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Anti-cancer, anti-biofilm, and anti-inflammatory properties of hen's albumen: A photodynamic approach

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ABSTRACT

The albumen plays a major role in the protection of eggs against microorganisms. It contains an arsenal of natural antimicrobial molecules and antibacterial proteins, including the well-known ovotransferrin and lysozyme, which exert their activities against a range of bacteria. In the present study, the hen's albumen extract treated with the dried insect body of blister beetle *M. pustulata* was assessed for antibacterial, antibiofilm, anti-inflammatory and anti-proliferative activity. The zone of inhibition against Gram positive *E. faecalis* and *S. aureus* was 10.8 mm and 12.1 mm respectively at 100 μg mL^{-1}. However, it was 13.6 mm and 15.3 mm for Gram negative *P. aeruginosa* and *P. vulgaris* respectively. The biofilm of tested bacteria was significantly inhibited at 100 μg mL^{-1}. The hydrophobicity of bacterial biofilms was considerably condensed after treatment with the hen's albumen extracts at 100 μg mL^{-1}. The anti-inflammatory activity of hen's albumen extracts was confirmed by the inhibition of cyclooxygenase (COX) enzyme to 84.91% at 100 μg mL^{-1} with the relative IC_{50} of 8.26 μg mL^{-1}. The albumen extract effectively inhibited the viability (23.61%) of HepG2 hepatic cancer cells at 100 μg mL^{-1}. The anti-proliferative activity of the albumen extracts was further revealed by the induction of HepG2 apoptotic cell morphology. This study concludes that the hen's albumen extract treated with *M. pustulata* is a natural therapeutic agent to treat biofilm associated clinical bacteria, inflammations and human hepatic cancer cells.

1. Introduction

Biofilms are bacterial exopolysaccharides that are formed on solid surfaces [1–10]. Inflammation is a biological defense against infection or injury [11]. It helps us to eliminate the foreign bodies or injurious agents. Furthermore, it removes damaged tissues and assists in healing process. However, many acute and chronic human diseases are due to uncontrolled inflammation [12].

Hepatocellular carcinoma (HCC) is the second leading cause of deaths worldwide [13–18]. Hence, the progress of an efficient anti-cancer agent is a vital need for administration of HCC. The study on the potency of animal-born drugs to treat hepatocellular carcinoma has received considerable attention due to their safety compared to chemotherapeutics.

Since ancient times, insects have been used to cure diseases. In traditional medicine, insect bodies, eggs and secretions was used for different ailments. Recent scientific studies have shown the pharmaceutical applications of insect extractions including anti-bacterial, anti-inflammatory and anti-tumor activities [19]. The blister beetles commonly called as ''oil beetles'' belong to the family Meloidae. They secrete yellow coloured, pungent smelling oily droplets of haemolymph from the leg joints when disturbed. The exudation contains the toxic material 'cantharidin' or cantharidin [20–24]. Cantharidin and its derivatives have been found to contain anti-tumoral properties against breast, colorectal, melanomas, bladder, pancreatic, hepatic cancers and leukemia [25]. The blister beetle of this study *M. pustulata* are black and

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red striped beetle, measuring about 26 mm in length, hence they are one of the largest species of the blister beetles. In this study, the anti-biofilm, anti-inflammatory and anti-proliferative effects of the dried insect body of *M. pustulata* soaked with hen' albumen extracts was reported.

2. Materials and methods

2.1. Preparation of hen's albumen (egg white) extracts

M. pustulata beetles were collected from Scott Christian College and Mylaudy. The beetles were anesthetized and were air dried in a hot air oven at 50–55 °C. The extraction was carried out following the procedure by Ma and Ruan [26]. The air dried insects body of *M. pustulata* was soaked in the hen's albumen in a conical flask for 24 h and then it was removed from the albumen. The albumen was then mixed with the chloroform (20 mL) and the extract was assessed for anti-biofilm, anti-inflammatory and anti-proliferative activity.

