ANTI-PROLIFERATIVE OF THE PHYTOSOME PROPOLIS, PHYTOSOME Lycopene AND SYNERGISTIC EFFECT ON THE BENIGN PROSTATIC HYPERPLASIA CELLS IN-VITRO

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Abstract

Propolis and lycopene compounds with potent pharmacological activities as anticancer, anti-inflammatory and antioxidant action. Propolis is a unique product produced by honeybees. Lycopene is phytochemical pigment belong to the group of carotenoids and found in different fruits and vegetative, as well as certain algae and fungi. This study was conducted to prepare and characterize phytosomal propolis and lycopene, then evaluate their in vitro anti-proliferative potential on rats prostatic hyperplasia cell line. Size of the nanoparticles determined by scanning electron microscope were 102.1±5.83 nm for phytosome propolis and 110.4±5.77 nm for phytosome lycopene. The encapsulation and loading efficiencies of phytosome formulations were 80.83 ± 4.41% and 64.66 ± 5.22% respectively for phytosome propolis and 75.82 ± 5.46% and 60.66 ± 4.37% respectively for phytosome lycopene. In order to ascertain the anti-proliferative activity of the phytosomal formulations, MTT assay was carried out on the prostate hyperplasia cells of rats. The half maximal inhibitory concentrations (IC50) of phytosome propolis was 58.46 μg/ml, phytosome lycopene was 89.74 μg/ml and their combination was 47.86 on the rats prostate hyperplasia cell line.

Key words: MTT, IC50, phytosome propolis, phytosome lycopene, Benign prostatic hyperplasia.

Introduction

Benign prostatic hyperplasia (BPH) is an age-related disorder of the prostate gland progressively developed in males (Aaron et al., 2016). This proliferation of the stromal and epithelial cells in the prostate increases linearly with age in all ethnic groups (McVary and Wefler, 2016). Prostate gland illness is relatively common in intact dogs but less common in other domestic animal species. Bacterial prostatitis (acute or chronic), prostatic abscesses, prostatic and para-prostatic cysts and progressively prostatic adenocarcinoma are seen much less frequently and could be sow in castrated males (Kutzler, 2013).

BPH pathogenesis still remains incomplete, Lately some demonstrated the role of some pathological conditions, such as chronic inflammation, abnormal wound repair, deregulation of circulating hormonal levels, altered expression of cytokines and chemokines (IL-8 in particular) (Gandaglia et al., 2013). Till now, There are no efficient therapies for BPH. Orthodox remedies include a-adrenergic blockers, 5α-reductase inhibitors and their combinations although has proven their efficacy in providing symptomatic benefits, excessive of side effects is associated with the use of these agents. Surgery, usually transurethral prostate resection is currently the most beneficial intervention for BPH but it’s also linked with a multiple postoperative complications commonly denoted to as the Transurethral resection of the prostate (TURP) syndrome (Ventura et al., 2011) hence, interest and awareness is growing in the development of efficient new drugs derived from natural sources for the treatment of BPH.

Propolis (bee glue) is produced by honeybees, is a resinous mixture derived from various plant sources. Different constituents of propolis have been identified such as, polyphenols, sesquiterpene quinones, coumarins, steroids and amino acids which have different activity as antioxidant, anti-inflammatory, anti-proliferating action, all these activities can be help in the alleviate the progression of the BPH (Khalil, 2006; Ibrahim, 2011). On the other
hand, Lycopene, a well-known red carotenoid pigment, has been drawing scientific interest because of its potential biological functions. It is a fat soluble carotenoid discovered by Ernest et al., in 1959. It is a natural constituent of red fruits and vegetables as well as certain algae and fungi. Tomatoes, tomato-based products and watermelon (Saaawarn et al., 2011, Nguyen and Schwartz, 1999). Lycopene have been found to inhibit cell growth and initiated the promotion of apoptosis as well as stimulate gap junction communication among cells and stop cell division (Pagano et al., 2013).

Targeted delivery of nutraceuticals is one of the new challenges in the treatment of neoplasms and BPH and attempted to develop strategies therapy for minimize the side effects of classical drugs that reflect to an active field of research. The nano-pharmaceutics of target delivery can be achieved and applied for, which is based on the development, application and characterization of nano-scale therapeutic systems with provide more an orchestrate controlled drug release and this predicted new approach of therapeutically relevant (Sakata et al., 2007). The solubility and oral bioavailability of propolis flavonoids have been reported to be increased by utilizing the phytosome forms and co-grinding technology. Nano form propolis was reported more effective than propolis in terms of antibacterial and antifungal activity (Afrouzan et al., 2012). This research, therefore aimed to investigate the in vitro anti-proliferative action of propolis, lycopene and their phytosomal formulations on the prostatic hyperplasia cells in culture media.

