Synthesis and Characterization of Nanoparticles Propolis Using Beeswax

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ABSTRACT: *In order to protection, convenient release, increase of antibacterial of capsules to treatment of diseases, propolis nanoparticles encapsulate. Beeswax is used for covering because of its special physical and chemical properties, ineffective and inactivity and ease of mixing with materials without any adverse reaction. In this study, nanotechnology and renewable natural compounds of beeswax were used in the process of encapsulating for protection against adverse environmental conditions. At first, propolis nanoparticles were mixed with chloroform then ammonia buffer and Tween -80 was added to it while stirring with speed rpm 300. The mixture was shocked to form the capsule. After filtration and washing produced capsules were dried for 48 hours at room temperature. Assessment of formation and performance of the capsules was done by changing parameters such as pH, time and temperature, the loading of nanoparticles by spectrophotometry method and increasing the antimicrobial properties using microbial culture. Also, FT-IR analysis was done to prove physical transplant of wax and propolis. According to TEM images, the size of produced capsules was estimated in the range of 200 to 500 nm with 95% distribution percentages. Based on Taguchi testing, the optimum time, temperature and pH for release of encapsulated nanoparticles were 10 minutes, 43ºC and 10, respectively.*

KEYWORDS: *Encapsulating; Propolis Nanoparticles; Capsule; Beeswax; Spectrophotometry*.

INTRODUCTION

Beeswax is a natural biological polymer [1] containing a mixture of several non-toxic and cheap substances (esters of fatty acids, alcohols, acids, etc.). The number of reported individual components have been contained beeswax exceeds 300 which are from various species of honeybees. Depending on the honeybee species and the geographical zone, the concentrations of individual components and substance classes may have only small differences [2]. In addition, from the point of view of chemistry, it is a stable and water-repellent substance [3]. Beeswax is a highly crystalline natural product that is used in pharmaceutical, cosmetics, food and other

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industries. It is also frequently used in the preparation of controlled release drug preparations [4]. In recent years, waxes have appropriate physical properties to prepare gastro resistant, biocompatible, biodegradable microspheres to release the entrapped drug in the intestinal lumen [5].

Propolis is a mixture of beeswax and resins gathered by the honeybee from plant buds, leaves, and exudates [6]. Bees use it like glue, a general-purpose sealer, and as draught-extruder for beehives [7]. Bees use the mechanical properties of this resinous substance applying it to blocking holes and cracks, fixing combs, strengthening the thin borders of the comb. On the other hand, they make use of its biological action: bee glue contains the putrefaction of "embalmed" intruders, killed in the hive which is too large to be carried out and is responsible for incidence lower microorganisms within the hive than in outside of the atmosphere [8].

Propolis is a bee product from plant origin, thus the source plants might vary at different geographic locations with respect to the local flora [6-9]. The propolis has a quite complicated chemical composition [7] and contains mainly waxes, resin, and volatiles. The main chemical groups of propolis resin comprise phenolic acids or their esters, flavonoids (flavones, flavanones, flavonols, dihydroflavonols, and chalcones), terpenes, aromatic aldehydes and alcohols, fatty acids, stilbenesand b-steroids [10, 11]. More than 300 compounds such as polyphenols, phenolic aldehydes, sequiterpene quinines, coumarins, amino acids, steroids, and inorganic compounds have been observed in propolis samples [7]. Propolis also contains several minerals like Mg, Ca, I, K, Na, Cu, Zn, Mn, and Fe as well as some vitamins such as B1, B2, B6, C and E, and a number of fatty acids [7].

Propolis has long been used in oriental folk medicine to curing infections; in European ethno-pharmacology also it is used as an antiseptic and anti-inflammatory agent to healing wounds and burnings. Propolis presents an array of biological and pharmacological features. immunomodulatory, antitumor, anti-inflammatory, antioxidant, antibacterial, antiviral, antifungal, antiparasite activities,[12-18] antibacterial, antifungal, antiviral, antiprotozoan, anti-inflammatory, antioxidant, hepatoprotective, immunostimulating, antitumor, and cytostatic activities are some properties have been reported for propolis; hence, it is recognized as a useful substance in medicine [6-8, 11, 19-21].