2.1.1. SDS-PAGE analysis of hen's albumen treated with dried body of M. pustulata

SDS-PAGE of albumen was electrophoresed according to Laemmli [27]. The molecular weight of albumen fractions was measured by comparison with standard molecular markers (Himedia, India). Briefly, the albumen was mixed with sample buffer (1:1 ratio) and boiled for 3 min. They were then cooled and loaded onto the wells. The samples were run at 150 V for 3 h. After staining with coomassie brilliant blue for about 3 h, the gel was viewed for photographed.

2.2. Bacterial culture

In this study, Gram negative and Gram positive bacteria were cultured in nutrient broth (121 °C for 15 min) and stored in glycerol (20% v/v) at −70 °C.

2.2.1. Antibacterial assay

The antibacterial activity of hen's albumen extract treated with insect body of *M. pustalata* was determined against test bacterial species by Disc diffusion method [28]. Different concentrations (50 and 100 μg mL^{-1}) of albumen extracts were used for antimicrobial activity. A negative control was maintained using distilled water. Ciprofloxacin disc (antibiotic) was as a positive control and incubated at 37 °C for 24 h.

2.2.2. Minimum inhibitory concentration (MIC)

The method described by Burt [29] was followed for MIC of albumen extract. Briefly, different concentrations of *M. pustulata* treated albumen extract (5 to 9.5 μg mL^{-1}) were added to 5 mL of Luria-Bertani (LB) broth (10^5 Cfu mL^{-1} bacterial suspensions) and rotating at 180 rpm for 24 h by orbital shaker. Then, 100 μL from each dilution tube was plated in MHA and incubated at 37 °C overnight. The inhibition was compared with ciprofloxacin (positive control).

2.2.3. Biofilm formation assay

Briefly, the bacterial suspension was adjusted to be equivalent to 0.5 McFarland's standard. Then, serial dilution was prepared (10^8 Cfu mL^{-1}–10^{10} Cfu mL^{-1}) and each well of the 96 well plate was saturated with 150 μL of bacterial suspension. After incubation for 24 h at 37 °C, the bacterial suspension was softly detached from each well and rinsed with phosphate buffer (0.2 mL) to remove planktonic bacteria. The plate was then stained with crystal violet (0.1%, w/v) and dried. Then, in the wells 95% ethanol was added and OD was determined at 590 nm. Triplicates were used for assay [30].

2.2.4. Biofilm inhibition assay

Glass pieces (1 × 1 cm) kept in 96-well tissue culture plates were used to grown the bacterial biofilms. Then, biofilms were treated by the albumen extract (50 μg and 100 μg mL^{-1}) and incubated overnight at 37 °C. Following treatment, the glass pieces were rinsed by PBS and stained by crystal violet (0.4%) and observed by microscope -ECLIPSE Ti 100 at 40 X magnification. One more set of biofilm grown above was rinsed with PBS, stained with acridine orange (0.1%) and observed by CLSM-Carl Zeiss LSM 710 [31].

2.2.5. Hydrophobicity index of bacterial biofilm

Both treated and control bacterial cells were re-suspended in MHB and the optical density was adjusted (1.0 ± 0.01at 595 nm), toluene (1 ml) was added and vortexed for 1 min. The OD was deliberated by UV–vis spectrophotometer (UV-1700 Shimadzu, Japan). The hydrophobicity index (HI) of bacterial cells was calculated according to Zhang et al. [32]

$$[(A0 - A)/A0 - 1] \times 100$$

Where, A0 and A are the initial and final optical densities of the aqueous phase, respectively.

2.3. Anti-inflammatory effect of albumen extracts

Human monocytic cell lines (THP1) were obtained from the National Centre for Cell Sciences (NCCS), Pune, India. THP1 cells was cultured in RPMI 1640 medium (Hi Media) in completely aseptic conditions. The cells were grown at 60% confluency followed by activation with LPS (1 μg mL^{-1}). The cells were then exposed to *M. pustulata* treated albumen extract at 10, 50 and100 μg mL^{-1} and incubated for 24 h. Then, they were centrifuged at 6000*g* for 10 min. The supernatant was discarded and the pellet was mixed with 200 μL of cell lysis buffer (1MTris HCl, 0.25 M EDTA, 2 M NaCl, 0.5% Triton) for enzyme inhibition assay.

