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Effect of the geographical origin on antibacterial and antifungal activity of Egyptian propolis as a natural product of honeybee colonies

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Abstract

Representative samples of propolis were collected from honeybee colonies established in four geographical regions of Egypt. Propolis ethanol extracts (PEE) were prepared in five concentrations (50, 100, 150, 200 and 250 ppm). The un-fractionated extracts were then used to find out their antimicrobial activities on eleven species of micro-organisms. The fungus *Aspergillus niger* was more resistant to the first three concentrations of propolis ethanol extracts (PEE) of all samples. Yeast, *S. cerevisiae* was more tolerant to the action of PEE than *Candida albicans. Staphylococcus aureus*, as a pathogenic bacterium, was found to be more sensitive towards PEE than *Actenomyces bovis*. The same trend was observed for the two thermophilic bacteria, (*Streptococcus thermophilus* and *Mycobacerium phlei*). Sporeforming *Bacillus subtilis, Lactobacillus casei*, *Sarcina* spp. and the Gram-negative *Escherichia coli*, were strongly affected by all PEE. A clear effect of the geographical origin of propolis was distinguished where the efficiency evaluation could be arranged according to its geographical origin as follows; North Delta, South Delta 2, East Delta, South Delta 1 and West Delta, respectively. In general, the results appeared the strong antimicrobial impact of the tested PEE samples obtained under Egyptian conditions.

Introduction

Propolis is a resinous natural product collected by honeybee workers from buds of different plants and trees. Bee workers gather and transport propolis as a pellet in their corbiculae after being moistened with the secretion of their salivary glands, (Ghisalberti, 1979 and Bankova *et al*., 2000). Honeybees use propolis in various purposes inside the nest such as closing hive cracks against wind and rain, smoothing out the internal walls and as a protective barrier against external invaders like snakes, lizards, and so forth, or for embalming big enemy corpses to preserve it from decay or subsequent moldiness (Lavie, 1978 and Burdock, 1998). Results of antimicrobial activities of propolis extracts against a wide range of bacterial strains and species, (Burdock, 1998; Santos *et al.*, 2002; Hendi *et al*., 2011 and Molna´r *et al*., 2017), yeasts, (Shub *et al*.,1978; Pepeljnjak *et al*.,1982; Kujumgiev *et al*.,1999; Sidra, 2010 and Bankova *et al*., 2016) and fungi, (Dziedzic

et al., 2013; Gavanji and Larki, 2017; Przybyłek and Karpi´nski, 2019 and Petruzzi *et al*., 2020) attained various efficiencies in different studies.

Many researchers such as Kujumgiev *et al.* (1999); Osés *et al*. (2016); Molna´r *et al.* (2017); AL-Ani *et al*. (2018); Kocot *et al*. (2018); Touzani *et al*. (2018 and 2021) and Mountford-McAuley *et al.* (2021) proved that the bioactivity of propolis extraction against wide range of microorganisms varied according to several factors mainly geographic origin that related to the changes in the environmental conditions. They also agreed that the essential principal compounds responsible for biological activities are polyphenols, aromatic acids, and di-terpenic acids, but very few different propolis types have been different in their main bioactive compounds. Different composition is also related to botanical sources of the region or even the season, as well as the production methods, processing, storage conditions and the types of microorganism (Haggag *et al*., 2006; Kumer *et al.*, 2008; Ophori *et al*., 2010; Dziedzic *et al*., 2013; Boisard *et al*., 2015; Woo *et al*., 2015 and Petruzzi *et al*., 2020).

The aim of this work is to study the efficiency of propolis samples, collected from different geographical regions and locations in Nile Delta of Egypt, towards eleven species of microorganisms. These microorganisms were selected based on their potential as spoilage organisms causing undesirable changes in food, their industrial importance and on their pathogenic activity.

Materials and methods

1. Gathering of propolis samples:

Five propolis samples were collected from the hybrid carniolan honeybee colonies housed in Langstroth hives and established in four different geographical regions in Egypt. These regions were: (a) South Delta which represented by two locations in Giza governorate,(Samples no. 1 from the apiary of Agricultural Experimental Station, Faculty of Agriculture, Cairo University and sample no. 2 from apiary established at Shabrament village about 25 Km. far from Giza city); (b) North Delta represented by one sample from El-Manzala province, Dakahlyia Governorate,(apiary located in the station of Agriculture Research Center). The region (c)was the East Delta which represented by Port Said Governorate, (location of Bahr El-Baker) and (d) was the West Delta that represented by one propolis sample collected from apiary established at new Nubaria, Nubaria Province, Behera governorate.