 Materials and Methods

Propolis collection and extraction

Raw local propolis samples were collected from honey bees colonies in the Al-Diwanya city/ Iraq then, certified in the honey division / department of plant protection / directorate of agriculture / ministry of agriculture in Al-Diwanya city, No. 2446, at date : 10/2/2019 after that conserved in dark containers at 4°C to prevent natural oxidation and transported to the laboratory and kept at -4°C.

The hydro-ethanolic propolis extract was prepared by frozen the propolis samples under -20°C for 24 hour, they were grinded by electrical grinder to obtain powder, 50 gram of the prepared powder was placed in the container with 500 ml of 70% ethanol and incubated at 37°C for 14 days. The mixture was shaken for short period throughout the incubation. After 14 time period, the obtained extract was filtered through Whatman filter paper No. 4, to remove waxes and less soluble substances, the suspension was subsequently frozen at -20°C for 24 hours, then filtered with Whatman No. 4 filter paper. The freezing filtration cycle was repeated three times. The final filtration led to represent the balsam (tincture) of propolis and is referred to as ethanolic extract of propolis (EEP). The hydro-ethanolic organic solvent was evaporated to near dryness via a rotary evaporator under reduced pressure at 60°C. The remaining extract was incubated at 37°C for two weeks till the remainder of the ethanol was evaporated and the resulting sticky like substance were kept at -20°C (Dziedzic et al., 2014).

Tomatoes collection and extraction

Fresh ripened bright red Tomatoes (10 Kg) were obtained from Karbala province during December 2018 and were transported to the laboratory and kept at -4°C. It was identified (authenticated) as lycopersicum esculentum Mill (solanaceae) in the Iraqi national herbarium / Directorate of seed testing and certification (D.S.T.C) / ministry of agriculture in Abou-grabi / Baghdad No. 216 at date : 17/1/2019. The tomatoes were thoroughly washed under running stream of tap water to remove dirt, dust and foreign materials attached to their surface. The seeds and skin were removed. The tomatoes then chopped into small pieces with stainless steel knife and grind in a mill and passed through a 200 micron stainless steel mesh sieve. To obtain tomatoes paste the sieved material was incubated at 45°C then stored at -20°C until use.

The extraction of lycopene was conducted according to the method developed by Motilva and his colleagues (15) 100 ml of triple organic solvents 50:25:25 (v/v) of hexane: acetone: ethanol was added to 15 gm of tomatoes paste that prepared in the previous step in conical flasks and mixed with a stirrer for 20 minute at 35°C. After that left stand for 10 minute in a cooling water bath, the solution was distinguished into bi-layers. Separating funnel was used to separate these layers. Upper organic layer was used to separate β-carotene and lycopene. The resulting crude lycopene extract was evaporated with reduced pressure to reach approximately 1% of the initial volume. The crystallization of lycopene was carried out by adding 100 ml methanol anti-solvent to the carotenoid mixture extract. Precipitation of lycopene from the mixture occurred within several minutes. The isolated lycopene was stored at -20°C until further analysis (Motilva et al., 2014).

liposome preparation.

• Preparation of the empty liposome and loaded phytosome:

The liposomes were prepared by dried thin lipid film technique as described by (Ramana et al., 2007) with
some modification. The lipid phase components include L- phosphatidylcholine : cholesterol in the ration of 0.25 mg : 0.25 mg and dissolved in 15 ml of organic solvent which consisting of chloroform : methanol (2:1) (v/v) mixing in the 25 ml glass test tube, vortexed for 30 minute (1500 rpm). Then, the mixture was incubated in a water bath 40°C for several minutes and transferred to a round bottom flask which was subsequently evaporated under vacuum in a rotary evaporator (80 rpm) attached to a vacuum pump. The round-bottom flask was immersed in a thermostatic water bath 45°C for 2 hours to affirm the dryness of the thin lipid film which deposited on the inner walls of the round bottom glass flask. Two milliliter of the diluted ethanol (30%) with or without propolis or lycopene added to the lipid thin film to formed free liposome, phytosome propolis, phytosome lycopene respectively under vacuum in rotary evaporator for 30 minutes.

