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RESEARCH ARTICLE

Immunological efficiency of microbial melanin on bacterial pathogenicity

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ABSTRACT

In our present study, the immunological efficiency of brown Mel1 extracted from *Streptomyces longisporoflavus* NR043926 and black Mel2 extracted from *Aspergillus niger* MT355517 on bacterial pathogenicity was investigated. Mel1 and Mel2 were extracted and purified from the dried microbial biomass. Chemical characterization of extracted Mel pigments was compared with standard melanin. *In vivo*, estimation of the protective action of these pigments against *Escherichia coli* infection in Swiss albino mice which was identified by molecular characterizations 16S rRNA gene sequences, Albumin content was reduced in +ve control group (infected non – treated mice), while liver enzymes (ALT & AST) were increased in comparison to normal values were detected in immunized mice with Mel pigments. Eosinophil, monocyte values showed a non-difference, while the changes in lymphocyte numbers were paralleled to the response of total leukocyte counts. Serum antibodies total IgM in the non-immunized (+ve control group) mice were markedly lower than those observed in immunized mice (prevention groups and treated groups) with microbial melanin which can stimulate immunity system. Mel2 had high immunoprotection defense against *E. coli* infection in comparison to Mel1.

Keywords: *Escherichia coli,* Melanin, Pigments, Swiss Albino Mice

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INTRODUCTION

Melanins are phenolic polymer compounds that can be classified into two common groups based on their molecular precursors and chemical structures: eumelanin (dark brown-black) and pheomelanin (orange-red). These compounds are highly complex cross-linked biological polymers (Herrera *et al.,* 2019). Melanin is dark-brown to black macromolecular pigments produced by oxidative polymerization of phenol and/or indolic compounds found in plants, animals, and microorganisms. These pigments showed a broad range of biological functions including antioxidants, antivenin, antitumor, anti-virus, liver protecting and radio protective activities (Hung *et al.,* 2002; Hung *et al.,* 2003; Sava *et al.,* 2003; Hung *et al.,* 2004; De Cássia *et al.,* 2005; Dadachova *et al.,* 2007).

Melanin can be produced by several biological sources for instance, *Sepia officinalis*, black-bone silky fowl, *Osmanthus fragrans* seeds, tea leaves, *Hypoxylon archeri, Auricularia auricular, Tuber mehnosporum, Ophiocordyceps sinensis, Ommastrephes bartrami, Streptomyces lusitanus, Aspergillus fumigatus, Lachnum singerianum, Paecilomyces variotii, Aspergillus carbonarius* (Zhang *et al.*, 2015). It has been reported that *Klebsiella sp* bacteria could produce Mel pigment in the presence of L-tyrosine. *Spissiomyces endophytica* strain SDBR-CMU319, was able to produce a brown-black pigment in mycelia, the physical and chemical properties of the pigment have demonstrated alkali solubility, acid precipitation, decolorization with oxidizing agents and insolubility in most organic solvents and water (Shrishailnath *et al.* 2010; Suwannarach *et al.,* 2019).

Display physical and chemical properties identical to these pigments. Such physicochemical properties include heavy light absorption, unusual solubility and extraordinary redox effects, as well as a range of beneficial functions and wide-ranging biological activities such as antioxidant, free radical-scavenging,

antimicrobial activity, antitumor activity, antivenin activity, anti-HIV activity, hepatic activity (Sun *et al.*, 2016a,b). Melanin was isolated from *Exidia nigricans* exhibited antibacterial and antioxidant activities (Lopusiewicz, 2018).