To obtain a good quality of propolis, the samples were gathered during summer season (June-August, 2020) as follows: (1) by scraping the small pieces of propolis presented above the hive frames and that stocked with the inner burlap cover, (Muszynska *et al*., 1983), (2) by using propolis trap which consisted of small pieces of hard wood,(Diameter of 20*20*3 mm.) fixed under the north side of the brood chamber with space of 3mm. between each of two successive pieces according to Mizis (1978). The yield of propolis was harvested at ten days intervals and each sample was preserved in plastic bag at 4 ±1 ºC until use.

2. Propolis extraction:

Ten grams from each crude propolis sample were prepared in fine slides and added to 100 mL of 96% ethyl alcohol (in a ratio of 1:10 w/v). Each solution was kept under agitation (200 rpm. using an orbital shaker for 8 hours daily) at ambient temperature (25 ± 1 ºC) for 3 days. After this time, the solution was filtered through Whatman No. 1 filter paper. After extraction, all propolis extracts were stored in amber flasks and evaporated in a water bath at temperature of 50±2 ºC until ethanol evaporated. The number of residual solids of each sample on filter paper weighed and subtracted from the weight of crude sample to obtain the real extracted mass, so the determination of required concentrations

from stock solution of each propolis sample was occurred, (Trusheva *et al*., 2007 and Pobiega *et al*.,2019).

3. Determination of antimicrobial activity of propolis ethanol extract (PEE):

3.1. The tested microbes:

The eleven tested microbial cultures were: (a) *Escherichia coli* as a Gram-negative bacteria;(b) *Bacillus subtilis, Lactobacillus casei, Sarcina* spp., *Streptococcus thermophilus* and *Mycobacerium phlei* as Gram-positive bacteria; (c) *Staphylococcus aureus* and *Actenomyces bovis* as pathogenic strains;(d) *Saccharomyces cerevisiae* and *Candida albicans* as cultures of yeasts and (e) one culture of fungi (*Aspergillus niger*).

All strains or species of these microorganisms were kindly obtained from "MIRCEN, Ain Shams University, Qalubiya, Egypt". All cultures were maintained on nutrient agar slants, of PH 7 while yeasts and fungus on potato-dextrose agar (PDA) slants.

3.2. Disc-diffusion method:

Antimicrobial activities of PEE were determined with a disc-diffusion method **(**Gavanji and Larki, 2017). One gram of each extracted propolis sample were dissolved in 100 mL of 80% ethanol.by shacking for 30 min. then filtered in a dark flask. Therefore, the final concentration for each sample was 10 mg / ml of ethanolic alcohol where the following five concentrations, (250,200,150,100 and 50 ppm) were prepared through serial dilutions to assay against the selected microorganisms. Sterile discs of Whatman paper No.1 (6 mm diameter) were individually impregnated with 30 µL of each PEE sample and ethanol (96 %) as control. Suspensions of tested bacteria were spread evenly on the surface of MHA, (Muller-Hinton agar medium), plates, and yeasts or mold spore suspensions on SDA plates. After 5 min, discs with PEE were placed on the surfaces of the inoculated plates. The plates with bacteria were incubated at 37 ºC for 24 hrs., (Or at 50ºC. in the case of thermophilic bacteria). Yeast plates were kept at 28 ºC. for 48 and those with mold at 25 ºC. for 72 hrs., (Standards Institute Clinical Laboratory (SICL), 2006 and Gavanji and Larki, 2017). After incubation, the diameters of inhibition zones (Included disc diameter) were measured using a ruler and expressed to the nearest millimeter after subtracting the effect of control. All tests were performed in triplicate and a new, inoculums were prepared for each replicate, and the standard deviations were determined.

4. Statistical analysis:

Statistical tests were performed using the MSTAT version 6.4 computer program. Two-way analysis of variance was carried out. The significance of differences between mean values was assessed using the DMRT at a significance level of 0.05.

Results and discussion

1. The antimicrobial effect of propolis ethanol extract (PEE) in different concentrations:

The antimicrobial effect of PEE in five concentrations was illustrated in Figures 1 – 5

1.1. Effect on Gram-negative and Gram – positive bacteria:

The obtained results showed that Gram – negative bacteria,(*E. coli*), was relatively more tolerant to PEE than *B. subtilis , L. casei* and *Sarcina* spp. as Gram-positive bacteria. However, they suffer in different categories from the inhibitory influence of PEE as shown in Figure 1(A – D). The ranges of inhibition zones for concentrations higher than 150 ppm were 22 – 48 mm, 31 – 63 mm, 22 – 69 mm and 30 – 53 mm, for the four mentioned bacteria, respectively. These inhibition zones reflected the strong suppression of propolis as an antibacterial substance.