2.3.1. Cox inhibitoryassay

The assay consists of reaction buffer, haem, enzyme and egg white extract pre-incubated at 37 °C for 20 min. This was followed by the addition of arachidonic acid and incubated at 37 °C for 2 min. The reaction was arrested by the addition of saturated stannous chloride solution at room temperature. The prostaglandins are measured by EIA. An aliquot of these reactions were added to the precoated plates together with AChE tracer and antiserum. They were kept in an orbital shaker for 18 h at room temperature. Finally, the plate was developed with Ellman's Reagent and kept in an orbital shaker in the dark for 60 min at room temperature. The absorbance was read at 420 nm. The standard drug diclofenacsodium was used as the positive control. The percent inhibition was calculated by the following formula:

$$\text{Percent Inhibition}(\%) = \frac{(\text{Activity of control} - \text{Activity of test})}{\text{Activity of control}} \times 100$$

The IC_{50} value was calculated by plotting the percent inhibition against the inhibitor concentration [33].

2.4. Anti-proliferative effect of M. pustulata treated albumen extracts

HepG2 hepatic cells were purchased from NCCS, Pune, India. Cells were developed in Dulbecco's modified eagles medium (Hi Media) supplemented with 10% FBS (Invitrogen). The cells were grown at 37 °C in 5% CO_2 (NBS, Eppendorf, Germany). Cells were trypsinized [500 μL of 0.025% Trypsin in PBS/ 0.5 mM EDTA solution (Hi Media)] for 2 min and transferred to T flask aseptically. Cells were then exposed to different concentrations of *M. pustulata* treated albumen extracts in DMSO (10, 50 and 100 μg mL^{-1}) and incubated overnight. The cell viability was determined by standard MTT assay after 24 h of incubation.

2.4.1. MTT assay

Briefly, HepG2 hepatic cells were cultured in DMEM in 75 cm^2 cell culture flasks at 37 °C in CO_2 incubator (95% air, 5% CO_2 and 100% relative humidity). The cells were then placed in 96 well plates (2×10^5 cells in eachwell) and incubated for 24 h. They were treated with various concentrations of albumen extracts (10, 50 and100 µg mL^{-1}). A respective control was maintained using DMSO for comparison of cytotoxic effects. Following treatment, 10 µL of MTT (5 mg mL^{-1}) was added to the plates and incubated for 4 h. The OD was calculated at 570 nm [34]. The cell viability was calculated using the following formula:

$$\text{Percentage of cell viability} = \frac{\text{OD value of experimental samples}}{\text{OD value of experimental controls}} \times 100$$

2.4.2. Hep G2 hepatic cell morphology using propidium iodide staining

Albumen extracts treated Hep G2 hepatic cells were stained by 50 µg mL^{-1} propidium iodide for 10 min. For cytotoxic effect the phase contrast microscope was used to observe the morphology of apoptotic cell nuclei [35].

3. Results

3.1. SDS-PAGE analysis

It is evident that the albumen of hen's egg treated with insect body of *M. pustulata* had an intense band separated at 30 kDa. However, the albumen of hen's egg had a band at around 25 kDa. On the otherhand, dried insect body of *M. pustulata* showed a strong protein band at 35 kDa (Fig. 1).

3.2. Antibacterial activity of albumen extract

The albumen extracts exhibited greater activity against bacteria comparable to that of commercial antibiotic ciprofloxacin. However, the albumen extracts showed higher activity against Gram negative *P. aeruginosa* and *P. vulgaris* compared to that of Gram positive *E. faecalis* and *S. aureus* (Table 1). The inhibition was 12.5 mm and 13.6 mm at 50 and 100 µg mL^{-1} respectively for *P. aeruginosa*. The inhibition was 13.4 mm and 15.3 mm at 50 and 100 µg mL^{-1} respectively for *P. vulgaris*. The zone of inhibition against *E. faecalis* was 9.4 mm and 10.8 mm at 50 and 100 µg mL^{-1}, respectively. Nevertheless, *S. aureus* exhibited