The role of melanin produced by one pathogenic bacteria (*Klebsiella pneumoniae*) isolated from an infected wound on host immune responses which showed the increasing of melanin concentration leads to decrease in the phagocytic index. *In vivo* tests are done by using different concentration of extracted microbial melanin which acts as virulence factor throughout suppress the most important pro-inflammatory cytokines and that squeal to counteract immune responses (Saud & Alaubydi, 2019). In *Aspergillus sp*, melanin could inhibit the apoptotic pathways in macrophages that have phagocytized conidia via blocking the production of cytokine and pathogen-associated molecular pattern (PAMP) recognition by the immune system, furthermore, melanin could modulate immune functionality in the experimental mouse model (Volling *et al.,* 2011). Researchers of (Vasanthabharathi *et al.,* 2011) reported that the crude melanin pigment from *Streptomyces* had shown antibacterial activity against *Escherichia coli,* and *Lactobacillus vulgaris* had the ability to inhibit Human Immunodeficiency Virus (HIV) replication in vitro. In addition, it acts as a virulence factor in the pathogenesis of *Burkholderia cepacia* which serves as an example of how melanin production increases bacterial virulence. Because is little known about the pathogenic *K. pneumoniae* melanin and its role in bacterial pathogenicity. Therefore, this study was aimed to investigate the effect of pathogenic *K. pneumoniae* melanin on some immune parameters in mice. Todar (2004) recorded that *E. coli* infection led to several diseases including urinary tract infection, gastroenteritis, and neonatal meningitis. Some cases, virulent strains of *E. coli* cause peritonitis, mastitis, septicemia and Gram-negative pneumonia. Food poisoning can be a long term problem. Pathogenic *E. coli* organisms make a variety of virulence factors, and among them are the groups of toxic proteins called RTX (repeats in toxin) cytolysis.

The current study aimed to compare between chemical characterizations of high quantity of brown Mel1 - black Mel2 and standard melanin. Estimation of the immunological efficiency of extracted Mel pigments against *Escherichia coli* infection in Swiss albino mice.

MATERIAL AND METHODS

Tested microorganisms

Streptomyces longisporoflavus NR043926 and *Aspergillus niger* MT355517 were kindly provided from Botany and Microbiology Department, Microbiology Unit, Faculty of Science, Tanta University, Egypt. *Escherichia coli* strain was provided from Microbiology Department, Faculty of Medicine, Tanta University, Egypt, and was identified in 2015 by molecular characterizations 16S rRNA gene sequences in GATC Company using ABI 3730xl DNA sequence using forward and reverse primers (Sigma Scientific Services Co., Cairo, Egypt).

Tested animals

Ninety males of old Swiss albino mice weighing 22-25g were obtained from the animal breeding unit, Department of Zoology, Tanta University, Egypt, Animal House at National Research Centre - Veterinary Division. Mice were bred in the house of the animals, Department of Zoology, Faculty of Science, Tanta University, Egypt. They were held at a temperature of 22±2°C and fed with a normal diet and water under a 12 h light-dark period. The research was performed in compliance with ethical guidelines accepted by Tanta University's animal ethics committee guidelines.

Extraction of melanin pigment:

Extraction of melanin pigment (Mel1) extracted from *Streptomyces longisporoflavus* NR043926

Streptomyces sp was grown on starch- nitrate medium at (pH 7.0), Petri dishes were prepared and inoculated each with a loop of this actinomycetes. The inoculated Petri dishes were incubated for (7 days at 30°C). Disks (10 mm diameters) were cut from the margin of the colonies, boiled for min in 5.0 ml distilled water and then centrifuged for 15 min at (5000 rpm). After washing and centrifuged, the pigment was extracted by autoclaving the pellets for (20 min

at 120°C). The pellets were dissolved in (3.0 m1 of 1.0 M NaOH). The alkaline extracted melanin was acidified to (pH 2.0) with concentrated HC1 for melanin precipitation. The precipitated melanin was washed in 3 changes with distilled water and dried overnight at 20°C in a dry atmosphere. Then, the extracted pigment was further purified by acid hydrolysis (5.0 m1 of 7.0 M HC1) in a sealed glass vial for (2h at 100°C). The precipitated melanin was washed three times with dis. water and dried overnight at 20°C in a dry atmosphere. The precipitated melanin was further purified by acid hydrolysis (5.0 ml of 7 N HC1) in sealed glass vials for (2 h at 100°C) according to Gadd (1988).

