

Original Research Article

Green Synthesis and Characterization of Libyan Propolis Nanoparticles and its Biological Activity

Sakina S. Saadawi^{1*}, Rokaya O. Amara², Hanin N. Mughrbi¹, Bushra M. Dakhil³, Riham M. El-Moslemany⁴, Khadija O. Turkman¹, Masarra A. Daraweel¹, Khairi A. Alennabi⁵, Rabia O Alghazeer⁶

¹Pharmacognosy Department, Faculty of Pharmacy, University of Tripoli, Tripoli, Libya

²Libyan Center for Biotechnology Research, National Nanoscience and Nanotechnology Project, Tripoli, Libya

³Microbiology and Immunology Department, Faculty of Pharmacy, University of Tripoli, Tripoli, Libya

⁴Department of Pharmaceutics, Faculty of Pharmacy, Alexandria University, Alexandria, Egypt

⁵Libyan Center for Biotechnology Research, Tripoli, Libya

⁶Chemistry Department, Faculty of Science, University of Tripoli, Tripoli, Libya

*Corresponding Author: Sakina S. Saadawi

Pharmacognosy Department, Faculty of Pharmacy, University of Tripoli, Tripoli, Libya

Article History

Received: 03.02.2022

Accepted: 11.03.2022

Published: 16.03.2022

Abstract: The ever-increasing demand for natural products and biotechnology, derived from bees and ultra-modernization of various analytical devices, has facilitated the rational and planned development of biotechnology products with a focus on human health to treat chronic and neglected diseases. This study aimed to prepare, characterize and examine the stability and evaluation of the antioxidant and the antibacterial activity of Libyan propolis. Propolis Nanoparticles PNP were prepared using particle size reduction, then Transmission Electron Microscope (TEM) at a magnification of X 25000, was used for accurate evaluation of the size distribution of NPs. Three different concentration (10, 5, 2.5 mg/ml) of propolis and nano-propolis powder were tested for their 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity. The quantitative antioxidant activity test results using UV Spectrophotometer absorbance at 517 nm. The antibacterial activities of propolis and prepared nano propolis at different concentrations (10, 5 and 2.5mg/ml) were tested on bacterial strain, Klebsiella, human mouth, skin, and surface bacteria using cup cut diffusion method. The findings exhibited that the prepared propolis Nanoparticles (PNPs) were generally non-spherical with a size 100-200 nm. The PNP was a nano-sized particle around 316 nm in diameter. Zeta potential of PNP showed a negative surface charge value (- 48 mV) which was sufficiently high to avoid NPs aggregation. This value represents a stable and dispersed suspension of NPs and disables the tendency of aggregations in a short in period of time. Poly dispersity index (PDI) of synthesized PNP was used as a measurement of the size distribution. PDI values for PrNP were generally uniform with PDI 0.3 indicating monodispersity of the prepared systems. The propolis and PNPs displayed good antioxidant activity with inhibition percentage (77%, 46% and 18%) for propolis and (82%, 66% and 37%) for PNPs. Propolis nanoparticles showed to have more antibacterial effect compared to propolis. Libyan propolis nanoparticles has shown to be potential candidates as antioxidant and antibacterial agent.

Keywords: Propolis, nanopropolis, antioxidant, antimicrobial, TEM.

INTRODUCTION

Propolis or bee glue is a substance obtained from honeybees that consist of resin, wax, essential oil, and a chemical compound with a complex composition secreted by the bees (*Apis mellifera*), collected from tree buds and sap, and changed with an enzyme to seal open spaces in the hive. Propolis contains natural bioactive compounds, such as polyphenols, flavonoids, and caffeic acid, with its esters. These various chemical components confer abundant pharmacological activities on propolis, including antioxidant, antibacterial, anticancer, antifungal, anti-inflammatory, and antiviral effects [1-3]. Propolis has been widely used in alternative and traditional medicine to treat several diseases. The

Copyright © 2022 The Author(s): This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International License (CC BY-NC 4.0) which permits unrestricted use, distribution, and reproduction in any medium for non-commercial use provided the original author and source are credited.

CITATION: Sakina S. Saadawi, Rokaya O. Amara, Hanin N. Mughrbi, Bushra M. Dakhil, Riham M. El-Moslemany, Khadija O. Turkman, Masarra A. Daraweel, Khairi A. Alennabi, Rabia O Alghazeer (2022). Green Synthesis and Characterization of Libyan Propolis Nanoparticles and its Biological Activity. *South Asian Res J Pharm Sci*, 4(2): 28-35.