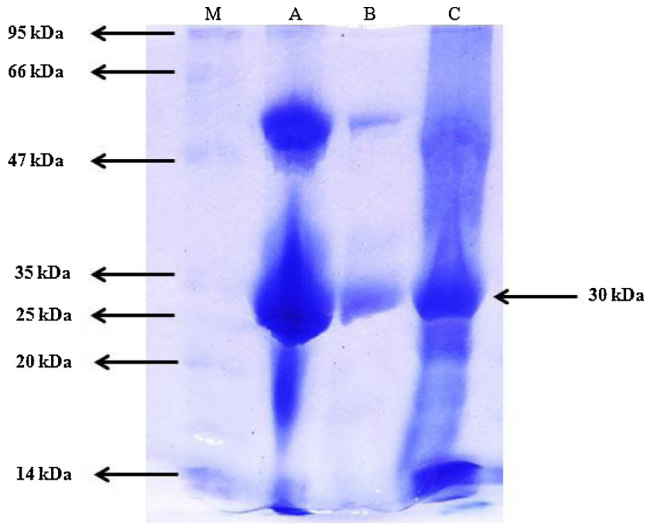

Fig. 1. SDS-PAGE of albumen. Lane M: Molecular marker; Lane A: albumen from hen's egg Lane B: albumen from *M. pustulata* and Lane C: hen's albumen treated with *M. pustulata* insect body.

Table 1
Antibacterial activity of hen's albumen extract treated with *M. pustulata* against tested bacteria.

Bacteria	Zone of Inhibition (mm)*		
	Albumen		Ciprofloxacin 5 µg/disc
	50 µg mL^{-1}	100 µg mL^{-1}	
Enterococcus faecalis	9.4[b]	10.8[c]	12.8[a]
Staphylococcus aureus	11.2[c]	12.1[a]	13.5[b]
Pseudomonas aeruginosa	12.5[a]	13.6[b]	15.1[d]
Proteus vulgaris	13.4[d]	15.3[e]	16.4[c]

* Values are mean ± SE of three replicates. Different alphabets in each column denote significant differences at $P < 0.05$ analyzed using ANOVA followed by Tukey's HSD test.

11.2 mm and 12.1 mm inhibition zone at 50 and 100 µg mL^{-1} respectively. The standard antibiotic ciprofloxacin showed 12.8, 13.5, 15.1 and 16.4 mm of inhibition zones against *E. faecalis*, *S. aureus*, *P. aeruginosa* and *P. vulgaris* respectively. The MIC of the albumen extracts against *E. faecalis*, *S. aureus*, *P. aeruginosa* and *P. vulgaris* were 3.4, 4.2, 2.6 and 2.8 µg mL^{-1} respectively (Table 2). The tested bacteria exhibited a well developed biofilm growth observed under light microscopy. The albumen extracts (100 µg mL^{-1}) treated bacteria had a poor biofilm growth compared to that of control (Fig. 2a). This was in accordance with the observation under CLSM where loose biofilm architecture of tested bacteria was recorded when treated with 100 µg mL^{-1} of albumen extracts (Fig. 2b). The hydrophobicity index of *E. faecalis* biofilm was also greatly reduced to 46% and 68% at 50 and 100 µg mL^{-1} respectively. The hydrophobicity of *S. aureus* biofilm was reduced to 56% and 75% at 50 and 100 µg mL^{-1} respectively. Nevertheless, a much greater inhibition of hydrophobicity was recorded against Gram negative *P. aeruginosa* and *P.* after treatment with the albumen extracts. At 50 µg mL^{-1}, *P. aeruginosa* and *P. vulgaris* hydrophobicity was inhibited to 74% and 90% respectively. However, the hydrophobicity was greatly inhibited at 100 µg mL^{-1} (88% and 94% against *P. aeruginosa* and *P. vulgaris* respectively) (Fig. 3).