Extraction of melanin pigment (Mel2) extracted from *Aspergillus niger* MT355517

Aspergillus niger was grown on Sabouraud's liquid medium at (pH 7.0 for 10 days at 30°C). Upon centrifugation, at the end of the incubation cycle (for 20 min at 8000 rpm). The precipitate obtained to remove carbohydrates and proteins was purified by acid hydrolysis using 6.0 M HCl at 100 °C for 2h. The precipitate melanin washed by distilled H_2O, after washing it was dried over anhydrous Na_2SO_4, then dissolved in 1M NaOH and centrifugation at 4000 rpm for 15 min for TLC separation, infra-red spectra analysis and using authentic melanin as reference according to Rowley and Pint (1972) and Sava (2001).

Preparation of standard melanin

Normal melanin was bought from Sigma Company (Cairo, Egypt) Dissolving 0.05g of melanin in 2.0 ml of 1.0 M NaOH prepared the normal melanin solution mg/ml.

Identification of MDR *E. coli* by 16S rRNA gene sequence

The 16S rDNA was an amplification of bacteria was prepared by PCR using universal primer 9F (5'- AGA GTT TGA TCC TGG CTC AG-3') and 1541R (5'- GGT TAC CTT GTT ACG ACT -3'). Thermal cycling conditions were as follows: DNA denaturation at 98 ˚C for 3 min followed by 30 cycles at 94˚C for 1 min, annealing of the primers at 52 ˚C for 1 min, and then extension at 72 ˚C for 5 min. The reaction mixture was held for 5 min at 72 ˚C and then cooled to 4 ˚C. Sequencing of the PCR product was carried out by using an automated sequencer and the same primers as above for sequence determination (Macrogen Inc., Seoul, Korea). The sequence was compared for similarity with the reference species of bacteria contained in genomic database banks, using the NCBI Blast. A phylogenetic tree based on 16S rRNA gene sequence was constructed with the neighbor-joining method in MEGA version 4 software (Singh *et al.,* 2012).

***In vivo*. The immunological efficiency of microbial melanin against *Escherichia coli* infection in mice**

Different treatments of Mel1 and Mel2 against *Escherichia coli* infection in Swiss albino mice

In vivo the productive efficiency of extracted melanin against *E. coli* infection in Swiss albino mice were divided into six groups (n = 6/group). The first group was let healthy without infection as a negative control. The sixth group was served as a positive control (6 mice) injected intraperitoneally with 200 µl of sterile phosphate-buffered saline (PBS) containing tested *E. coli* at $1x10^8$ CFU/ml. The second group and the fourth group (treated groups) each contain nine mice were injected intraperitoneally with 200 µl of sterile phosphate-buffered saline (PBS) containing tested *E. coli* at $1x10^8$ CFU/ml and then treated with injected intraperitoneally (i.p.), in three successive doses, with 200 µg\24 h of the following preparation: 600 µg suspension of Mel1 and Mel2 respectively in 1 ml phosphate-buffered saline (PBS), The third group and fifth group (prevention groups), each group contain nine mice were injected intraperitoneally (i.p.), in three successive doses, with 200 µg\24 h of the following preparation: 600 µg suspension of Mel1 and Mel2 respectively, in 1 ml phosphate-buffered saline (PBS), then were injected intraperitoneally (i.p.), with 200 µl of sterile phosphate-buffered saline (PBS) containing tested *E. coli* at $1x10^8$ CFU/ml). The treatment was repeated every five days and then five days of the final dose injection, the mice liver and kidney were removed under aseptic condition. The blood was collected from retro-orbital plexus. The blood samples were allowed for clotting, the serum was separated by centrifugation (at 2500 rpm for 15 min at 37°C)

and the serum was used for estimation of biochemical parameters immunoglobulin M (IgM), complete blood picture, AST, ALT, ALP, urea, and creatinine. Serum aspartate aminotransferase (AST) and serum Alanine aminotransferase (ALT) activities *(*Reitman and Frankel, 1997). The total protein concentration, albumin, urea and creatinine (Mallay and Evelyn, 1987). Blood samples were also collected from mice after bleeding for blood count (total and differential blood counts). All analyses were performed using commercially available kits from Span Diagnostics Ltd.