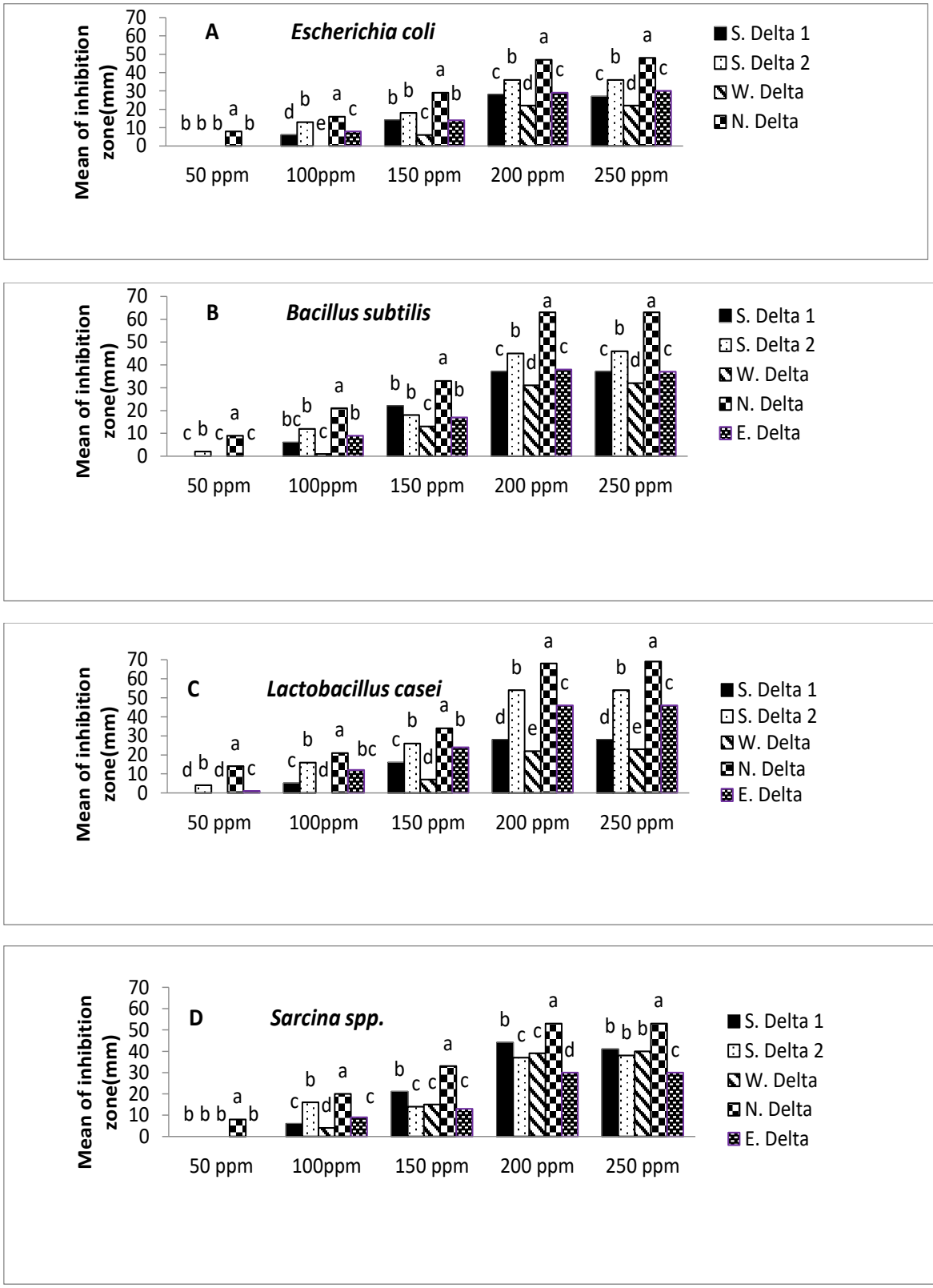

Figure (1): Effect of the geographic origin on the bioactivity of propolis against Gram negative (A= *E. coli*) and Gram positive bacteria (*B= B. subtilis; C= L. casei* and D= *Sarcina* spp.). Columns with the same small letter in the same PEE concentration do not.

1.2. Effect on pathogenic bacteria:

S. aureus as a pathogenic bacterium was found to be more sensitive towards PEE than *A. bovis*. The inhibition zones ranged between 43 – 66 mm. and 12 – 37 mm. in presence of PEE concentrations ranged between 200 - 250 ppm, as illustrated in Figure 2(A and B), respectively.