presence of secondary metabolites from plants like phenolic acids, phenolic esters, flavonoids, clerodanes, lupeones, propolones, and prenylated benzophenones have been identified in different propolis around the world and they are responsible for the biological activities in propolis raw material [4]. Propolis have presented diverse pharmacological activities amongst them antioxidant [5], antimicrobial [6, 7], antifungal [8, 9], antiviral [5], antiparasitic [10, 11], anti-inflammatory [5, 6], anticancer [4, 12-18] and wound healing [19, 20], but its biological activity depends on its particular chemical composition and some favorable conditions such as (bee species and its genetic variability, geographic area, biodiversity of plant species around hives, climate and seasonality, and presence of water around the hives specially in semiarid area). Propolis has an efficacy against the inhibitory effects of free radicals and as an antibacterial [21, 22]. Research study determined that the ethanol extract of propolis is 70% effective at inhibiting the growth of microorganisms. It was reported that propolis has antibacterial effects [23]. Propolis can be used as an alternative in the prevention of dental caries reducing the number and growth of *Streptococcus mutans* [24]. Another study reported that propolis can be used as an antibacterial agent for *Salmonella thipymurium* [25]. Besides, propolis has the ability to inhibit *E. coli* and *Staphylococcus aureus* [26]. Other study hypothesized that some constituents in propolis could limit the bacterial enzyme RNA polymerase ability to attach to the DNA [27]. Consequently, bacterial DNA replication does not occur. It was noticed that flavonoids and tannins are the most effective Propolis components on bacteria [28]. In addition, it was stated that the hydroxyl group of flavonoids may be able to decrease toxic effects of bacteria by changing the transport system of nutrients and structure of organic compounds [27]. Nano-propolis is a nano-sized (1–100nm in diameter). Propolis particles are prepared in form to be more effective without changing its properties by changing the size of propolis using different methods. Nano-propolis can result in better efficacy in the fields of medical science and biology [29]. Propolis does not have a good solubility in water. Nano-propolis would raise the ability to dissolve a substance achieving better solubility compared to propolis. Nano-propolis can more easily enter through the outer membrane of bacteria in order that the active antibacterial compounds can harm bacterial cell walls. Researchers stated that micro- and nano propolis might be used as antimicrobials agents or for other purposes in food or healthcare products [30]. Propolis-based nanoparticles, which are a natural material with a homogeneous distribution in the polymer matrix, were synthesized by the green nonchemical method. The low cost, nontoxic and natural materials attract the attention of nanotechnology studies. For this reason, the propolis is a green candidate for these new nanomaterials. Having many important effects, propolis dissolves in water slightly, on the other hand, NP may prove more effective by increasing the dissolvability of propolis. Studies on NP usually remain limited to studies on bacteria. As a matter of fact, in studies on *E. coli*, it has been reported that even a very small amount of NP inhibits the development of *E. coli* [31, 32]. It was reported that antimicrobial activity of NP was much more effective than propolis [29].

This study was designed for the purpose of prepare, characterize and examine the stability and evaluation of propolis nanoparticles, in addition to comparing the antioxidant and the antibacterial effect of Libyan propolis and prepared PNP.

MATERIAL AND METHODS

Propolis Sample Collection

A total of sixteen grams of raw propolis was collected in march 2021 from of honeybees located in Tripoli, Libya. Sample was kept in sterile polyethylene bag and kept in the freezer.

Extraction and Preparation of Nanopropolis

Sixteen grams of pure propolis bulk was dissolved in 100 ml of ethanol. The solution was stirred at room temperature for few hours, and the product was filtered using filter papers (Whatman, 40 Ashless, Germany) to remove impurities. The filtered solution was added at 1:10 ratio to distilled water to isolate pure propolis particles. The suspension was placed in an ultrasonic bath for 20-30 minutes to obtain PNPs. Afterwards, nano propolis was acquired in the colloid state.

Colloid nano propolis was subjected to freeze-drying (Freeze dryer) for 24 hours at -70°C. After this time, PNPs were obtained in powder form [33-35].

Characterization of Propolis Nanoparticles PNP

The morphology of Propolis Nanoparticles PNP were observed using a Transmission electron microscope (TEM), Diluted samples of PNP dispersions were negatively stained using 2% w/v aqueous uranyl acetate solution and placed on copper grids. Samples were air-dried at room temperature. TEM images were then taken at a magnification of 25,000 kV. The z-average particle size (PS), polydispersity index (PdI) and zeta potential (ZP) of Propolis Nanoparticles were measured by photon correlation spectroscopy (PCS) at a fixed angle (173°) at 25 °C using (a Zetasizer Nano ZS, Malvern Instruments, Malvern, UK) at zero time and after 2 weeks. Samples were diluted 1:30 v/v with filtered deionized water prior to measurements. Three measurements with automatic measurement duration were carried out per sample. Results are the average size, PdI and zeta measurements of at least three different samples.

Free radical scavenging activity test

Three different concentrations (10, 5, 2.5 mg/ml) of propolis and Nano propolis powder were dissolved in methanol [36], Equal amounts of samples (different concentrations) and DPPH methanolic solution (80 µg/ml) were mixed Then all samples were incubated for 30 min in dark place. Absorbance was measured using UV Spectrophotometer absorbance at 517 nm.

The percentage inhibition of DPPH radical was calculated by comparing the results of the test with those of the control (not treated with extract) using the formula [36]:

$$\text{Percentage of DPPH inhibition} = \frac{(\text{Absorbance of control} - \text{Absorbance of test})}{\text{Absorbance of control}} \times 100$$

Test was in triplicates, Vitamin C used as positive control and DPPH in Methanol used as negative control.