• Characterization of phytosomal formulations

• Morphology of liposomal formulations

Optical microscope examination

The prepared empty liposome, liposome propolis and liposome lycopene were inspected under a light microscope for confirming the vesicle formation. A 50 µl drop of prepared phytosome spread on a glass slide and covered with cover slip and examined under optical microscope at 40 X magnification (Bibi et al., 2011).

Scanning electron Microscope (SEM)

A phytosome was examined in Scanning electron microscope technique to characterize the morphology and size of the prepared vesicles. The prepared phytosome samples 0.5 gram, 1% were kept in a Eppendorf at 4°C and transported to the RAZI applied research foundation for examination (Lankalapalli et al., 2015).

Preparation stock solution of propolis and lycopene

Propolis extract and crystalized lycopene stock solutions each one alone was prepared by weighed 250 mg of each substance using analytical balance and dissolved in the 5 ml ethanol and chloroform respectively, vortexed for 30 seconds using vortex mixer, the final concentration of each compound was 5 gm/100 ml and the solution filtered through 0.25 µm syringe filter and kept at 4°C until use.

Determination absorption curve and lambda max

Accurately weighting of 0.2 mg of propolis, lycopene and phytosomal formulations and 3.6 mg of empty liposome were dissolved in 1 ml of ethanol, hexane or BPS. the prepared solutions were then vortexed for 3 minutes examined in a wavelength range of 200-800 nm, then depicted the absorbance curve.

Calibration curve preparation by UV.

Standard curve for propolis and partially purified lycopene solution was performed and estimated by UV-visible spectrophotometer at 320, 480 nm respectively.

A stock solution of 1mg/ml of each standard substance in ethanol or hexane were prepared, then the serial dilutions of each stocked propolis and lycopene in the range of 0.025, 0.05, 0.1, 0.2, 0.4 mg/ml concentration by diluting the stock solution with appropriate volumes of ethanol or hexane. The absorbance values of these solutions were measured at respective wave length of maximum absorbance, using 1 cm quartz cuvette in UV-visible spectrophotometer. Calibration curve were plotted with concentration against absorbance.

Encapsulation efficiency and loading

The entrapment efficiency and percentage measurements were performed on UV-visible spectrophotometer technique by using centrifugation method. The liposomal suspension was taken and centrifuged at 5000 rpm for 15 minute to separate the supernatant containing liposomal vesicles from the sediments containing the unentrapped drug. The clear supernatant was collected and recentrifuged at 5000 rpm for 15 minute.

The liposome were soaked in 10 ml of methanol and then vortex for 10 minute. The phytosomes were dissolution to release the drug. In order to quantify the content of each propolis and lycopene in supernatant and sediments in the samples, series of standard solutions were prepared as mentioned in the previous paragraph. Encapsulation efficiency of phytosomal propolis and phytosomal lycopene were calculated by the ratio of encapsulated drug to the initially added drug, the measurements were done in triplicate. The encapsulation efficiency and percentage were calculated using the following equations (Wasankar et al., 2012).

\[
EE\% = \frac{\text{Amount of the encapsulated drug}}{\text{Initial amount of drug added}} \times 100
\]

Percent substance loading was calculated by the ratio of encapsulated drug to liposomal lipid amount as according to the following equation:

\[
\text{Drug loading} \% = \frac{\text{Amount of the encapsulated drug (mg)}}{\text{Amount of the liposomal lipid (mg)}} \times 100
\]

In vitro challenged of phytosomal formulations

• Prostate rats isolation.

It was accomplished as described by (Montpetit and Tenniswood, 1989) and (Tsugaya et al., 1996) as
following steps:

1. The male rats were sacrificed by cervical dislocation, sterilized the rats with 70% ethanol at the site of the incision.

2. The whole prostate was removed aseptically and washed with phosphate buffer saline to remove all traces of blood, then transport to ice-cold hank balanced salt solution (HBSS).

3. The prostates were minced finely approximately 1-3 mm$^3$ using forceps and scissors and transferred to 75 cm$^2$ T-flasks for three rinses for 5 min. with HBSS. The tissue fragments were allowed to settle and the supernatant removed.

4. The tissue was dissociated by digestion in 10 ml contain 1% collagenase, 1% trypsin and 1% chicken serum (CTC) in HBSS for 20 min. at 37°C with slow agitation.

5. One hundred µL 0.4% DNase 1 in HBSS also added to dissociate the DNA matrix that could be formed from damaged cells and the incubation was continued for additional 5 min.

6. To separate prostate cells (sediment), the mixture was decanted into a centrifuge tube and centrifuged at 775 g for 10 min.