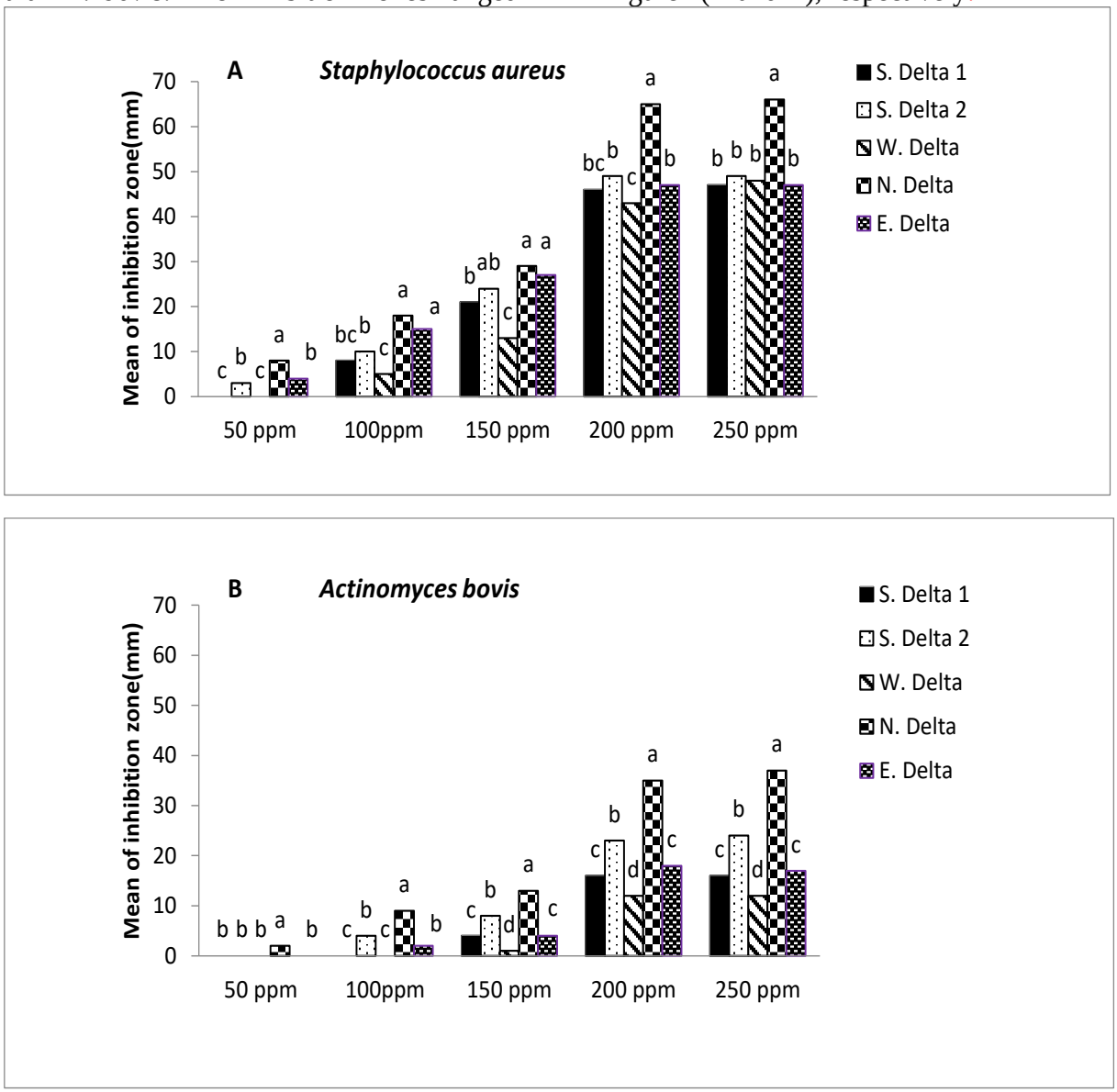

Figure (2): Effect of the geographic origin on the bioactivity of propolis against certain pathogenic bacteria (A= *S. aureus and B= A. bovis*). Columns with the same small letter in the same PEE concentration do not significantly differ according to DMRT at 0.05 probability.

3.1. Effect on thermophilic bacteria:

The effect of PEE on *S. thermophilus* and *M. phlei* as thermophilic bacteria took the same trend as in pathogenic ones. The first microbe was more sensitive (The inhibition zones ranged from 0 - 60 mm.) than the second ones (the inhibition zones ranged from 0 – 37 mm.), respectively as shown in Figure 3 (A and B).