Antibacterial activity test

The antibacterial activities of Propolis and prepared nano propolis at different concentrations (10, 5 and 2.5mg/ml) against bacterial strain, *Klebsiella*, were determined by the agar well diffusion method [37]. A swab from oral cavity in addition to skin of hands were used to determine propolis and PNP antimicrobial effect against human mouth and skin bacteria. *Klebsiella*, oral cavity sample and hand sample were swabbed uniformly by cotton on plates containing sterile Muller Hinton Agar (MHA) and 3 wells were prepared using a cup borer with a diameter size of 6 mm. 50 µl of propolis and PNPs were poured into each well and incubated the plates for 24 hours at 37°C. Zone of inhibition (ZOI) was observed around each well with the diameter in millimeter. Gentamicin was used as a positive control.

Statistical Analysis

All measurements were repeated at least three times and the data were reported as the mean ± standard deviation (S.D.) using (IBM SPSS statistics 20). The data of DPPH assay was statistically analyzed by one-way ANOVA with Tukey's post hoc test. The bacterial activity was analyzed by independent T test. The *P* values less than 0.05 were considered as statistically significant.

RESULT AND DISCUSSION

Propolis extraction result

Propolis was obtained in three different forms (Photo 1). A clear golden methanolic solution of propolis, the second form was a white emulsion after adding water indicating the ability of propolis to work as emulsifying agent. The last sample was a white powder after freeze drying of propolis.

Characterization of propolis nanoparticles (PNPs)

Propolis Nanoparticles PNP were observed using a Transmission Electron Microscope (TEM) at a magnification of 25,000, the result was shown in (Figure 1), Propolis Nanoparticles were generally non-spherical with a size 100-200 nm.

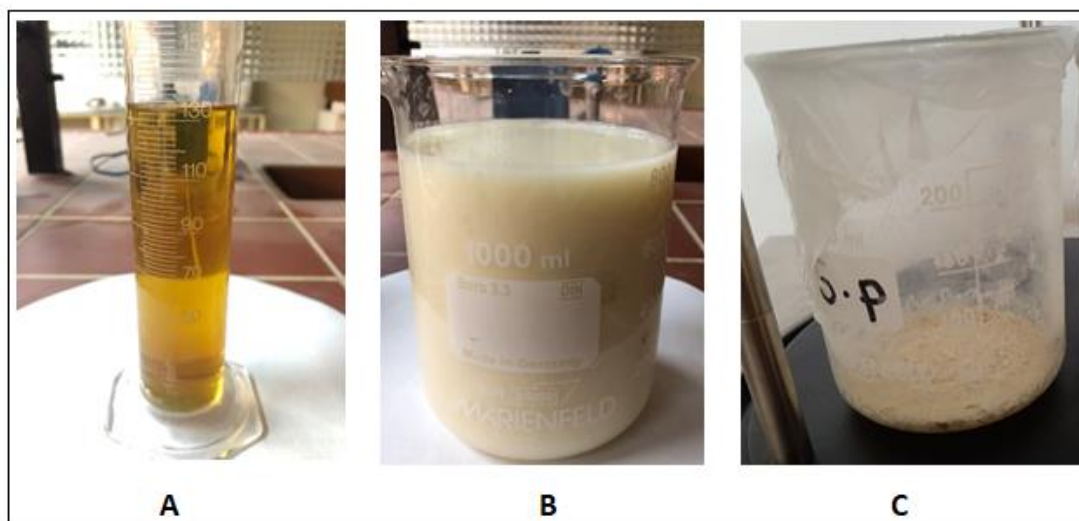


Photo 1: The different Propolis form: A: propolis methanolic solution, B: propolis emulsion, C: propolis powder

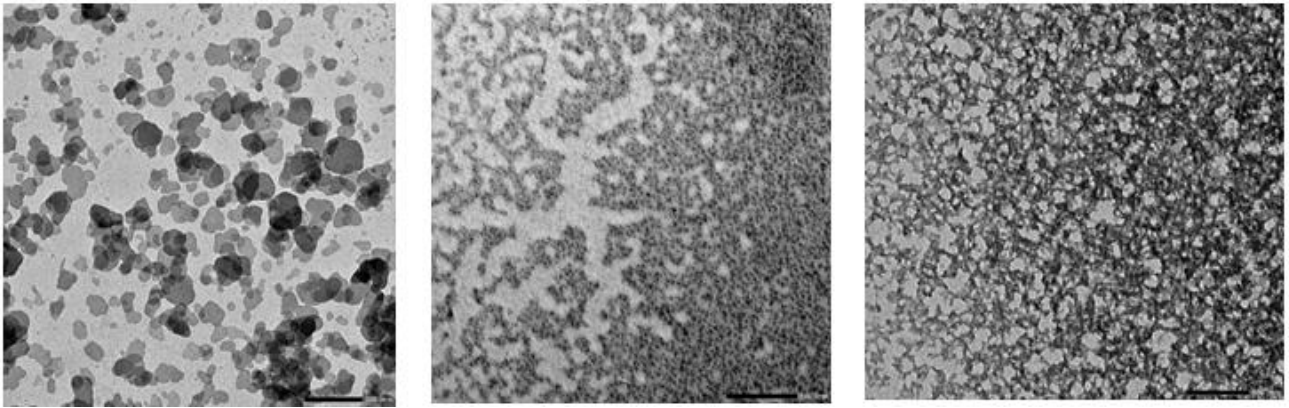


Figure 1: TEM images of Propolis Nanoparticles at X 25000 magnification. The scale bar represents 100nm and 200nm