7. To wash out the collagenase, The deposits were re-suspended in 20- mM hydroxyethyl-piperazine ethaneesulphonic acid (HEPES) buffer solution at pH 7.6.

8. The cells were sedimt again by centrifugation and the process was repeated twice

9. The cells pellets was re-suspended in 5 ml RPMI medium and the suspension was finally centrifugated at 135 g for 20 min. the small aggregates of fibroblasts and single cells were dispersed in the supernatant.

10. For the primary culture of the fibroblasts, the supernatant containing the fibroblasts was centrifuged at 775 g for 10 min. and the deposited were inoculated into a 75 cm$^2$ T-flask containing 15 ml of the fibroblast growth media of RPMI 1640 medium supplemented with FCS(10%), L-glutamine (1%), penicillin (100 IU/ml) and streptomycin (100 µg/ml).

11. The culture medium was changed on days 3 and 10. The fibroblasts spread on the bottom of culture flask 10 days later. On reaching confluence, fibroblast growth medium was removed and the cells were passaged by detaching from the flask with a 5 ml trypsin solution (0.05%) and EDTA 0.01% and placing the pure fibroblasts in 24 well plates for subsequent experimentation.

**Assay procedure (MTT assay)**

The MTT assay was performed as reported by (Unthong et al., 2011) and (Jayshree, 2013) cultured cells with final plating density (1×10$^4$ cells) in 200 µl of complete RPMI 1640 culture media were seeded into each well of a flat 96-well plate and then incubated for 24 h at 37°C in a 95% humidified air atmosphere enriched with 5%(v/v) CO2 to allow the cells attach to the bottom of each well.

The prepared cultured cells were then treated with different concentrations of test compounds include (liposome alone, propolis ethanol extract, partially purified lycopene, phytosome propolis, phytosome lycopene and their combination 50/50 concentration) each one alone. This done by addition of 2 µl of the serial dilutions of the tested compounds dissolved in appropriate vehicle (BPS, DMSO) to give a final concentration of 100, 50, 25, 12.5, 6.25 and 3.125 µg/ml. In addition, 2 µl of BPS, DMSO alone were added to another set of cells as the solvent control. The triplicate was maintained for all concentrations. The plates were then incubated as above conditions for another 72 h prior to the addition of 10 µl of a 5 mg/ml solution of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) into each well. The incubated cells was continued for another 4 hrs. before the media was removed. A mixture of DMSO (150 µl) and glycine (25 µl) was added to each well and mixed to ensure cell lysis and dissolving of the formazan crystals, before the absorbance at 492 nm was measured by using micro-plate reader. Three replications of each experiment were performed and the half maximal inhibitory concentration (IC50) of each extract was calculated as detailed below.

**Determination of IC$_{50}$**

The obtained absorbance at 450 nm of the tested compounds and vehicle control was used to determine the rate of relative percentage of inhibited prostate cells assuming that 100% survival was obtained from the vehicle only control. Under these assumptions, the percentage inhibition of the treated prostate hyperplasia cells was calculated according to the formula below:

$$\text{The relative Percentage of inhibited cells} = 100 - \left( \frac{\text{Abs. of treated cells}}{\text{Abs. of control cells}} \right) \times 100$$

Where (Abs. of treated cells ) and (Abs. of control cells) are represented as the absorbance at 540 nm of the treated cells and the control cells, respectively.

The IC50 values were graphically obtained by plotting the absorbance obtained against the corresponding different concentrations of the test compounds used and are reported as the mean ± standard error (SE).
### Results

#### Propolis and lycopene extraction

The yield percentage of ethanolic propolis extract and lycopene extract for ten patches was 45.7±1.56%, 0.063±0.002% respectively for each 50 gm of crude propolis powder and 100 gm of crude tomatoes respectively as shown in table 1. The yield of final product of propolis after complete dryness was viscous consistency and dark brown color with distinguish odor whereas, partially purified lycopene after complete dryness was slightly sticky and red in color fig. 1.

The optical microscope was showed the vesicle forming field phytosomal formulations. The phytosomal propolis and lycopene were found to be discrete, spherical in shape and un-lamellar fig. 2.

The description of surface morphology and shape for phytosomal formulations were done by scanning electron microscope. SEM images of the tested phytosomal samples exhibits that all prepared phytosomal formulations were fine spherical and smooth vesicular structures. The values of average particle size in the Nano-metric scale determine through measure the size of 100 particles were 102.1 for phytosome propolis and 110.4 for phytosome lycopene. There was no significant difference (P>0.05) in the size of particles between two phytosomal formulations table 2. Scanning electron photographs of the phytosomal propolis and lycopene were shown in the fig. 3.