Today propolis is widely used in foods and drinks to maintain or enhance human health [7]. No side-effects have been seen in propolis administration for humans or rats [22, 23], it could potentially be an appropriate inexpensive cancer treatment [24] and has some positive effects on diabetic complications [25].

In the last years, many researches have showed that different encapsulation systems, such as Nano emulsions, liposomes, micelles, polymeric micro or nanoparticles, have potential to be applied in different biological and medical applications, mainly as targeted drug delivery systems to minimize and the negative effects of different chronic degenerative diseases as delay [26]. Hence, a wide variety of colloidal delivery systems including micro emulsions, Nano emulsions, solid lipid nanoparticles, multilayer emulsions, polymeric nanoparticles, inclusion complexes, and filled hydrogel particles, have been developed to encapsulate, protect, and target releasing different bioactive compounds in diverse sites of action [27-29]. As a case in point, the synthesis, physico-chemical characterization, and cancer-related application of a nanoparticle-encapsulated formulation of propolis, 'propolis Nano food'. Cross-linked polymeric nanoparticles with a hydrophobic core and a hydrophilic shell were used to encapsulate propolis to generate propolis Nano-food with a size consistently less than 100 nm [30]. The main bacteria found in recurrent Urinary Tract Infections (UTI) which is *Escherichia coli*, now frequently resistant to several currently used antibiotic treatments to make new solutions essential [25].

In this research, nanoparticles of propolis were used because of its disinfectant, photochemical, anti-fungal, anti-parasitic, anti-oxidant and anti-bacterial properties in the strengthening of the immune system. Because of the difficulty of working with beeswax and propolis, we were succeeded in providing a new method after much effort. Using the new method, the problems related to immiscible, adhesion and waxiness of beeswax and propolis were solved. Thus, the use of these two materials and their properties become readily available.

Beeswax is a suitable material as a protective coating against air and moisture due to its high resistance. It can also be used as covers without any unwanted reactions because of its special physical and chemical properties, inactivity and easy mixing it with materials.

Table 1: Equipment of the research.

No	Equipment	Model
1	Digital Balance	GERMAN-FEW
2	Incubator Shaker	IRAN-AC
3	Oven	3491-50 Lit
4	pH Meter	ENGLAND GP-353
5	FTIR	GERMAN-100 Spectrum
6	TEM	JEOI-JSM-5800
7	Spectrophotometer	2100-UV
8	Heater electromagnetic	GERMAN-MSB

Table 2: Materials of the research.

No	Equipment	Model
1	Propolis	SIGMA,20-50nm
2	Beeswax	IRAN
3	Polysorbate 80	SIGMA
4	Bertani Broth (99%)	MERCK
5	Bertani Agar (99%)	Merck
6	Chloroform	MERCK
7	E.Coli	PTCC1998

As a result, the above method prevented the agglomeration and particles didn't get out of the Nano scale. Nanoparticle capsules released in the appropriate place and were not ruptured along the way.

Considering the above data, similar methods and materials can be used to achieve the best treatment. These capsules have not specifically produced yet and it can be one of the best and most practical cases. Hence, it is better to do necessary actions to know the materials and cure the diseases.

EXPERIMENTAL SECTION

Materials and methods

All equipment used in the study is shown in Table 1.

All materials used in this study are shown in Table 2.

The encapsulation of propolis nanoparticles with beeswax

In order to encapsulate propolis nanoparticles, 3g propolis plus 10 mg/mL chloroform were mixed and stirred until complete dissolution. Bees wax was melted at a temperature 80°C and added to the resulting mixture of nanoparticles and then stirred for 15 min with 300 rpm. During the mixing process, 300-mL ammonia buffer with pH = 10.9 was added to the article and stirred until the mixture uniformed. Subsequently, 1.8 g/mL liters Tween-80 was added and the mixture of water and ice got into the cold to be shocked in order to decrease the temperature to 10°C instantaneously for formation and aggregation of capsules. The capsules were isolated from the mixture by filtration using Buchner funnel and vacuum pump and rinsed with water to remove any remaining residue in two phases. Finally, capsules were dried at room temperature for 48 hrs as shown in Fig. 1.