These results were confirmed by evaluation of the size distribution of nano-propolis NPs (Figure 2) indicates the DLS (Dynamic Light Scattering) data of the PrNP at 25 °C. As shown, the PrNP was a nano-sized particle around 316 nm in diameter.

Zeta potential of PNP (Figure 3) showed a negative surface charge value (− 48 mV) which was sufficiently high to avoid NPs aggregation. This value represents a stable and dispersed suspension of NPs that there is no tendency to form aggregates in a short period of time [38].

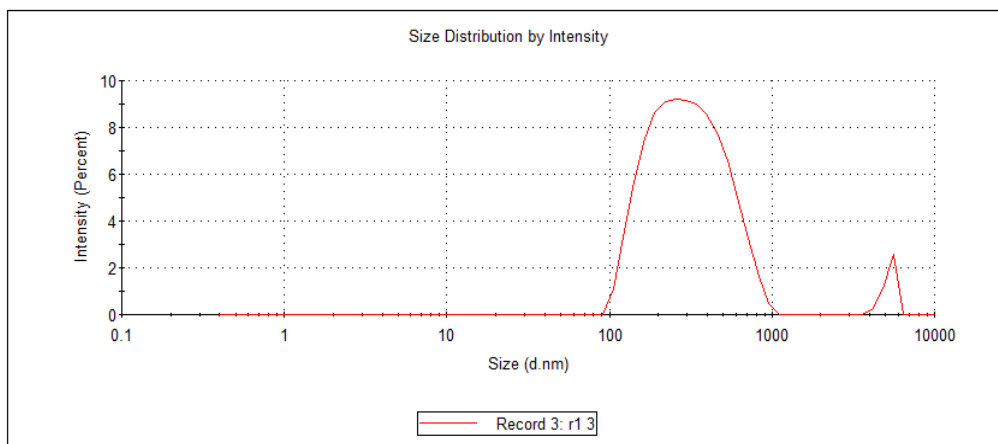


Figure 2: Size distribution by intensity curves (3 records) of Propolis Nanoparticles

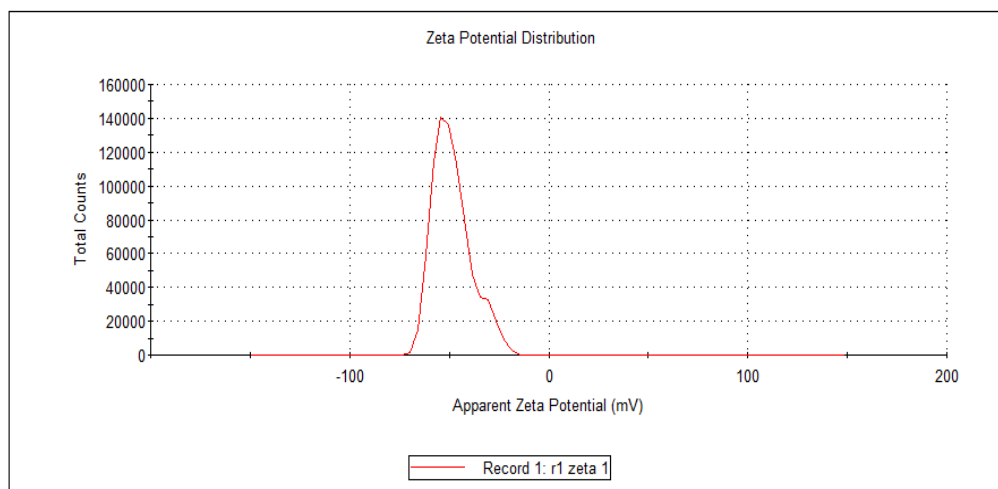


Figure 3: Zeta potential of Propolis Nanoparticles

Poly dispersity index (PdI) of synthesized PNP was used as a measurement of the size distribution. PdI values for PNP are shown in Table 1. were generally uniform with PdI 0.3 indicating mono dispersity of the prepared systems. A small value for PdI (<0.1) indicates a monodisperse size distribution while a PdI above 0.3 indicates a broad distribution [39]. The colloidal properties of the propolis nanoparticles did not change significantly after 2 weeks. There were insignificant differences in particle size, regarding size distribution, the formulation remained monodisperse with PdI<0.3 (Table 2).