#### Lambda max and Calibration curve of drugs

The λ max. of propolis extract, crystalized lycopene extract, liposome alone, phytosome propolis and phytosome lycopene were determined by scanning the prepared solutions in the wavelength ranging from 200 to 800 nm by using Uv-visible spectrophotometer as shown in fig. 4. All reading were done at 37°C at 2 nm intervals. The maximum absorbance wavelength was found to be 324, 478, 290, 330, 480 nm respectively. The linearity of the calibration curve was plotted for absorbance versus concentrations ranging from 0.025 to 0.4 mg/ml. The regression coefficient value for propolis extract was R²=0.961 with the slope equation y=4.1629x+0.6528 and R²=0.940 y=3.1129x+0.4035 for partially purified lycopene. The absorbance values and standard cure shown in the table 3, fig. 5.

The entrapment efficiency and loading of phytosomal propolis and lycopene formulations are listed in the table 4 for three patches. The percentage of propolis and lycopene incorporated in the liposome nanoparticles relative to the initial amount of propolis and lycopene in the solution were 80.83, 75.82 respectively whereas the percentage of propolis and lycopene incorporated in the liposome nanoparticles relative to the content of the total lipid used were 64.66, 60.66 respectively. There was no significant difference (P>0.05) in encapsulation efficiency.

### Table 1: Yield of extraction and percentage of studied materials.

<table>
<thead>
<tr>
<th>Type of material</th>
<th>Crude materials</th>
<th>Yield weight</th>
<th>Min-max yield weight</th>
<th>percentage (%)</th>
<th>Min-max %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propolis</td>
<td>50 gm</td>
<td>22.85±0.78gm</td>
<td>19.3-27.2 gm</td>
<td>45.7±1.56</td>
<td>38.6-54.4</td>
</tr>
<tr>
<td>Lycopene</td>
<td>100 gm</td>
<td>63.45±2.92mg</td>
<td>48-79 mg</td>
<td>0.063±0.002</td>
<td>0.048-0.079</td>
</tr>
</tbody>
</table>

The values represent the means of the ten patches for each material.

### Table 2: Particle size of the phytosomal propolis and lycopene.

<table>
<thead>
<tr>
<th>Formula type</th>
<th>Size of phytosome</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range</td>
</tr>
<tr>
<td>Phytosome propolis</td>
<td>38.6-178.2</td>
</tr>
<tr>
<td>Phytosome lycopene</td>
<td>41.3-187.2</td>
</tr>
</tbody>
</table>

### Table 3: Calibration curve data of propolis and partially purified lycopene.

<table>
<thead>
<tr>
<th>Concentration mg/ml</th>
<th>Absorbance value (mean± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Propolis extract</td>
</tr>
<tr>
<td>0.025</td>
<td>0.588±0.013</td>
</tr>
<tr>
<td>0.05</td>
<td>0.868±0.025</td>
</tr>
<tr>
<td>0.1</td>
<td>1.191±0.023</td>
</tr>
<tr>
<td>0.2</td>
<td>1.608±0.034</td>
</tr>
<tr>
<td>0.4</td>
<td>2.236±0.028</td>
</tr>
</tbody>
</table>

Values are means of three independent experiments ± SEM.
and loading efficiency between phytosome propolis and phytosome lycopene.

### Anti-proliferative effects

To examine the potential anti-proliferative action of different concentrations of propolis extract, partially purified lycopene and phytosomal formulations each one alone on rats BPH cells as compared to the effect of an BPS, DMSO and empty liposome, the MTT cell viability assay was applied. Accordingly, data were normalized and expressed as percentage of growth inhibition over controls. The results showed that all drug formulations exhibited inhibitory effect against the prostate cells growth in tissue culture and the growth inhibition % increased as the concentration increased for all tested compounds. The cells inhibition percentage were 5.43, 3.06, 7.13, 5.2, 8.2% for propolis extract, crystalized lycopene, phytosomal propolis, phytosomal lycopene and their combination at concentration 3.125 µg/ml respectively increased at the concentration 100 µg/ml to the 41.33, 34.1, 59.93, 54.03, 61.58% respectively.

On the contrary, the BPS, DMSO and empty liposome were observed no significant effect (P>0.05) on the rat BPH cells in tissue culture compared with other tested drug fig. 6 and 7. Statistical difference of each dose of phytosomal formulations compared to the control was significant important (P<0.05).

