.

FTIR analysis: The fourier-transform infrared spectroscopy (HORIBA JOBIN YVON Fluoromax-4), used for molecule distribution. FT-IR spectral analysis of propolis emulsion and pure propolis was carried out using KBr tablets (1% w/w of product in KBr) with a resolution of 4 cm−1 and 100 scans per sample on a Perkin Elmer Spectrum RX1 apparatus. The results were expressed in infrared transmittance percentage.

Anti-mycological effect of pure propolis and propolis nano-emulsion on A. fumigatus growth

The strains A. fumigatus were cultured on Potato Dextrose Agar (PDA) for 5 days at 25˚ C, the fungal growth was washed with sterile saline and the fungus suspension was prepared according to 0.5 MacFarland standards. 1 ml of fungus suspension was dissolved in Muller Hinton agar medium and distributed into Petri dishes, and then wells were made. Add 0.1ml (200µg) of PP and PPE extracts to wells and left for 48hrs, their effect was evaluated by the diameter of the formed zone around each well [10].

RESULTS

Characterization of PNE

Our novel modifications used in the PNP preparation yielded a newly nano-compound with specific properties. The average dynamic nano-size by DLS- zetasizer reached to 16.66 nm diameter. The prepared propolis nano-emulsions showed a good stability condition of nano-solution as the poly-disparity index (pdi) is lower than 0.5 (Figure 1). Zeta potential of PNE showed a negative surface charge value (-44 mV) which was sufficiently high to avoid PNE aggregation. This value represents a stable and dispersed suspension of PNE that there is no tendency to form aggregates in a short period of time. The TEM image reveals that the PNE morphology is nearly spherical with has an average size of 32.8 nm in diameter (Figure 2). The results of FTIR analysis revealed that PNE are

showing new bonds formed by the conversion of the pure propolis to nano-propolis compound (Figure 3).

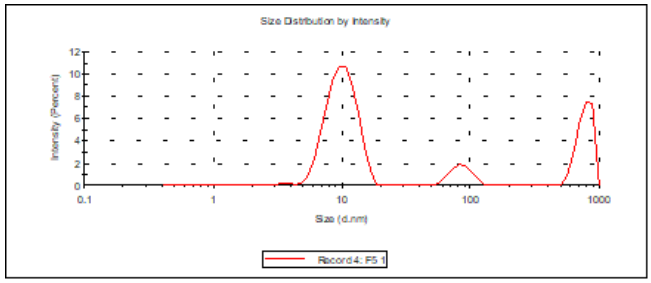

Figure 1: Zetasizer peaks showed an average dynamic nano-size of the propolis nano- emulsion.

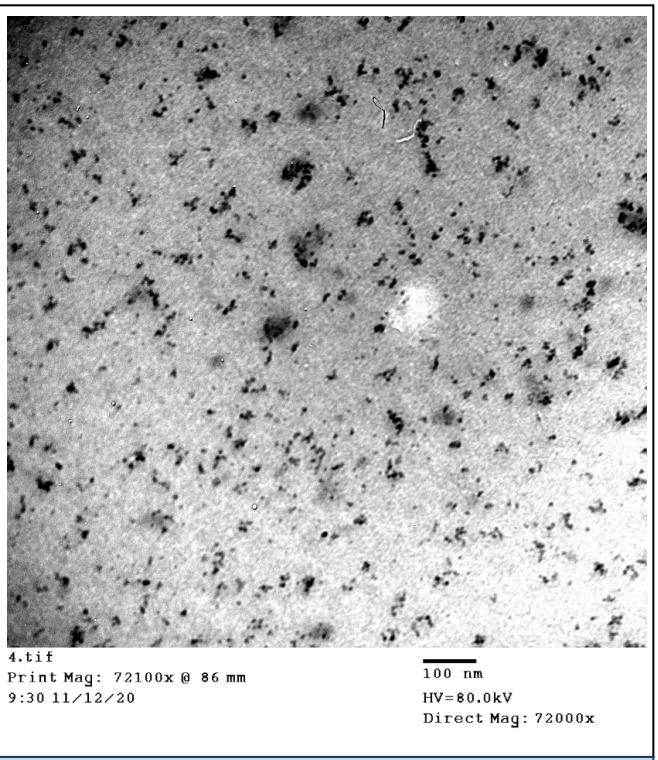

Figure 2: TEM image showed the propolis nano-emulsion with average size of 35 nm diameter.

Anti-mycological potency of pure propolis and propolis nano-emulsion

Inhibitory effect of pp and PNE on the growth of A. fumigatus isolates: The addition of 0.1ml (200µg) PP showed a peripheral inhibited growth zone of A. fumigatus isolates on PDA media (Figure 4c). A whitish- green inhibited peripheral growth zone of A. fumigatus isolates on PDA media appeared after administration of 200µg/ml nano- propolis concentration (Figure 4d). The inhibition area zone recorded the average diameters of 27.55 ± 7.975 and 24.133 ± 8.324 mm, respectively, with a significant difference at $p > 0.01$ between pure propolis and propolis nano-emulsion Table 1.