Table 1: Colloidal properties of Propolis Nanoparticles

Code	Mean size nm (n=2)	PdI	ZP mV(n=2)
PrNP	325.8	0.35	-44.8

Table 2: The colloidal properties of the Propolis Nanoparticles upon storage at 4°C for 2 weeks

Code	Storage time 2 weeks	Mean size nm	PdI
PrNP	0	325.8	0.35
	2	326.8	0.259

Antioxidant activity test result

Three different concentration (10, 5, 2.5 mg/ml) of propolis and Nano propolis powder were tested for their free radical scavenging activity. DPPH is a violet-colored, stable free radical which turns yellow when gets scavenged by a scavenger typically by an antioxidant compound. Radical scavenging assay by DPPH free radical was used to evaluate and compare the antioxidant activity of P and synthesized PNPs.

Results in photo 2 showed a clear change in color from violet to yellow which was an initial indication of antioxidant activity. The color changes in concentration dependent manner.

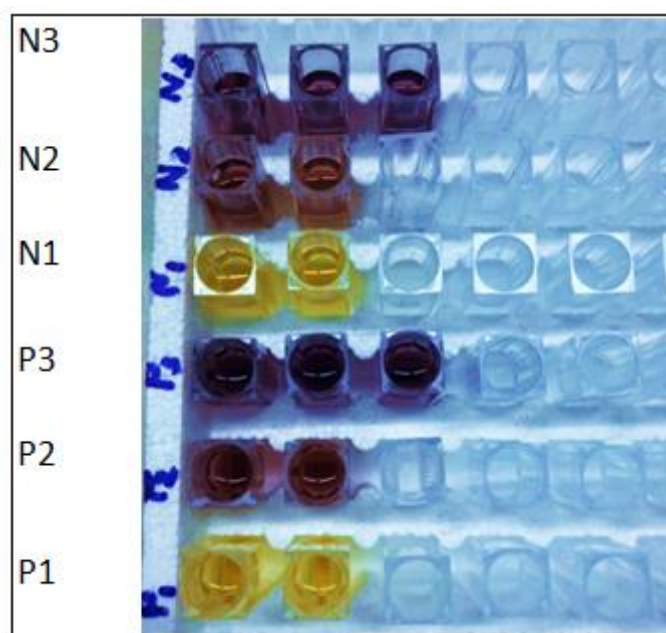


Photo 2: Free radical scavenging activity of propolis (P1=10, P2= 5 and P3=2.5 mg/ml) and nanopropolis (N1=10, N2= 5 and N3=2.5 mg/ml)

The quantitative antioxidant activity test results using UV Spectrophotometer absorbance at 517 nm were shown in Figure 4. The results confirmed the qualitative findings, that the DPPH scavenging activities is directly proportional with the increase in the concentration by ($r =98%$) for P and ($r =93%$) for PNPs. The propolis and PNPs displayed worthy antioxidant activity with inhibition percentage (77%, 46% and 18%) for propolis and (82%, 66% and 37%) for PNPs. Tukey's post hoc test revealed that the PNPs at the concentration (2.5mg/ml) exhibited a statistically significant ($P <0.05$) superior antioxidant activity than propolis. While at the concentrations (5, 10mg/ml) the DPPH scavenging activity differences between P and PNPs were not significant ($P >0.05$).

The propolis phenolics and flavonoids contents [4] play an important role to neutralize harmful free radicals in the human body. In this work. PNPs displayed a superior antioxidant activity than P. It is recommended to formulate the propolis as nano particles due to more bioavailability and distribution and consequently more efficacy.

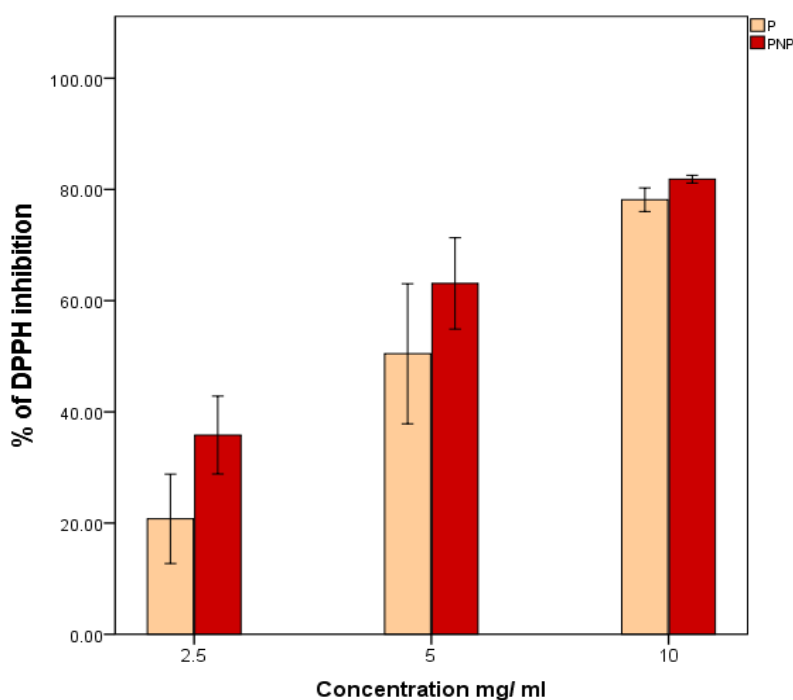


Figure 4: The percentage of free radical scavenging activity of propolis P (10, 5, 2.5mg/ml) and nano-propolis NP (10, 5, 2.5mg/ml). Error bars represent the S.D (n=3)

Antibacterial activity test result

The antibacterial activities of Propolis and prepared nano propolis at different concentrations (10, 5 and 2.5mg/ml) against bacterial strain, *Klebsiella*, oral bacteria, surface bacteria, and hand bacteria results were shown in Table 3.