Evaluation of anti-bacterial feature of capsules

In order to study the antibacterial properties of the capsules and propolis, six tubes of propolis and capsules were prepared with a Saline solution of percentages (W) 10%, 30%, 50%. Then 0.1 mL of each test tube's solution was taken and streaked on agar medium. At last, plates were located in the incubator for 24 h in temperature 37 ° C, then colonies were counted using a colony counter.

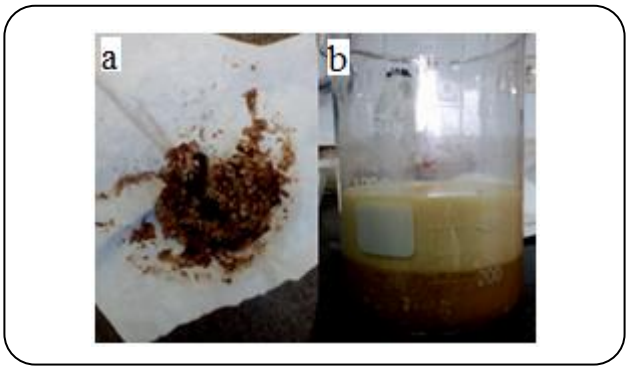

Fig. 1: a) Capsules powder, b) Capsules solution.

Dilution of E. coli

In order to count bacteria, five test tubes of 9 mL solution of Saline were prepared. 1 mL bacteria from the first test tube was taken and added to the second one and from the second to the third and so on. Each test tube was measured at an optimal wavelength of 620nm by spectrophotometer. Finally, 0.1, 0.3 and 0.5 g of capsule were added to three test tubes. 4.5 mL Saline solution and 0.5ml of test tube with dilution of 10^{-4} mL were added and mixed for two hours at 25°C and speed of 130 rpm. At the final stage, 0.1ml from each test tube's solution was given and cultured on plate's surface. In order to complete the process of cultivation, all the plates were placed in an incubator at temperature of 37° C for 24 h and number of colonies was counted by a colony counter.

Design of the experiments

According to the conditions of the human body, the capsule ingredients and their effectiveness with other ingredients, three important factors including temperature, time and pH were considered. Optimal experimental conditions at three levels were calculated by three factors and by the Taguchi method.

Depend on the ambient temperature, the normal temperature of the human body and maximum effective temperature of capsules ingredients, in this experiment three temperature range (25, 37 and 43 °C) were considered because the most optimal possible results from the tests on the capsule ingredients were obtained in this temperature range. On the other hand, according to the minimum and maximum time of releasing capsule inside the human body, minimum temperatures were selected as 10, 30 and 60 °C.

Considering the two factors mentioned before, the amount of capsules release in the acidic, neutral, and alkaline pH ranges were tested and evaluated 5, 7 and 10, respectively. For this purpose, 0.5 g of the capsule was added to 5 mL into test tubes and placed in beaker containing water then located on the heater in order to adjust the temperature.

27 experiments were planned based on the values of Table 3 that are shown in Table 4.

RESULTS AND DISCUSSION

Assessment of antimicrobial properties of propolis produced in medium containing E. coli; Anti-microbial count results

The result of the microbial tests show antibacterial properties of the capsules justifies the very high anti-bacterial properties of capsules and can be used as an edible capsule to destroy microbes including *E.coli*.

The results show that the percentage of contamination in a germ-free environment is negligible and the rate of change was the same for all samples. Dilution did not change the test results and the rate of contamination was the same. As a result, it could be noted that materials are without inherent contamination.

Results of the tube culture method

Results of the mentioned method were evaluated after 24 h incubation and counting colonies with a colony counter and the percentage rate of antibacterial properties was calculated.

The results show that the more concentration of propolis leads to the more antibacterial effect of material in environment which has been contaminated with *E.coli* and also more elimination of the bacteria. On the other hand the less capsule dilution, the less antibacterial effect. Percentages of remove pollutants based on dilution for propolis are (95-10)%, (206.4-30)% and (327.5-50)% and for the capsules are (408-10)%, (324-30)% and (400-50)%, respectively. Produces capsules will be verifiable for use in terms of anti-bacterial properties because they have a significant role in removal of harmful bacteria.

Results of infrared spectroscopy (FT-IR)

In addition to providing information about the chemical structure of a sample, Infrared spectroscopy (FT-IR) is also used to identify organic groups and indicates the physical or mechanical presence of particles.