Serum chemistries

Determination of liver function enzymes (ALT and AST)

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) catalytic concentrations were determined from the rate of decrease of NADH by means of the lactate dehydrogenase (LDH) coupled reaction which measured at 340 nm according to Gella *et al.* (1985) method.

Assurance of Total Protein

About 20 µl of each serum of the sixth groups of studied mice were transferred to a clean tube, 1 ml of protein reagent were pipette to the serum sample. For celebration pipetted 20 µl of total protein STD. cat. No. 1618 to 1.0 ml of the reagent in a clean tube. The blank was prepared by pipetting 20 µl of distilled water to 1.0 ml of the reagent. All test tubes were shacked well and incubated for 5 min at 37°C. The absorbances were measured at 545 nm against the blank (Merck, 1974).

Total protein conc. (g/dl) =
(Sample reading absorbance/
Standard reading absorbance) × 6 (Standard conc.)

Assessment of Albumin

Albumin was determined according to Doumas *et al.* (1971). A volume of 10 µl from each serum of the sixth groups of studied mice was transferred to a clean test tube containing 2.5 ml of albumin reagent (R2). For celebration pipette 10 µl of albumin stander reagent (R1) to 2.5 ml of R2 in a clean tube. The blank was prepared by pipetting 2.5 ml R2 in a clean tube. All tested tubes were shacked well and incubated at 37°C for 5 min. The absorbance was measured at 620 nm against the blank.

Albumin conc. (g/dl) =
(Sample reading absorbance /
Standard reading absorbance) ×5 (Standard conc.)

Kidney function biomarkers (urea and creatinine) assessment

Quantitative determination of urea and creatinine in plasma were enzymatically assayed by spectrophotometrically measured at (578 nm) according to Fawcett and Scott (1960).

Hematology

Measuring of red blood cell count (Egyptian Diagnostic Media Company, Egypt) according to (Cheesbrough, 2005) method

Briefly, 4 ml of R.B.Cs were added to the narrow stoppered pipeline, counting soluting solution. Using Automatic pipette draw 0.02 (20 micron) capillary or venous blood, add the blood to 4 ml of R.B.Cs counting soluting solution and mix well, securely attach the special cover glass supplied to the counting chamber. Fill the chamber with the well-mixed diluted blood using a fine bore Pasteur pipette, and allow the cells to settle for 5 minutes. Using a 40 X target. Count the number of cells in 1/5 sq.mm; use 5 of the large center square's small squares. Number of R.B.Cs /mm^3= number of R.B.Cs counted in five small squares x 10.000.

Measure of Hemoglobin

This method is done according to (Cheesbrough, 2005) method by spectrophotometer at Wavelength: 540 nm & Temperature 37 c and Cuvette: 1 Cm light path.

$$\frac{\text{Length of red cell column (mm)}}{\text{Length of total column (mm)}} = \text{PCV}$$

Measuring of red cell indices

Red cell indices most frequently used in the investigation of anemia are: Mean cell hemoglobin concentration (MCHC) & Mean cell volume (MCV) & Mean cell hemoglobin (MCH).

$$\frac{\text{PCV (l/l)}}{\text{RBC} \times 10^{12}/\text{l}} = \text{MCV fl*} \quad \frac{\text{Hbg/l}}{\text{PCV (l/l)}} = \text{MCHC g/l} \quad \frac{\text{Hb in g/l}}{\text{RBC} \times 10^{12}/\text{l}} = \text{MCH pg*}$$

Count of platelets

The blood is diluted 1 in 20 in an ammonium oxalate reagent filtered solution that lyses the red cells. Platelets are counted microscopically using a counting chamber ruled by Improved Neubauer and the number of platelets per liter of blood calculated using Cheesbrough (2005) method.