In this research, PNPs revealed a significant higher ($P < 0.05$) antibacterial activity (against *Klebsiella*) in comparison to P, whereas the PNPs did not exhibit a significant ($P > 0.05$) antibacterial activity against the oral bacteria, surface bacteria, and hand bacteria. Although, there are increase in the ZOI as showed in the Table 3, but this increase was not significant. In comparison to P and PNPs, gentamicin (positive control) showed a significant superior ($P > 0.05$) antibacterial activity.

The presence of nutrients, epithelial debris, and secretions makes the mouth a favorable habitat for a great variety of bacteria. Oral bacteria include *Streptococci*, *Lactobacilli*, *Staphylococci* and *Corynebacteria* with a great number of anaerobes, especially *bacteroides* [40]. Skin provides good examples of various microenvironments *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Micrococci* and *Streptococci* [41]. The principal mechanism by which NPs exhibit antibacterial action may be through oxidative stress created by reactive oxygen species (ROS). This factor can destroy proteins and DNA in bacteria. PNPs have good stability, increased surface area, and small size, which boost faster association with microorganisms.

Table 3: Zone of inhibition by propolis and PNPs (10, 5, 2.5 mg/ml) against *Klebsiella*, oral, hand and surface bacteria

Parameter	<i>Klebsiella</i>			Oral bacteria			Hand bacteria			Surface bacteria		
	10	5	2.5	10	5	2.5	10	5	2.5	10	5	2.5
Propolis												
Zone of inhibition in mm	8	6.5	/	7.5	7	/	8	7	/	8	7	/
Nano propolis												
Zone of inhibition in mm	19	12	10	8	7	6.5	10	7	6.5	9.5	6.5	/
Gentamicin ZOI	20			21			20			22		

CONCLUSION

Nanotechnology is effective in almost every area of concern to human and animal health. Nanomedicines against various pathogens in medicine could be developed. Natural nanoproducts such as nano-propolis are useful to medicine in terms of health, performance, and reliable food production. There is a need to increase research because of

the paucity of research currently being done on this topic. In this study, it can be concluded that propolis nanoparticles are more effective than propolis as antibacterial and antioxidant agent which require more studies.

ACKNOWLEDGMENT

Authors of this study would like to present their gratitude to Faculty of Pharmacy, Alexandria University, Alexandria, Egypt and Mrs. Najat Megrahi, microbiology and immunology department, faculty of pharmacy, University of Tripoli, Libya because of their valuable assistance during this research.