Table 3: Taguchi experimental factors in this project.

No	Factor	Surface No 1	Surface No 2	Surface No 3
1	pH	5	7	10
2	Time (min)	10	30	60
3	Temp (°C)	25	37	43

Table 4: Results of designing experiments with the Taguchi Method.

Number	pH	Time(min)	Temp(°C)
1	5	10	25
2	5	30	37
3	5	60	43
4	7	10	37
5	7	30	43
6	7	60	25
7	10	10	43
8	10	30	25
9	10	60	37

Table 5: Results of the culture medium without microbes.

Test materials in the plates (w/v)	First Repetition	Second Repetition	Average
Control sample	Colony layer of the surface (cfu)	Colony layer of the surface (cfu)	%100
% ١٠Propolis	100	100	100
% ٣٠Propolis	100	100	100
% ٥٠Propolis	100	100	100
% ١٠Capsule	100	100	100
% ٣٠Capsule	100	100	100
% ٥٠Capsule	100	100	100

FT-IR studies are used for determining absorption bands of important functional groups of pure nanoparticles and loaded capsules with the nanoparticles. In Fig. 4 and in FT-IR spectrum of propolis nanoparticles, a strong absorption band centered on the 666 to 1636 cm^{-1} can be seen which is related to the transplant of propolis. As mentioned above, the bar in the FT-IR spectrum of Nano capsules is easily visible at a wavelength of 1545cm^{-1} which has shifted slightly to the spectrum of nanoparticles. Also, the release of propolis can be seen between the spectrums of 1900 to 700 cm^{-1}. These physical effects cannot be seen in spectrum of Beeswax and represent the physical presence of nanoparticles of propolis in its Nano capsules which is consistent with standard range available in resources.

Results of Transmission Electron Microscope (TEM)

Imaging of produced Nano capsules was done by TEM. Since the images are obtained by scanning the entire surface of the capsule, they can also represent the size of the produced capsule. According to the shape, size range of produced capsules is between 200 to 500 nm, and more than 95% of capsules have appeared in this size range. The surface of the capsules is also smooth and free of charge gatherings.

Table 6: Results of the culture medium with microbes.

Test materials in the plates (w/v)	First Repetition (cfu)	Second Repetition (cfu)	Average (cfu)
Control sample	6240	5760	6000
Propolis 10%	722	1178	950
Propolis 30%	718	658	688
Propolis 50%	520	790	655
Capsule 10%	3840	4320	4080
Capsule 30%	1120	1040	1080
Capsule 50%	640	960	800