The anti-proliferative efficacy of the all tested formulations were further quantified by IC$_{50}$ calculation by the linear regression analysis. The inhibition of rats prostatic cells proliferation for all tested formulations was dose dependent, with the level of inhibition activity increased at high concentrations fig. 6A 50% mortality of benign prostatic cells were obtained after 72 h incubation period was 250.61 µg/ml for propolis extract, 528.44 µg/ml for

### Table 4: The entrapment efficiency and loading of phytosomal propolis and lycopene.

<table>
<thead>
<tr>
<th>Formula type</th>
<th>Total amount</th>
<th>Liposome weight (mg)</th>
<th>Free drug (mg)</th>
<th>Encapsulated efficiency (%)</th>
<th>Loaded efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phytosome propolis</td>
<td>400 mg</td>
<td>500</td>
<td>76.66±17.63</td>
<td>80.83±4.41</td>
<td>64.66±3.52</td>
</tr>
<tr>
<td>Phytosome lycopene</td>
<td>400 mg</td>
<td>500</td>
<td>96.66±21.85</td>
<td>75.82±5.46</td>
<td>60.66±4.37</td>
</tr>
<tr>
<td>T test value</td>
<td></td>
<td></td>
<td>0.712</td>
<td>0.712</td>
<td>0.712</td>
</tr>
<tr>
<td>P value</td>
<td></td>
<td></td>
<td>0.516</td>
<td>0.516</td>
<td>0.516</td>
</tr>
</tbody>
</table>

Values represent means ± SEM of three independent experiments

Fig. 3: Scanning electron microscope (a) phytosome propolis (b) phytosome lycopene.

Fig. 4: Uv-visible spectrum of the (a) empty liposome (b) propolis ethanolic extract (c) crystalized lycopene (d) phytosome propolis (e) phytosome lycopene.
partially purified lycopene, 50.46 µg/ml for phytosome propolis, 89.74 µg/ml for phytosome lycopene and 47.86 µg/ml for their combination, whereas liposome alone exhibit limited effects on the BPH cells inhibition table 5.

Discussion

The active ingredients present in the crude propolis was extracted by using cold maceration at room temperature with ethanol alcohol (70%) for 14 days and solvent elimination under reduced pressure. Ethanolic extract of propolis was dark brown in color and percentage yield was found to be 45.7±1.56% within the range of 38.6-54.4 for each 50 gm of crude propolis and this result was in the line with the range reported in the previous studies (Paviani et al., 2009; Biscaia and Ferreira, 2009; Al-Hasnaoui, 2013) that presented values of extraction 39.45%, 46% and 38.5% respectively. Lycopene was extracted from local tomatoes by maceration using solvents of varying polarity. Partially purified lycopene was slightly sticky and red in color with percentage yield 0.063% ± 0.002 % ranging 0.048-0.079% for each 100 gm of crude tomatoes.

Several tests were performed on the prepared phytosomal formulations to make sure that these formulations have the desired properties as particle size estimation, encapsulation efficiency, pH tolerance and osmolarity tolerance. Particle size and polydispersity are consider the most important properties of Nano-carrier systems (Danaei et al., 2018). They help to determine the targeting ability of nanoparticles and toxicity, while they greatly influence the drug loading, drug release and the stability of nanoparticles (Jain and Thareja, 2019). The small size of the propolis and lycopene loaded liposome resulted in an enhanced cellular entry and greater bioavailability. Moreover, liposomal formulations can protect the drug compound from enzymatic degradation, renal clearance and uptake by the immune system, resulting in prolonged blood circulation (Gentile et al., 2004). In the present study, SEM was used to investigate the morphology and size of tested nanoparticles As showed in the fig. 3 synthesized phytosome propolis nanoparticles successfully prepared with the thin film hydrated method and exhibited the

<table>
<thead>
<tr>
<th>Tested substance</th>
<th>IC50 (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empty Liposome</td>
<td>4.59 × 10^9</td>
</tr>
<tr>
<td>Propolis extract</td>
<td>250.61</td>
</tr>
<tr>
<td>Partially purified lycopene</td>
<td>528.44</td>
</tr>
<tr>
<td>Phytosome propolis</td>
<td>58.46</td>
</tr>
<tr>
<td>Phytosome lycopene</td>
<td>89.74</td>
</tr>
<tr>
<td>Combination</td>
<td>47.86</td>
</tr>
</tbody>
</table>

Fig. 5: Calibration curve of (a) propolis extract (b) crystalized lycopene.