REFERENCES

1. Laskar, R. A., Sk, I., Roy, N., & Begum, N. A. (2010). Antioxidant activity of Indian propolis and its chemical constituents. *Food Chemistry*, 122(1), 233-237.
2. Osés, S. M., Pascual-Maté, A., Fernández-Muiño, M. A., López-Díaz, T. M., & Sancho, M. T. (2016). Bioactive properties of honey with propolis. *Food chemistry*, 196, 1215-1223.
3. Sampietro, D. A., Vattuone, M. M. S., & Vattuone, M. A. (2016). Immunomodulatory activity of *Apis mellifera* propolis from the North of Argentina. *Lwt*, 70, 9-15.
4. de Mendonça, I. C. G., Porto, I. C. C. D. M., do Nascimento, T. G., de Souza, N. S., Oliveira, J. M. D. S., Arruda, R. E. D. S., ... & Barreto, F. S. (2015). Brazilian red propolis: phytochemical screening, antioxidant activity and effect against cancer cells. *BMC Complementary and Alternative Medicine*, 15(1), 1-12.
5. Ozan, F., Sümer, Z., Polat, Z. A., Er, K., Ozan, U., & Deger, O. (2007). Effect of mouthrinse containing propolis on oral microorganisms and human gingival fibroblasts. *European Journal of Dentistry*, 1(4), 195-201.
6. Dausch, A., Moraes, C. S., Fort, P., & Park, Y. K. (2008). Brazilian red propolis—chemical composition and botanical origin. *Evidence-based complementary and alternative medicine*, 5(4), 435-441.
7. Grenho, L., Barros, J., Ferreira, C., Santos, V. R., Monteiro, F. J., Ferraz, M. P., & Cortes, M. E. (2015). In vitro antimicrobial activity and biocompatibility of propolis containing nanohydroxyapatite. *Biomedical materials*, 10(2), 025004.
8. Dota, K. F. D., Consolaro, M. E. L., Svidzinski, T. I. E., & Bruschi, M. L. (2011). Antifungal activity of Brazilian propolis microparticles against yeasts isolated from vulvovaginal candidiasis. *Evidence-Based Complementary and Alternative Medicine*, 2011.
9. Pippi, B., Lana, A. J. D., Moraes, R. C., Güez, C. M., Machado, M., De Oliveira, L. F. S., ... & Fuentefria, A. M. (2015). In vitro evaluation of the acquisition of resistance, antifungal activity and synergism of Brazilian red propolis with antifungal drugs on *Candida* spp. *Journal of applied microbiology*, 118(4), 839-850.
10. Nina, N., Lima, B., Feresin, G. E., Giménez, A., Salamanca Capusiri, E., & Schmeda-Hirschmann, G. (2016). Antibacterial and leishmanicidal activity of Bolivian propolis. *Letters in applied microbiology*, 62(3), 290-296.
11. Pontin K, da Silva FA, Santos F, Silva M, Cunha W, Nanayakkara NP, Bastos JK, Albuquerque S (2008) In vitro and in vivo antileishmanial activities of a Brazilian green propolis extract. *Parasitol Res* 103:487–492. doi:10.1007/s00436-008-0970-z
12. Franchi, G. C., Moraes, C. S., Toret, V. C., Dausch, A., Nowill, A. E., & Park, Y. K. (2012). Comparison of effects of the ethanolic extracts of Brazilian propolis on human leukemic cells as assessed with the MTT assay. *Evidence-based complementary and alternative medicine*, 2012.
13. Li, F., Awale, S., Tezuka, Y., & Kadota, S. (2008). Cytotoxic constituents from Brazilian red propolis and their structure–activity relationship. *Bioorganic & Medicinal Chemistry*, 16(10), 5434-5440.
14. Begnini, K. R., Moura de Leon, P. M., Thurow, H., Schultze, E., Campos, V. F., Martins Rodrigues, F., ... & Seixas, F. K. (2014). Brazilian red propolis induces apoptosis-like cell death and decreases migration potential in bladder cancer cells. *Evidence-Based Complementary and Alternative Medicine*, 2014.
15. de Mendonça, I. C. G., Porto, I. C. C. D. M., do Nascimento, T. G., de Souza, N. S., Oliveira, J. M. D. S., Arruda, R. E. D. S., ... & Barreto, F. S. (2015). Brazilian red propolis: phytochemical screening, antioxidant activity and effect against cancer cells. *BMC Complementary and Alternative Medicine*, 15(1), 1-12.
16. Li, F., Awale, S., Tezuka, Y., & Kadota, S. (2008). Cytotoxic constituents from Brazilian red propolis and their structure–activity relationship. *Bioorganic & Medicinal Chemistry*, 16(10), 5434-5440.
17. Pinheiro, K. S., Ribeiro, D. R., Alves, A. V. F., Pereira-Filho, R. N., Oliveira, C. R. D., Lima, S. O., ... & Albuquerque-Júnior, R. L. C. D. (2014). Modulatory activity of Brazilian red propolis on chemically induced dermal carcinogenesis. *Acta Cirúrgica Brasileira*, 29, 111-117.
18. Begnini, K. R., Moura de Leon, P. M., Thurow, H., Schultze, E., Campos, V. F., Martins Rodrigues, F., ... & Seixas, F. K. (2014). Brazilian red propolis induces apoptosis-like cell death and decreases migration potential in bladder cancer cells. *Evidence-Based Complementary and Alternative Medicine*, 2014, 639856.
19. Olczyk, P., Wisowski, G., Komosinska-Vassev, K., Stojko, J., Klimek, K., Olczyk, M., & Kozma, E. M. (2013). Propolis modifies collagen types I and III accumulation in the matrix of burnt tissue. *Evidence-Based Complementary and Alternative Medicine*, 2013, 423809.