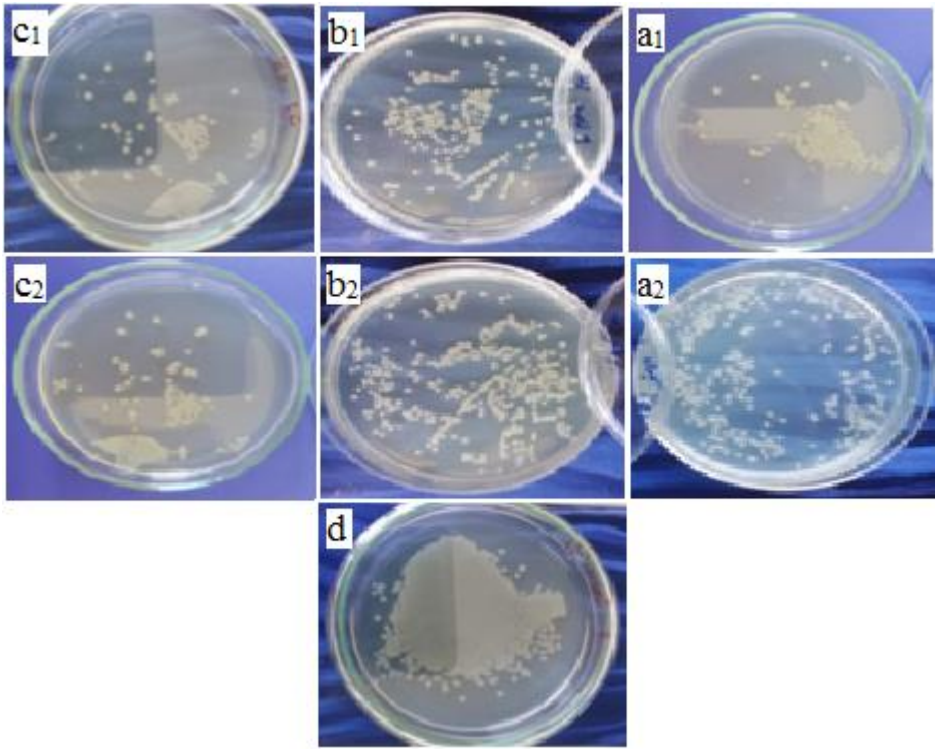

Fig. 2: Culture medium of microbes free is obtained; a1: propolis (50%), b1: propolis (30%), c1: propolis (10%), a2: capsule (50%), b2: capsule (30%), c2: capsules (10%), d: control (10-6).

Most binary combinations are formed in either hexagonal wurtzite structure or cubic compound where each cation is surrounded by four anions at the corners of a four-sided shape and vice versa. This tetrahedron coordination is an example of a covalent bond, but these substances also have significant ionity and appear in two main forms of hexagonal and cubic wurtzite.

Wurtzite cubic structure is only stable under ambient conditions and thus is more common. Zinc-blende form can be stabilized with the growth of propolis nanoparticles on the beds with a cubic lattice structure and cubic may be obtained at relatively high pressure. Crystal structures are shown schematically in Fig. 6. According to the TEM images of produced capsule, it can be said that the propolis nanoparticles used in the production of Nano capsules were cubic.

Results of statistical analysis of the of spectrophotometry; Analysis of S / N release

As described before, the values of S / N for each test are calculated by the software and given in Table 7.

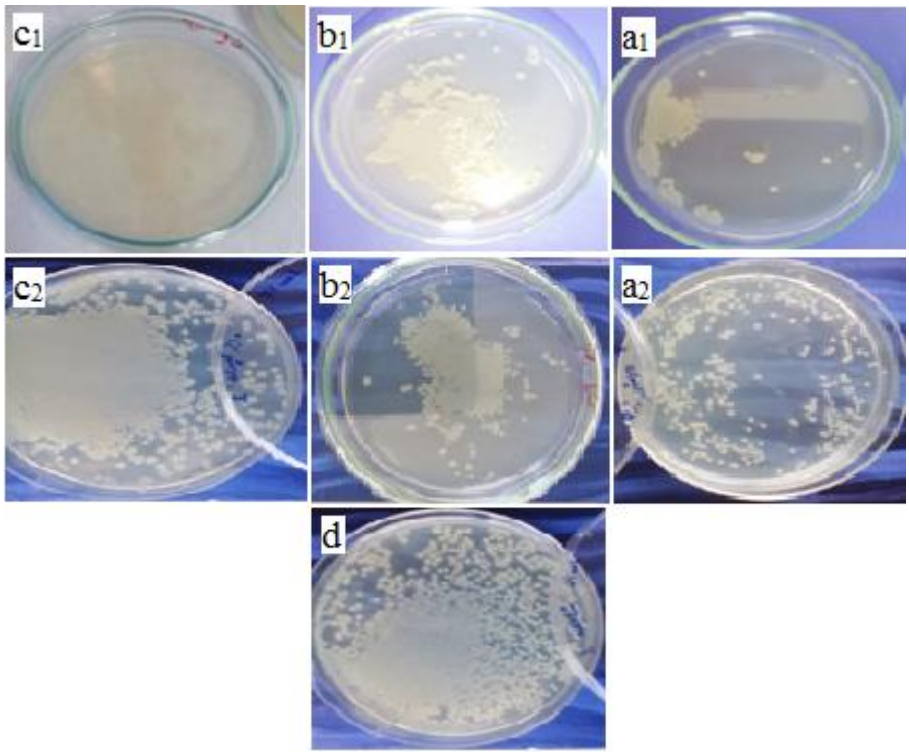

Fig. 3: Microbial cultures medium are obtained; a1: propolis (50%), b1: propolis (30%), c1: propolis (10%), a2: capsule (50%), b2: capsule (30%), c2: capsules (10%), d: control (10-6)