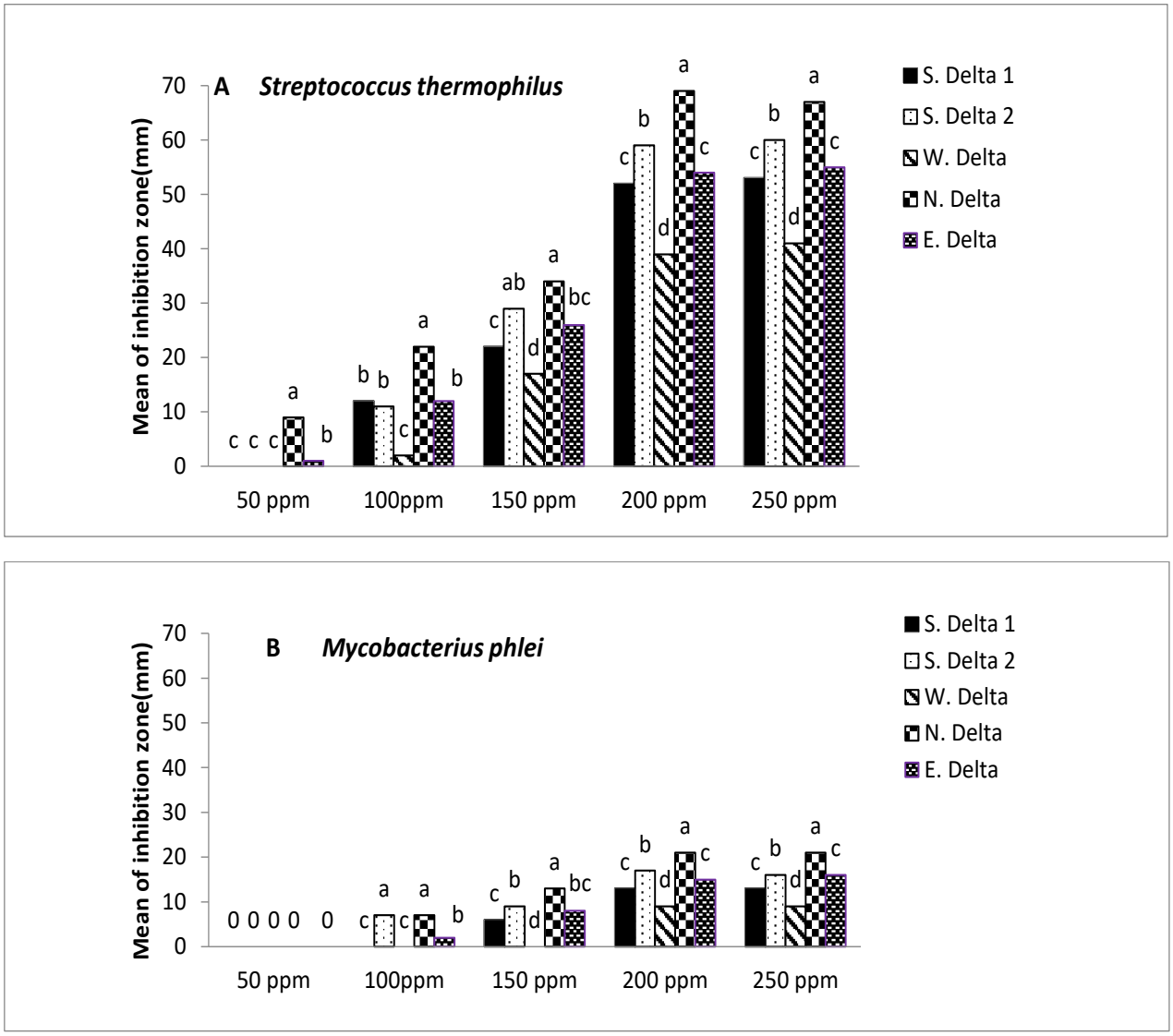

Figure (3): Effect of the geographic origin on the bioactivity of propolis against certain thermophlic bacteria (A= *S. thermophilus and B= M. phlei*). Columns with the same small letter in the same PEE concentration do not significantly differ according to D.