3.3. Anti-inflammatory activity of albumen extracts

The anti-inflammatory activity of *M. pustulata* treated albumen extracts was determined by cyclooxygenase (COX) inhibitory assay. The human monocytic cell lines treated with LPS alone were used as the negative control in which the cells were grown with the influence of LPS and its OD value at 632 nm was found to be 0.2590. The standard drug diclofenac sodium was used as the positive control in which the drug inhibited the LPS treated cells with the percentage inhibitions of 43.28%, 67.25% and 69.61% at 10, 50 and 100 µg mL^{-1} respectively with a relative IC_{50} value of 231.86 µg mL^{-1} and the relative OD values decreased from 0.102 to 0.048. The albumen extracts at 10 µg mL^{-1} inhibited cyclooxygenase to 68.12% and its OD value at 632 nm was 0.1469. Similarly, the albumen extracts at 50 µg mL^{-1} inhibited

Table 2
MIC of hen's albumen extract treated with *M. pustulata* against tested bacteria.

Bacteria	MIC (µg mL^{-1})*
Enterococcus faecalis	3.4 ± 0.6[c]
Staphylococcus aureus	4.2 ± 0.4[a]
Pseudomonas aeruginosa	2.6 ± 0.3[b]
Proteus vulgaris	2.8 ± 0.5[b]

MIC-Minimum inhibitory concentration.
*Values are mean ± SE of three replicates. Different alphabets in each column denote significant differences at $P < 0.05$ analyzed using ANOVA followed by Tukey's HSD test.

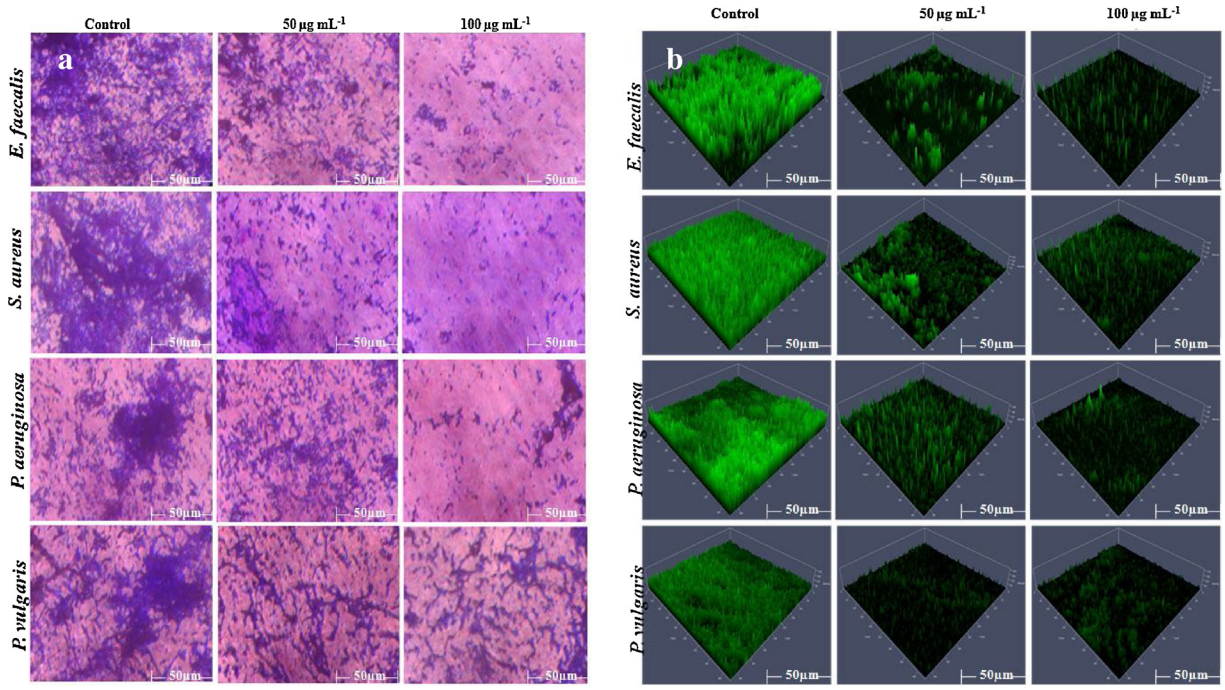

Fig. 2. The inhibition of Gram positive and Gram negative bacterial biofilms treated with hen's albumen extracts at different concentrations. (a) Light microscopic images (40× magnifications) (b) Confocal laser scanning microscopic images (40× magnifications).