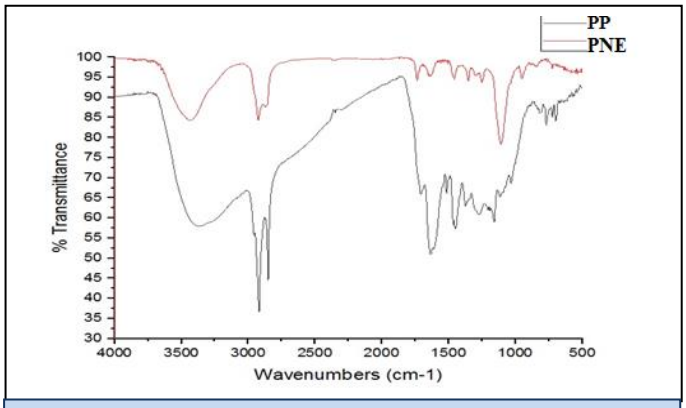

Figure 3: FTIR revealed the PNE has new bonds and active chemical groups formed by the conversion of the pure propolis to nano-propolis compound.

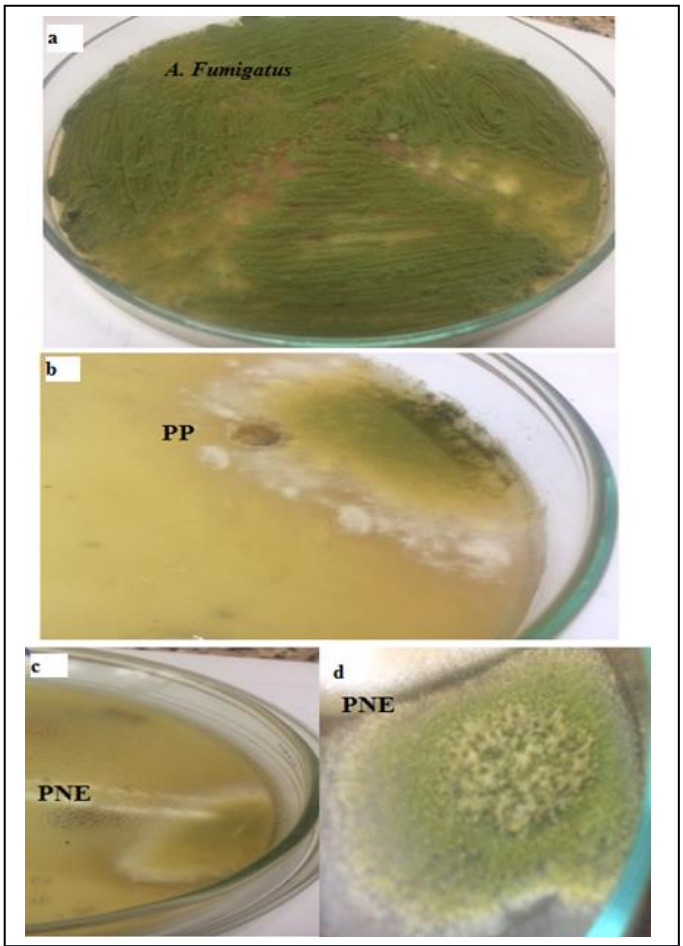

Figure 4: a. Showed the full growth of A. fumigatus isolates on PDA agar medium.
b. Showed a peripheral inhibited growth zone of A. fumigatus isolates on PDA media after 0.1ml of PP administration.
c. Showed a clear zone of inhibition of A. fumigatus isolates on PDA agar media 0.1ml with addition of 0.1ml nano- concentration of PNE.
d. Showed a core of whitish- green inhibited growth zone of A. lumigatus isolates on PDA media with 0.1ml nano- concentration of PNE.

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Table 1: Inhibitory effect of pure propolis and propolis nano-emulsion on A. fumigatus growth on PDA agar.

Fungal *spp*	Inhibition zone (mm)						*P* value
	PP			PNE			
	Min	Max	Mean ± SD	Min	Max	Mean ± SD	
A. fumigatus **(n=40)**	14	43	29.55 ± 6.776	10	34	23.031 ± 5.322*	0.05*

*significant difference at P > 0.05.

DISCUSSION

This article declares the methodology that connects to a propolis nano-emulsion oral solution and the preparation method that not include an ethyl extract solution. Basically, PNE extract includes an organic surfactant and injection water. It contains 200 mg by weight of propolis pure powder, the oil-water phase comprised a hydrophilic surfactant Tween 80 in (3%) concentration and the balance of high-purity water of 100 ml double deionized distilled water. Novel modifications were added to this basic manufactured methodology by our team where this preparation was kept on a magnetic stirrer at 3000 rpm for 7hrs at 40°C. In the same context of our result Kubiliene et al. [9] showed that non-ethanol solvent complex and the effect of higher temperature allows more effective extraction of active compounds from propolis. Concentration of total phenolic compounds in these extracts does not differ significantly from the concentration found in ethanol extract. Propolis non-ethanol extracts have radical scavenging and antimicrobial activity. The propolis nano-emulsion oral solution has the advantages of higher stability, higher absorption rate and better health care treatment effect [11].