In these circumstances, it is demonstrated that the efficiency of PEE as antimicrobial natural substance against most microorganisms was positively correlated with its concentrations. It is obvious that the Gram negative and Gram-positive bacteria, being spore forms, (Thermophilic or pathogenic bacteria), were significantly affected by, in vitro, the unfractionated PEE. These antimicrobial activity results are consistent with other previously experiments conducted on antibacterial activity of propolis, (Grange and Davey,1990; Burdock, 1998; Park *et al*., 1998 and Molna´r *et al*.,2017). Santos *et al.* (2002) and Kumar *et al.* (2008) proved that EEP has antimicrobial activity against Gram-positive bacteria, but lower activity against Gram-negative ones. Hendi *et al.* (2011) observed that ethanolic extract of Al-Museiab propolis, (EEMP), had a bacteriostatic effect on *S. aureus* and other bacterial species and its effects were elevated when the concentration increased to 20% and 30%. They also, found that the Gram – positive bacteria was comparatively more sensitive to propolis activity than Gram – negative ones, but the standard *E. coli* strain was highly sensitive to EEMP than other

Gram negative bacteria. In the same trend, Touzani *et al.* (2021) found that the Minimum Inhibitory Concentrations (MICs) values ranged from 0.31 to 2.50 mg/mL for Gram-negative bacterial strains and from 0.09 to 0.125 mg/mL for Gram-positive bacterial strains.

4.1. Effect on yeasts:

S. cerevisiae was more tolerant to the action of propolis than *C. albicans.* The respective inhibition zones ranged from 3 – 15 mm. and from 1 – 25 mm. at the tested concentrations, Figure 4 (A and B).

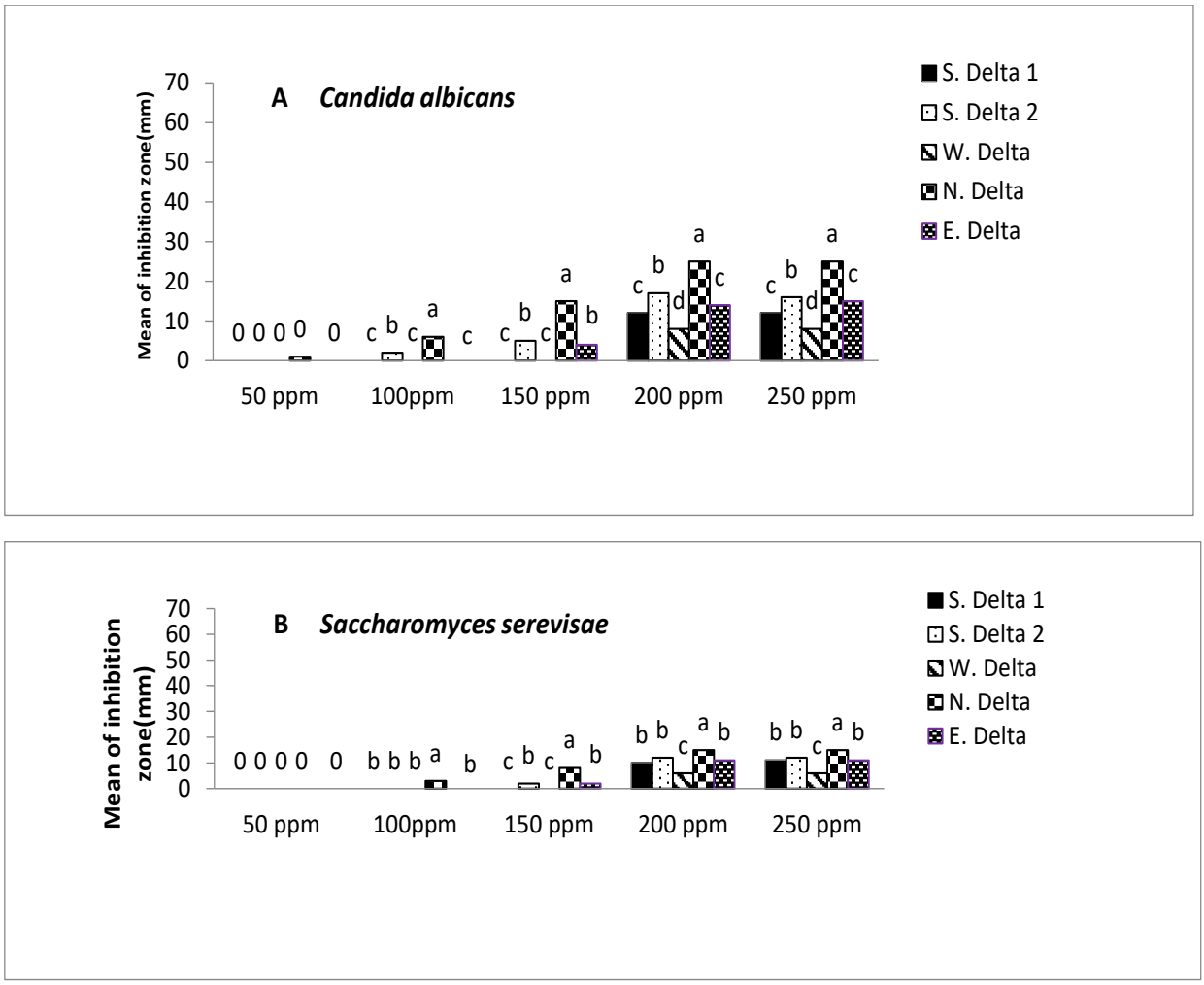

Figure (4): Effect of the geographic origin on the bioactivity of propolis against certain yeasts, (A= *C. albicans and B= S. serevisae*). Columns with the same small letter in the same PEE concentration do not significantly differ according to DMRT at 0.05 probability.