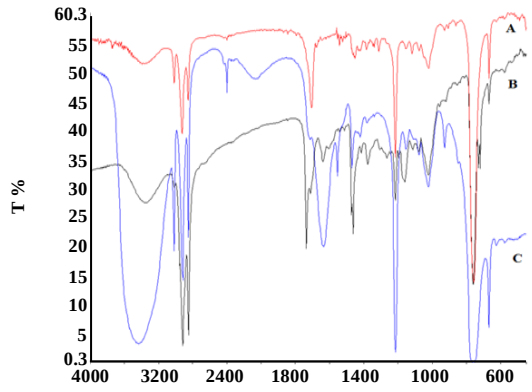

Fig. 4: FT-IR spectra of produced Nano-capsules; a: produced beeswax, B: produced capsules C: produced Propolis.

Given that the largest amount of S / N represents the most favorable test, the 7th test is the best one. In the test pH=10, temperature= 43 °C and time is 10 min which are shown in Table 7.

After obtaining the values of S / N in the above table, optimal condition is always the maximum level of each factor which is also consistent with ANOVA analysis results that are shown in Table 8.

As Table 8 shows, factor (B) of time in level 1 has the maximum amount. The importance order of factors is 2>1>3 (Fig. 7).

According to Fig. 7 the most optimal value for factor B is Level 1 which represents the optimum conditions. Also, the slopes of the graph indicate that the effects of the time on the release rate in Level 1 are higher than levels two and three.

ANOVA Analysis of the release

According to Table 8, ANOVA Analysis was conducted which is shown in Table 9.

P represents the percentage of factors share in the distribution of dispersion of answer. Any factor that has a higher percent share the response is more effective on the answer. In this analysis percent share of time is more than pH and temperature. Therefore, it can be said that the impact of the ammonia buffer time on release is more. Fisher coefficient (f) indicates that the factor is

Table 7: Results of produced capsule obtained using spectrophotometer.

No	Experiment No	S/N (nm)	First Repetition (nm)	Second Repetition (nm)	Third Repetition (nm)
1	1	2.229	0.69	0.701	0.91
2	2	-0.89	0.749	0.949	1.49
3	3	-3.656	1.549	1.53	1.493
4	4	1.343	0.758	0.894	0.91
5	5	-1.179	0.979	1.22	1.22
6	6	-0.777	0.896	1.18	1.18
7	7	9.438	0.22	0.49	0.23
8	8	2.938	0.523	0.521	0.99
9	9	-4.755	1.576	1.964	1.62

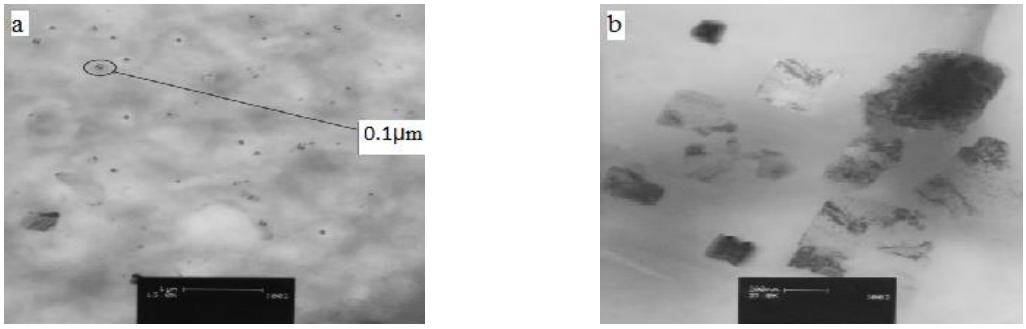

Fig. 5: TEM image of Nano propolis produced capsules; a: 13X magnification of, b: 35X magnification.

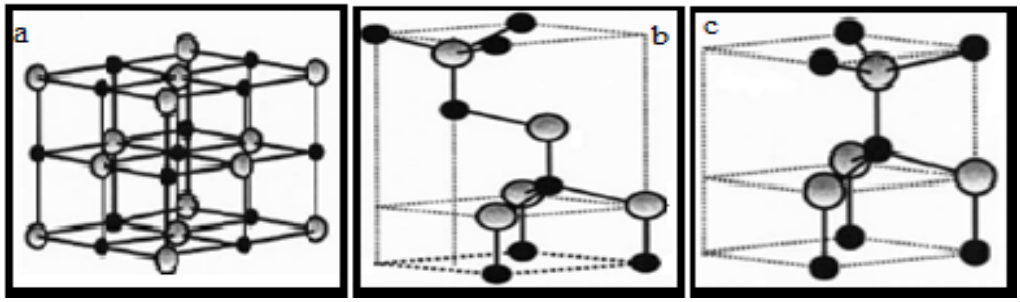

Fig. 6: Structure of propolis; a: hexagonal wurtzite structure, b: Cubic zinc-blende structure, c: Cubic rock salt structure.