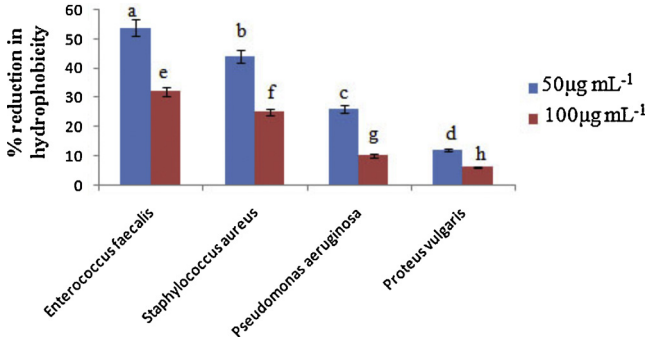

Fig. 3. Hydrophobicity index of Gram positive and Gram negative bacteria biofilms treated with the hen's albumen extracts at different concentrations. Each bar indicated mean ± SD of three replications. Bars not labeled by the same letter represent statistical significance at $p < 0.05$ using ANOVA followed by Tukey's HSD test.

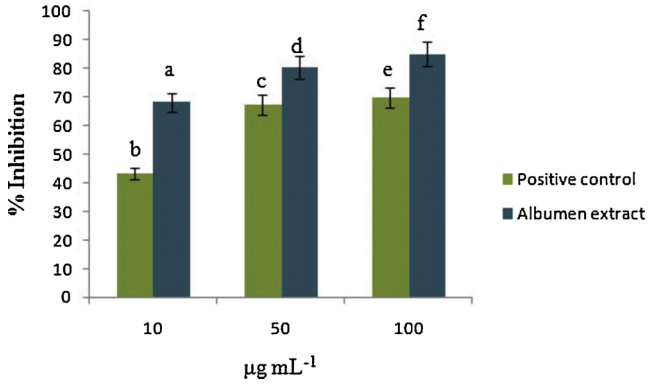

Fig. 4. Inhibition of cyclooxygenase enzyme revealing the anti-inflammatory activity of *M. pustulata* treated hen's albumen extracts. Bars not labeled by the same letter represent statistical significance at $p < 0.05$ using ANOVA followed by Tukey's HSD test.

cyclooxygenase to 80.19% and its corresponding OD value at 632 nm was 0.0848. However, the albumen extracts elicited an inhibitory level of 84.91% at 100 µg mL^{-1} (Fig. 4). The relative IC_{50} value was found to be 8.26 µg mL^{-1}.

3.4. Anti-proliferative activity of *M. pustulata* treated albumen extracts

The anti-proliferative activity of the albumen extracts was tested on cultured Hep G2 cancer cells by MTT assay. The untreated HepG2 cells were used as negative control and it showed 100% viability and the OD at 540 nm was 0.9723. The drug doxorubicin was used as the positive control which showed an effective anti-proliferative activity with the viability of HepG2 cells were reduced to 79.81%, 50.33% and 44% at 10, 50 and 100 µg mL^{-1} respectively with the corresponding IC_{50} value of 67.03 µg mL^{-1}. The corresponding OD values were decreased from 1.961 to 0.4582. Treatment with the albumen extracts at 10 µg mL^{-1}, the viability was reduced to 69.65% and the OD at 542 nm was 0.7760. The viability of HepG2 was reduced to 31.16% at 50 µg mL^{-1} and the OD at 542 nm was 0.4894. A reduction in the viability (23.61%) of HepG2 cells was observed at 100 µg mL^{-1} of albumen extracts (Fig. 5) and the OD at 542 nm was 0.4279. The corresponding IC_{50} value was 36.16 µg mL^{-1}. To authenticate the cytotoxic effects of *M. pustulata* treated albumen extracts, the cells were stained with propidium iodide and observed under the phase contrast microscopy. Interestingly, HepG2 cells showed morphological changes in the nucleus and loss of membrane stability at 100 µg mL^{-1} after 24 h (Fig. 6). This study revealed that the *M. pustulata* treated hen's albumen extracts were effective in controlling the proliferation of HepG2 cancer cells.