1.5. Effect on mold *Aspergillus niger:*

Values illustrated in Figure (5) appeared that the fungus *A. niger* was more resistant to the first three concentrations of PEE in all examined geographical propolis samples. Little inhibitory reaction (Ranged from 2 – 9 mm.) was observed for the two higher concentrations, (200 and 250 ppm). Concerning the anti-yeasts and mold effect, results appeared that they were more tolerant, (For yeasts) or resistant, (For mold) to the lower concentrations of PEE until 150 ppm. However, low inhibition zones do not exceed 25 mm for *C. albicans*, 15 mm for *S. serevisiae* and 9 mm for *A. niger* were attained at the highest tested concentration, (250 ppm).

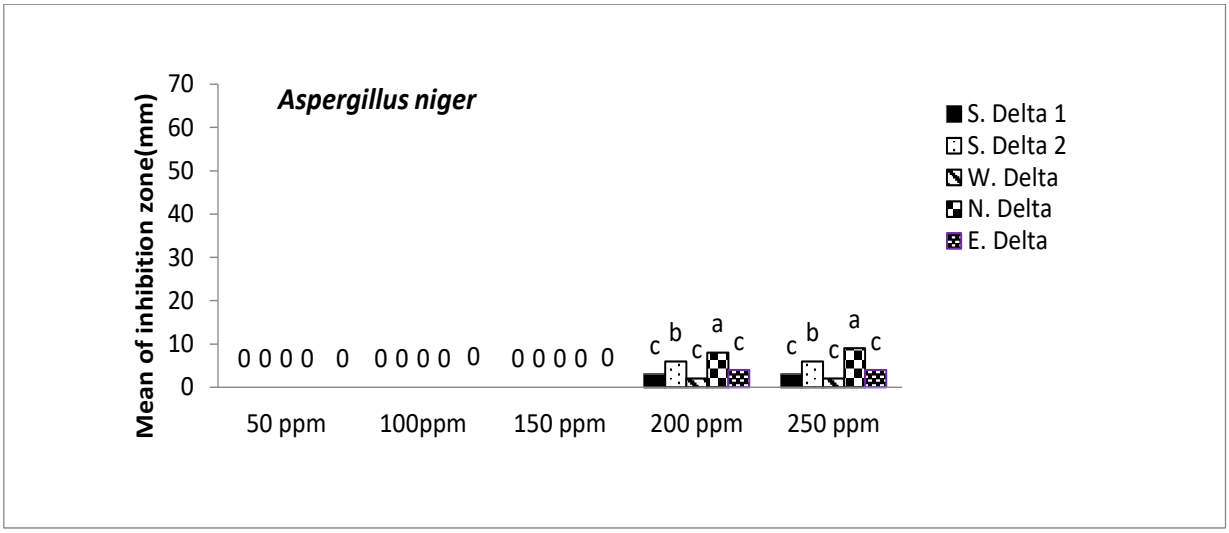

Figure (5): Effect of the geographic origin on the bioactivity of propolis against the fungus of *Aspergillus niger*. Columns with the same small letter in the same PEE concentration do not significantly differ according to DMRT at 0.05 probability.

These findings are in agreement with Ashour (1989) who found that the yeasts of *C. albicans, C. tropicalis, C. utilis* and *S. cerevisiae* were influenced by the high concentrations of propolis ethanol extracts, (4.36%), where the zones of inhibition reached 11, 9, 8 and 7mm., respectively. The preceding studies by Shub *et al.* (1978) confirmed the present findings where they reported that propolis at rather high concentrations of 125 – 500 mg / L. does not inhibit C. albicans even at concentrations above 1000 mg. / L. Kumar *et al.* (2008) registered moderate zone of inhibition for *C. albicans* but not against *A. niger* at concentration of 200 ppm. of PEE. De Castro *et al.* (2011) suggested that 1250 ppm propolis could be an adequate choice as a sub-inhibitory concentration for *S. cerevisiae*.