Table 5: IC 50 values (µg/ml) of propolis extract, partially purified lycopene and phytosomal formulation on BPH cells.

Fig. 6: Percentage of growth inhibition of tested formulations on the rats prostate cell lines.

Fig. 7: Anti-proliferative activity of tested compounds against BPH cells. (a) Control untreated cells. (b), Cells treated with PBS. (c) Cells treated with DMSO. (d) Cells treated with Liposomes. (e) Cells treated with Propolis. (f) Cells treated with lycopene (g) Cells treated with phytosome propolis (h) Cells treated with phytosome lycopene (i) Cells treated with combination.
average particle size around 102.1 nm within the range of 38.6-178.2 nm whereas phytosome lycopene exhibited 110.4 nm average particle size with range of 41.3-187.2 nm. The difference in the particle size of the two phytosomal formulations was statistically non-significant (P>0.05). These nanoparticles with diameter less than 200 nm for drug delivery purposes can have high potential for permeability to prostate cells due to more diffusion in extracellular space of prostate tissue (Nance et al., 2012). Nanoparticle drugs with diameter less than 20 nm may be removed quickly through kidney by glomerular filtration (Sunoqrot et al., 2014) whereas nanoparticle drugs with diameter more than 200 nm may be cleared using Reticuloendothelial Systems (RES) (Lorenzer et al., 2015). Therefore, it seems that nanoparticle drugs with the diameter between 20 and 200 nm may be considered suitable. (Maleki et al., 2017). The scanning electron microscope analysis of the phytosomal formulations of propolis and lycopene also exhibited structures fine spherical shape and smooth vesicles. In previous studies where liposomes were loaded by same method in a BPS solution, similar morphological observations were also reported (Wasankar et al., 2012).

Encapsulation efficiency is another important property of the nanoparticle system and represent the measure of the fraction of input agent found in the liposomal product (Demirbag, 2011). The encapsulation and loading efficiencies of prepared phytosome formulation batches with constant phosphatidylcholine:cholesterol ratio were 80.83 ± 4.41% and 64.66 ± 3.52% for phytosome propolis and 75.82±5.46% and 60.66±4.37% for phytosome lycopene, respectively as represented in the table 4. The good entrapment efficiency of the prepared phytosomal preparations in our study are most probably due to the composition of the formulations as amount of cholesterol used for prepared phytosome which tighten fluid bilayers and reduce the leakage of the contents from the liposome (Onyeson, 2014; Tseng et al., 2007) other factors as preparation method and drug solubility are also affected significantly (Tiwari, 2013). The encapsulation efficiency is important because when its high, the amount of liposome that has to added is low, thus, it is a matter of economics and efficiency (Demirbag, 2011).

Imura and his colleagues demonstrated high percentage encapsulation efficiency of liposome made from Lecinol S-10EX which contains 95% PC while low % encapsulation efficiency of liposome made from Lecinol S-10 contains 32% PC. Based on these preliminary experiments PC was selected to formulate the lipid vesicle formulations due to its higher percentage encapsulation efficiency (Imura et al., 2003). However, encapsulation efficiency of hydrophilic drugs is affected by the rigidity of the bilayer which is affected by CHOL content. Takahashi and his colleagues reported that the entrapment of the drug is dependent on the composition of the liposomes and partly on the starting amount drug used in preparation of liposomes (Takahashi et al., 2009). Moreover, (Hussian, 2010) observed that the PC/Chol (2:1) liposomes had the highest amount of the active ingredient whereas PC only liposomes had the lowest.

To determine the biological efficacy of propolis, lycopene and their phytosomal formulations on the in vitro growth inhibition of the prostate cells, MTT based colorimetric assay was performed. The experimental finding for this study demonstrated that the treatment with increasing concentrations of Propolis extract and partially purified lycopene showed a considerable dose dependent inhibition of cell proliferation on the prostate cells as shown in the fig. 6 but interestingly, phytosomal formulation of these compounds and their combination significantly outperformed on the activity of the propolis extract and lycopene extract. In the same time, Blank liposome induced minor inhibition of prostate cells proliferation. In contrast, DMSO and BPS did not show significant activity on the prostate cells proliferation. The decrease in the density of the prostate cell accompanied with increasing concentration of tested formulations as revealed by the cell viability study is return to the direct effect of the anti-proliferative action of these formulations. According to the previous finding the propolis extract and crystalized lycopene produce prostate cell growth inhibition mostly due to both direct cytostatic and cytotoxic effect (Touzani et al., 2018; Ivanov et al., 2007). The concentration response curve with a different concentration of phytosomal NPs was shown in fig. 6.