4. Discussion

In recent times, antibiotics are gradually losing their antibacterial effectiveness, as bacterial pathogens develop resistance against them. The antibiotic resistant microorganisms have the ability to spread infections [36]. Natural products have been demonstrated to be an alternative source for antibiotics as there has been significant progress in the exploration of new bioactive compounds from natural sources with

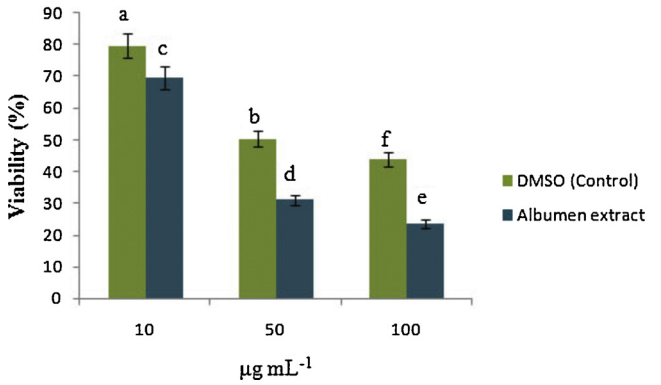

Fig. 5. Effect of the *M. pustulata* treated hen's albumen extracts on the viability of HepG2 hepatic cancer cells in comparison with the control (100 µg mL^{-1} of DMSO). Each bar indicates mean ± standard deviations of three replications. Bars not labeled by the same letter represent statistical significance at $p < 0.05$ using ANOVA followed by Tukey's HSD test.

greater antimicrobial activity [37–41]. Insects are the important source of antimicrobial peptides and the first antimicrobial peptide was isolated in 1981 from the giant silk moth *H. cecropia* [42]. The antibacterial activity of the albumen extracts of *M. pustalata* was higher than that of the antibiotic sparfloxacin against some pathogenic bacteria [43]. The whole body extracts of the red velvet mite *T. Grandissimum* and the larvae of the insect *Z. morio* showed antibacterial activity against all the pathogens tested [44,45]. In accordance to these reports, in the present study, a greater antibacterial activity was noted. It is suggested that the greater antibacterial activity may be due to the effectiveness of albumen extract. Biofilm is a key factor for bacterial resistance to antibiotics [46,47]. In the present study, effective inhibition of biofilm of Gram negative bacteria was noted compared to that of Gram positive bacteria. The hydrophobicity of bacterial biofilms was reduced significantly at 100 µg mL^{-1} of albumen extracts. Macromolecules are abundant in the egg white, and might play a role in the defense against bacterial contaminations. The effect of most of these compounds is mediated according to three mechanisms: direct lysis of the microorganism, chelation of essential nutrients and/or inhibition of microbial enzymes [48].

Inflammation helps to maintain the integrity of the organism during chemical, physical and infectious damages to the cell [49]. The anti-inflammatory activities of various insects have been extensively studied nowadays. The various extracts such as from Chinese medicinal ants *P. lamellidens,* the insect *U. dermestoides* and the venom from the Samsum ants *P. sennaarensis* were found to have remarkably anti-inflammatory activity in mice [50–52]. In the present study, the *M. pustulata* treated hen's albumen extracts showed anti-inflammatory effect on cyclooxygenase. Previously, it was reported that the egg white-chalcanthite and purple bamboo salts (EC + PBS) mixture had a potential anti-inflammatory effect against osteoarthritis [53]. *Mylabris* species are traditionally used in Chinese medicine and the potential of cantharidin as an antitumor drug has been growing rapidly because of its cytotoxicity against human tumor cell lines [54,55]. In accordance to these reports, in the present study, hen's albumen extracts treated with the dried insect body of *M. pustulata* showed excellent anti-proliferative activity. Treatment with the albumen extracts, the viability of HepG2 hepatic cancer cells was significantly reduced to 23.61% at 100 µg mL^{-1}. The