Fungi appeared to be more resistant against the tested concentrations of PEE and may need higher levels to induce strong inhibition. The findings of many researchers confirmed this conclusion where Pepeljnjak *et al.* (1982) found that concentrations of 1500 – 3000 µg / ml. from pure propolis extracts were needed to inhibit the growth of *C. albicans* and A. flavus. Ôzcan (1999) reported that the concentration of 4% of propolis extract can inhibit *F. oxysporum f.* sp. *melonis* by 50%. In another study, Shehu *et al.* (2016), noticed that 5 mg/mL (5000 ppm) of EEP completely inhibited the radial growth of *F. oxysporum* on solid media (PDA). Petruzzi *et al.* (2020) found that 3000 ppm of PEE are needed to delay *F. oxysporum* growth where they attained a half of the maximum diameter on PDA medium after 8.29 ± 0.09 days.

The effectiveness of propolis against microorganisms attributed to various causes such as inhibition of cell division, protein synthesis and bacterial motility, disruption of cell walls, enzyme inactivation, and bacteriolysis (Fernandes Júnior *et al*., 2005 and Takaisi-Kikuni and Schilcher, 1994). The polyphenols of propolis affect microbial proteins by forming hydrogen and ionic bonds (Wink, 2015). In addition, the inhibition of Gram- positive and Gram – negative bacteria by propolis has been ascribed to phenolic esters and flavonoids (Pinocembrin, galangin and their derivatives) as reported by Boisard *et al.* (2015) and Touzani *et al.* (2018).

2. Effect of geographical origin on the antimicrobial activity of propolis ethanol extract (PEE):

Concerning the role of the geographical origin of propolis samples on their efficiency as antimicrobial substance, data illustrated in Figures (1 - 5) revealed that propolis of North Delta region, (The sample collected from Dakahlia Governorate) exceeded those from other regions against all the tested microbes. Moreover, such activity differed among propolis samples collected from various locations in the same region such as South Delta 1 and 2. The best action of the lowest PEE concentration was recorded for North Delta sample. Therefore, the efficiency of PEE under evaluation could be arranged according to its geographical origin as follows; North Delta, (Dekahlia Governorate); South Delta 2,(Location of Faculty of Agriculture, Giza Governorate); East Delta, (Port Said Governorate); South Delta 1,(Location of Shabrament village, Giza Governorate) and West Delta,(Reclaimed lands at Nubaria province, El-Behera Governorate), respectively.

Results of geographical effects on the quality of propolis were agreed with those of many authors who stated that the antimicrobial activity of propolis samples differ according to their origin, (Geographical and consequently botanical origin). These natural factors strongly affect the chemical components of this substance. Shub *et al*. (1978) observed that the antimicrobial activity of alcoholic extracts of propolis collected in 18 regions of Russia varied from high active (From Odessa, which inhibited *S. aureus* at 125 ppm and *C. albicans* at 250 ppm.) to the low active (From Moscow and Crimea, which inhibited the *S. aureus* at 1000 ppm. Meresta and Meresta (1983), in Poland, found that the minimal inhibitory concentration (MIC) of 149 propolis samples as such ranged from 60 – 430 ppm., minimal bactericidal concentration, (MBC) of their extracts ranged from 110 – 1380 ppm. Fawzy and Al-Deeb (2016) evaluated four different types of propolis, (Saudi, Turkish, Chinese, and Egyptian) in different concentrations (From 1.25% to 10%) against various species of Gram-negative and Gram-positive bacteria. They showed that there was highly significant effect of Saudi, Egyptian and Turkish than Chinese propolis on tested bacteria. They concluded that the antibacterial activity of propolis was concentration depends and depends upon its botanical origin. Dziedzic *et al.* (2013) reported for seventeen samples of propolis collected from south of Poland that the mean MIC and MBC values of EEP were 0.025 mg/ml and 25 mg/ml, for the mutants streptococci group bacteria,0.7 mg/ml and1.10 mg/ml for *Lactobacillus* spp., respectively. Many researchers proved that the variance in propolis composition is related, beside botanical sources of the region, to the season, the production methods, processing, as well as storage conditions, (Kujumgiev *et al*.,1999; Sforcin *et al*.,2000; Park *et al*., 2002;Kartal *et al*., 2003; Osés *et al*., 2016; Molna´r *et al*., 2017; AL-Ani *et al*., 2018; Kocot *et al*., 2018 and Mountford-Mc Auley *et al*., 2021).

The results showed that the potential capacities of various propolis samples are not similar against the tested microbes, and this may be due to their different compositions depending on region or area of collection and its predominant plants. This conclusion is confirmed by the findings of Touzani *et al.* (2018 and 2021) who reported that various propolis samples collected from different regions, or even from the same region, have different compositions, and they are different in their antimicrobial activity.

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