20. Olczyk, P., Komosinska-Vassev, K., Winsz-Szczotka, K., Stojko, J., Klimek, K., & Kozma, E. M. (2013). Propolis induces chondroitin/dermatan sulphate and hyaluronic acid accumulation in the skin of burned wound. *Evidence-Based Complementary and Alternative Medicine*, 2013, 290675.
21. Hasan, A. E. Z., Ambarsari, L., Widjaja, W. K., & Prasetyo, R. (2014). Potency of nanopropolis stingless bee *Trigona* spp Indonesia as antibacterial agent. *IOSR Journal of Pharmacy*, 4(12), 1-9.
22. Schmidt, E. M., Stock, D., Chada, F. J. G., Finger, D., Christine Helena Frankland Sawaya, A., Eberlin, M. N., ... & Torres, Y. R. (2014). A comparison between characterization and biological properties of Brazilian fresh and aged propolis. *BioMed Research International*, 2014, 257617.
23. Barud, H. D. S., de Araújo Júnior, A. M., Saska, S., Mestieri, L. B., Campos, J. A. D. B., De Freitas, R. M., ... & Berretta, A. A. (2013). Antimicrobial Brazilian propolis (EPP-AF) containing biocellulose membranes as promising biomaterial for skin wound healing. *Evidence-based complementary and alternative medicine*, 2013, 703024.
24. Dzedzic, A., Kubina, R., Wojtyczka, R. D., Kabała-Dzik, A., Tanasiewicz, M., & Morawiec, T. (2013). The antibacterial effect of ethanol extract of polish propolis on mutans streptococci and lactobacilli isolated from saliva. *Evidence-Based Complementary and Alternative Medicine*, 2013, 681891.
25. Hasan, A. E. Z., Artika, I. M., Fatoni, A., Kuswandi., & Haryanto, B. (2011). Antibacterial activity of propolis *Trigona* spp. From Bukittinggi West Sumatera against *Salmonella* sp. *Chem Prog*, 4, 55-59.
26. Popova, M., Dimitrova, R., Al-Lawati, H. T., Tsvetkova, I., Najdenski, H., & Bankova, V. (2013). Omani propolis: chemical profiling, antibacterial activity and new propolis plant sources. *Chemistry Central Journal*, 7(1), 1-8.
27. Sabir, A., Gigi, B. K., & Kekokterun, F. (2005). The inflammatory response on rat dental pulp following ethanolic extract of propolis (EEP) application. *Maj Ked Gigi (Dent J)*, 38, 77-83.
28. Fatoni, A. (2008). Effect of propolis *Trigona* spp. *Bukittinggi against several bacterial the small intestine of cattle and searches active component*. Bogor (Indonesia): Bogor Agricultural University.
29. Afrouzan, H., Amirinia, C., Mirhadi, S. A., Ebadollahi, A., Vaseji, N., & Tahmasbi, G. (2012). Evaluation of antimicrobial activity of propolis and nanopropolis against *Staphylococcus aureus* and *Candida albicans*. *African Journal of Microbiology Research*, 6(2), 421-425.
30. Sahlan, M. U. H. A. M. A. D., & Supardi, T. O. N. Y. (2013). Encapsulation of Indonesian propolis by casein micelle. *Int. J. Pharma Bio Sci*, 4(1), 297-305.
31. Prasetyo, R., Hasan, A. E. Z., & Siregar, R. (2011). Application of nanoparticle technology *Trigona* spp propolis from Bogor as an antibacterial *Escherichia coli* in vitro. *Ekol*, 11, 36-43.
32. Hasan, A. E. Z., Artika, I. M., Fahri, V. R., & Sari, N. (2012). Application of nanoparticle technology for medicine preparate (Antibiotic base on natural product, Propolis *Trigona* spp.). *Chem. Prog*, 5, 1-7.
33. Sawicka, D., Car, H., Borawska, M. H., & Nikliński, J. (2012). The anticancer activity of propolis. *Folia Histochemica et Cytobiologica*, 50(1), 25-37.
34. Todd, M. A., Staley, R. N., Kanellis, M. J., Donly, K. J., & Wefel, J. S. (1999). Effect of a fluoride varnish on demineralization adjacent to orthodontic brackets. *American journal of orthodontics and dentofacial orthopedics*, 116(2), 159-167.
35. Ahn, S. J., Lee, S. J., Kook, J. K., & Lim, B. S. (2009). Experimental antimicrobial orthodontic adhesives using nanofillers and silver nanoparticles. *Dental Materials*, 25(2), 206-213.
36. Kumarasamy, Y., Byres, M., Cox, P. J., Jaspars, M., Nahar, L., & Sarker, S. D. (2007). Screening seeds of some Scottish plants for free radical scavenging activity. *Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives*, 21(7), 615-621.
37. Naika, H. R., Lingaraju, K., Manjunath, K., Kumar, D., Nagaraju, G., Suresh, D., & Nagabhushana, H. (2015). Green synthesis of CuO nanoparticles using *Gloriosa superba* L. extract and their antibacterial activity. *Journal of Taibah University for Science*, 9(1), 7-12.
38. Afrasiabi, S., Pourhajbagher, M., Chiniforush, N., & Bahador, A. (2020). Propolis nanoparticle enhances the potency of antimicrobial photodynamic therapy against *Streptococcus mutans* in a synergistic manner. *Scientific Reports*, 10(1), 1-16.
39. Qiao, H., Chen, L., Rui, T., Wang, J., Chen, T., Fu, T., ... & Di, L. (2017). Fabrication and in vitro/in vivo evaluation of amorphous andrographolide nanosuspensions stabilized by d- α -tocopheryl polyethylene glycol 1000 succinate/sodium lauryl sulfate. *International Journal of Nanomedicine*, 12, 1033-1046.
40. Kenneth, T. (2015). The Normal Bacterial Flora of Humans. University of Wisconsin Department of Bacteriology. www.textbookofbacteriology.net
41. Baron, S. (1996). Medical Microbiology. 4th edition, Chapter 6: Normal Flora. Galveston (TX): University of Texas Medical Branch at Galveston.