significant and in the case of not zero and according to the values obtained for this parameter, it can be concluded that selected factors are significant and time has the greatest impact on changes in the experiments.

The release of optimal conditions

Based on the results of S / N and ANOVA analyses the optimal conditions for the test are obtained in Table 10.

Based on software predictions some values are proposed for the current and S / N that should be compared with experimental results in optimum condition. Table 11 shows the obtained and predicted values.

Based on the above table the differences between the predicted and measured values are low and in the value of 92.45% it was acceptable and 128.12% was measured as the maximum amount of S/N which this can confirm

Table 8: Influence of the factors in the obtained analysis of S / N release.

Row	Caption	Factor	Surface No 1	Surface No 2	Surface No 3	L_2-L_1
1	A	pH	-0.772	-0.205	2.54	0.567
2	B	Time (min)	4.336	0.29	-3.063	-4.047
3	C	C)(°Temp	1.463	-1.434	1.534	-2.868

Table 9: ANOVA analysis obtained from the release.

No	Factor	Degree of freedom	Total Sum of Squeres	Fisher factor	Net Square	Portion (%)(P)	Variance
1	pH	2	18.829	0.84	0	0	9.414
2	Time (min)	2	82.392	3.678	59.97	42.495	41.181
3	C)(°Temp	2	17.209	0.768	0	0	8.604
4	Error	2	22.391	-	-	57.406	11.195
5	Total	8	14.793	-	-	100.00	-

Table 10: Release of the optimal conditions based on Software's envision.

Row	Factor	Surface	Value	Collectivity
1	pH	10	3	2.019
2	Time (min)	10	1	3.815
3	Temp(°C)	43	3	1.013

Table 11: Pnticipated results of the release in optimal conditions

No		Predicted value(nm)	Measured value (nm)
1	Time (min)	0.53	0.49
2	S/N(nm)	7.36	9.43

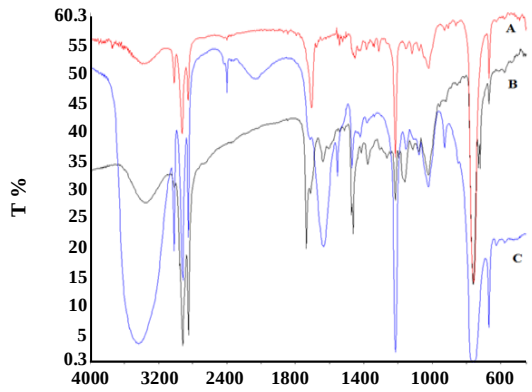

Fig. 7: The release in optimum conditions in the three factors: A (pH), B (time), C (Temp).

the accuracy of the test results and the low value of the error.

CONCLUSIONS

Propolis is one of the most important natural plant materials and can be used in the fields of pharmaceuticals, toiletries, cosmetics, etc. The best way to use the material is encapsulation. It can be also encapsulated with other materials but beeswax is the best due to its features that finally will produce herbal capsules. Based on tests and production of nanoparticles, the antibacterial properties of propolis can be increased which is the most important property of the material. TEM was used to prove the hypothesis and particles with the size of 50 to 100 nm was obtained. The test was done

for one of the most important germs named E.coli that its culture medium was confirmed according to the tests. The FT-IR was used to determine the amount of capsules production. Release of nanoparticles from the walls of the capsules was controlled by the Taguchi method which is of the most prestigious programs of designing experiments to determine the optimal answer. These results are usable and operational for other substances with similar size and chemical properties. Hence, release and production of the capsules were conducted with more than 95% of efficiency. Finally, given the extreme conditions of temperature and costly work with propolis, this approach can be used to achieve the best release in 10 minutes, 43° C and pH 10 with low cast as well.

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