The emulsions formed in this work used Spontaneous Emulsification (SE) with food-grade ingredients, the emulsions formed using SE described by McClements [3], but our methodology has some modification as mentioned in the previous paragraph. Our invention idea connects to a propolis nano emulsion solution and a formation technique is based on producing nano-emulsions using low-energy way. Recently, there has been growing interest in producing nano-emulsions utilizing low-energy means due to the fact that expensive specialized equipment [12]. Emulsifiers act a critical role in facilitating the preparation of nano-emulsions by lowering the interfacial tension, and thereby lowering the free energy penalty accompanied with droplet formation [13]. Emulsifiers are surface-active agents capable of adsorbing to the oil-water interface and forming a protective coating around droplets [14]. This protective coating prevents droplet aggregation during and after emulsion formation. Low-energy formation of nano-emulsions as it applies to other fields of study such as pharmaceuticals have reviewed in many studies [15,16].

Nanoparticle's propolis has unique data indicated that PNE morphology is spherical with has an average diameter size of 35 nm by the TEM image. The PNE-zetasizer, data recorded the nano-sized particle reached to 16.70 nm in diameter. The newly compound PNE revealed a good stability status of nano-solution as the polydispersity index (pdi) is lower than 0.5. Zeta potential of PNE had a negative surface charge value (-44 mV) which was sufficiently high to avoid PNE aggregation. This value indicates a stable and dispersed suspension of PNE that there is not tendency to form aggregates in a short period of time [17]. The data of FTIR assay conducted that propolis nanoparticles are having new bonds added by the conversion of the pure propolis into newly nano-propolis form.

The present work proved the anti-mycological properties of pure propolis and nano- propolis extracts against A. flumigatus isolate growth as a natural bioactive substance. The application of PP or PNE in a concentration of 0.1ml (200µg) indicated an inhibitory peripheral zone of growth of A. fumigatus isolates on PDA media. The mean diameters of the area zone of inhibition were reported as 29.55 ± 6.776 and 23.031 ± 5.322 mm, respectively, with a significant difference at p > 0.05 between PP and PNE. These data are in agreed with the results of previous literatures on the antifungal effect of propolis against food-borne or plant-origin molds. It was reported that the administration of 1%, 5%, and 10% concentrations of ethanol-extracted propolis inhibited in vitro growth of Penicillium digitatum [18,19] and decreased the growth of Botrytis cinerea on strawberry [20]. Özcan [21] stated that treatment with 4% water-extracted propolis produced more than 50% inhibition of some plant pathogens, including P. digitatum and B. cinerea, *in vitro*. The administration of 2% and 5% concentrations of methanol-extracted propolis inhibited in vitro growth of Alternaria alternata and Fusarium oxysporium f. sp. Melonis [22]. Mattiuz et al. [23] found that propolis (7:3 v/v) limited up to 75% the growth of the phytopathogenic fungus Diplodia seriata. The

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extract of nano- propolis, at the highest concentration of 40% produced a notable inhibition on the spore germination and principally, on the aflatoxin production of A. flavus [24]. Hosseini et al. [25] reported that the higher the extract concentration, the lower the mycelium of A. parasiticus devlopment, and the highest fungal production inhibition by propolis was, in the concentration 100µg/ml. Our findings are in agreement with the data of other similar literatures reported around the world, Velazquez et al. [26] showed that the antibacterial and free-radical scavenging activities of propolis. The antifungal impact of propolis against fluconazole-resistant Candida glabrata isolates obtained from women with recurrent vulvovaginal candidiasis was reported with Shokri et al. [27]. The effect of propolis extract from different regions of Argentina on dermatophytes and yeast strains was recorded and showed that the minimum inhibitory concentration was between 125 and 31.25µg/ml [28]. Also, Ownagh et al. [29] obtained that in the concentration 125µg/ml of propolis extract could inhibit the production of Aspergillus niger, Microsporum canis, Trichophyton rubrum and Epidermophyton floccosum.

CONCLUSION

The study idea relates to a preparation method of a nano-propolis solution without addition of the ethanol and with lower, simple and inexpensive techniques. The method succeeded to add propolis into a non ethanolic solution and using a hydrophilic surfactant. Then apply stirring or performing ultrasonic treatment to obtain a nano-propolis emulsion of which the fine particle diameter is 35 nanometers. The newly properties obtained of nano-propolis emulsion greatly promised that improve disparity and absorptivity of propolis and the physiological and biochemical processes of propolis as antimicrobial are improved, and the bioavailability of propolis is achieved.

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