It is well known that benign prostatic hyperplasia is a proliferative process of the both stromal and epithelial cells (Nahta and Dixit, 2011) this process was able to stimulated by both local paracrine and autocrine growth factors as bFGF (Lawson, 1990). Propolis is a unique product produced by honey bees with wide range of biological activity (Kaskoniene et al., 2014). Many studies have reported anti-proliferative activity of both propolis and various bioactive compounds derived from it in vivo and in vitro (Mouse et al., 2012). The propolis samples from different regions contain varying amounts of the biologically active chemicals especially phenolic compounds and were found to be more potent anti-proliferative activity against several cancers cell lines (Grunberger et al., 1988; Teerasripreecha et al., 2012; Khacha-ananda et al., 2013). The growth inhibition produced by propolis extract and its phytosomal
formulation on the prostate hyperplasia cell line may indicate a potential capability of these agents to exert an inhibitory effect to prostate tumor cell proliferation and this action may be connected with content of different type of flavonoids compounds including tectochrysin, galangin, pinocembrin and pinocembrin7-methylether in propolis samples. Such activity of flavonoids and other phenolic compounds of propolis has been verified in tumor cell growth (Barbaric et al., 2011). Apoptosis induction and cell cycle arrest are recommended as main mechanisms of the anti-proliferative activities of propolis (Sawicka et al., 2012). On the other hand, the lycopene, is one of the major carotenoids in the diet and is believed to have a number of biological and health beneficial effects (Story et al., 2010) the lycopene and phytosomal formulation is the other showed potent capability to inhibit prostate cell growth in tissue culture. This finding confirmed other studies that reported role of lycopene for ameliorate cell proliferation in vitro (Rahmat et al., 2002) Lycopene was recorded to interact with various cellular processes, including cell cycle progression and the modulation of signal transduction pathways (Kelkel et al., 2011). Hantz and his colleagues recorded that lycopene produced apoptosis in androgen sensitive prostate cancer cells and this action is mediated via the disruption of mitochondrial membrane and generation of ROS. Androgen signaling pathway play a key role in prostate cancer and benign hyperplasia growth and development (Hantz et al., 2005) So androgen administration revealed increased in protein level of proliferation and abnormal expression of androgen receptors and 5α-reductase (Wu et al., 2017). 5α-reductase inhibitors can block the conversion of testosterone to the more potent dihydrotestosterone, thereby inhibiting prostate growth (Kramer et al., 2009) Additionally, lycopene was proven its activity for inhibition of 5α-reductase enzyme and consequently inhibit androgen signaling (Kao et al., 2014). The inhibition or activation of the androgen receptors can produce alterations in the growth and proliferation of the prostate tissue (Lee et al., 1995). Venier and his colleagues found that the apoptosis and anti-proliferative actions of capsaicin are enhanced in the presence of lycopene (Venier et al., 2012). Beside this, the anti-proliferative and pro-apoptotic activity of lycopene has been observed in malignant T-lymphoblast cells (Amir et al., 1999).

IC_{so} is the concentration of agent that inhibits fifty percent of cell proliferation was calculated for all prepared formulations table 5. Resulting indicate that anti-proliferative effects steadily strengthen with an increase in the concentration of tested compounds. IC_{50} was found to be 50.46 µg/ml for phytosomal propolis compared with 250.61 µg/ml for propolis extract and 89.74 µg/ml for phytosomal lycopene compared with 528.44 µg/ml for partially purified lycopene whereas combination displayed IC_{so} value 47.86 µg/ml. This overcome in the activity of nano-formulations compared with non-nano-formulation of propolis and lycopene in the IC_{so} value on prostate cells may attributed to the advantage of nanoparticles for enhanced permeability of drugs and retention effect allows to the extravasations of phytosomal propolis and phytosomal lycopene nanoparticles preferentially in the prostate area and reduced toxicity.

The results show that the anti-proliferative potential of the formulation of propolis-loaded liposome nanoparticles was more effective than lycopene-loaded liposome on the BPH cells, probably due to more aqueous dispersion after nano-encapsulation. More recently, studies have been directed towards the use of a different types of NPs in the diagnosis and treatment of diseases. Among the different NPs in their sources of synthesis, liposome nanoparticles are promising products that can be applied in the nanoscale because they have unique properties and their non-toxic nature (Danaei et al., 2012).

### References


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