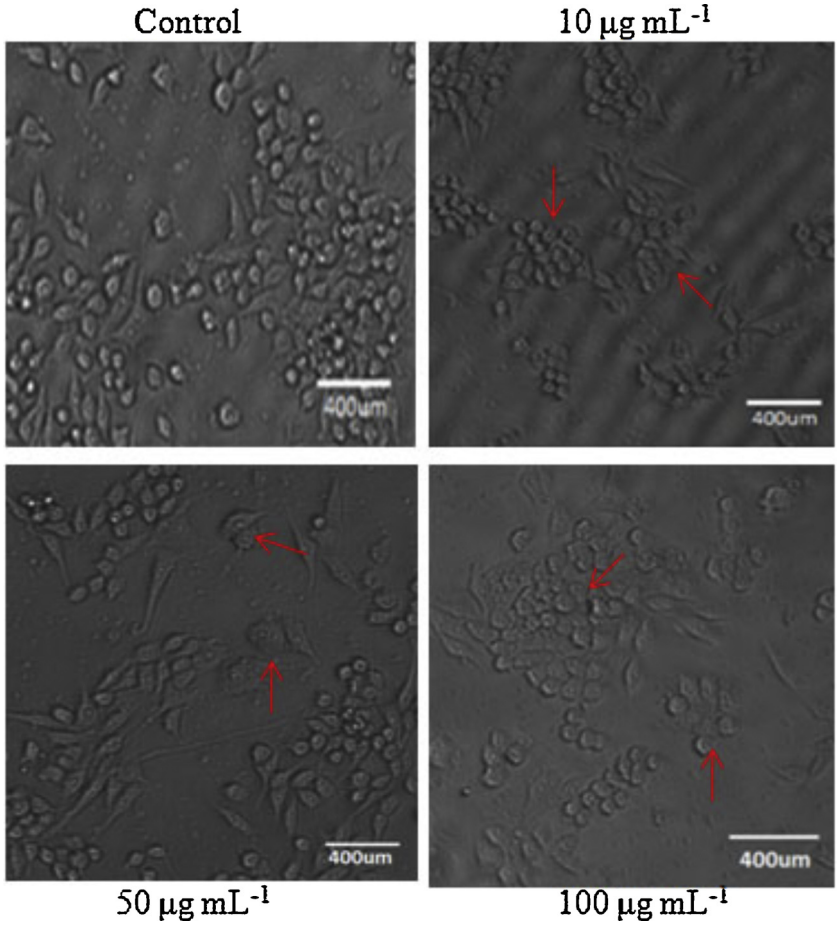

Fig. 6. Effect of the *M. pustulata* treated hen's albumen extracts on the morphology of HepG2 hepatic cancer cells in comparison with the control (100 µg mL^{-1} of DMSO). Arrow indicates morphological changes in apoptotic cells.

peptide fraction derived from hen's egg white induced apoptosis in mouse lymphoma cell line [56]. Furthermore, morphological changes in the HepG2 hepatic cancer cells were observed after treatment with the albumen extracts. Earlier studies demonstrated the anti-cancer activity of *M. cichorii* against Ehrlich ascites Carcinoma (EAC) and Murine ascites Dalton Lymphoma [57,58]. The bioactive compound cantharidin isolated from *M. cichorii* is a potential antitumor agent and inhibited the proliferation of EAC (Ehrlich ascites carcinoma) cancer cells with an IC_{50} of 25.8 μg/mL [57]. The present study concludes that the *M. pustulata* treated hen's albumen extracts showed effective antibacterial, anti-inflammatory and anti-proliferative activity.

5. Conclusion

Current investigation forms the primary attempt to exhibit the antibacterial, antibiofilm, anti-inflammatory and anti-proliferative activity of *M. pustulata* treated hen's albumen extracts. The hen's albumen extract showed effective inhibition of tested bacteria. The biofilm of tested bacteria was greatly inhibited at 100 μg mL^{-1}. Apart, the albumen extracts possessed anti-inflammatory activity by inhibiting the cyclooxgenase. In addition, the hen's albumen extracts treated with *M. pustulata* reduced the viability of HepG2 hepatic cancer cells and induced apoptotic cell morphology. This study concludes that the hen's albumen extracts treated with insect body of the blister beetle, *M. pustulata* may be used as a natural therapeutics for diseases associated with clinical bacteria, inflammations and cancer cells.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.pdpdt.2019.07.026.

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