Immunological examinations

The immunological experiment was designed to harvest the total and differential count of blood leukocytes and estimation of total IgM.

The total and differential count of blood leukocytes in mice
Count of white blood cells

By using WBC diluting fluid, the whole blood is diluted 1 in 20 in acid reagent which haemolyzes the red cells (not the nucleus of nucleated red cells), Letting the white cells count. White cells are counted microscopically using an improved counting chamber (haemocytometer) controlled by Neubauer and the amount of WBCs per liter of blood determined according to the Cheesbrough, 2005 method.

Blood films. The technique of making a thin blood film by Cheesbrough (2005).

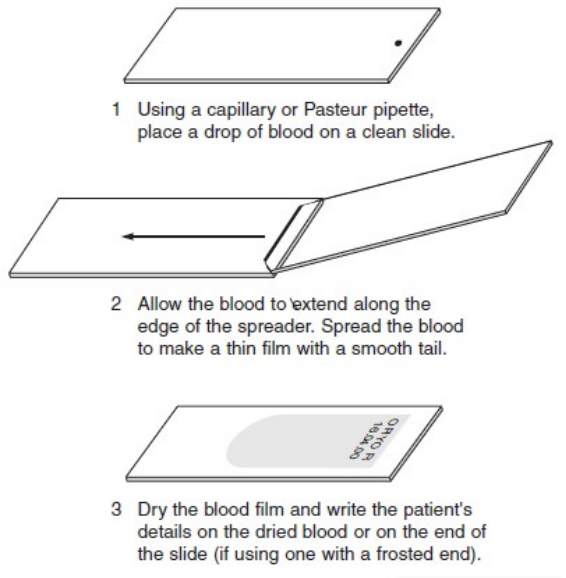

Figure 1. Spreadin g a thin blood film.

Estimation of serum antibodies immunoglobulin M (total IgM)

IgM is a standardized, turbidimetric assay used to calculate total IgM in the serum or plasma mice. When combined with samples containing IgM, the anti-human IgM antibodies form insoluble complexes. The dispersing light of the immune complexes depends on the IgM concentration in the mice sample, and can be quantified by comparison from a calibrator of known IgM concentration due to the methods of Friedman and Young (1997).

Determination of bacterial load in infected organs (liver, kidney, spleen, small intestine and large intestine in mice

In a beaker containing 70 % ethanol, mice were anesthetized with ether, killed and surface sterilized to fully wet them. Growing mouse had been dissected to obtain the target organs under aseptic conditions. In a tissue grinder or homogenizer, each organ was homogenized. The growing organ was homogenized in a saline solution sterilized to 5.0 ml. The homogeneous was diluted serially, and 0.1 ml was plated on EMB paper. The slabs were incubated for 18 h at 37 °C. And the number of colonies to record the number of Colony Forming Units per organ (CFU / organ) was counted. All the tests were performed triplicates and the graph was plotted with the mean values (Neuenhahn *et al.,* 2010).

Number of bacteria (CFU/organ) = (average number of bacterial colonies/ amount plated) x dilution.

Histopathological examination

Histopathological examination was carried out for positive control, prevented, treated and negative control group**.**

Separation of mice's liver tissues

Treated and control liver tissues were exercised from the liver under aseptic sample were immediately soaked in 10% formaldehyde fixative solution and let for 24 h.

Staining of liver sections

Principle dye was hematoxylin; hematoxylin solution is composed of 2.0 g hematoxlin +100 ml methanol + 100 ml glycerol +3 g ammonia alum + 100 ml distilled water + 0.24 g Na-iodate. The counter dye is eosin; eosin solution is composed of 1.0 g eosin (YC. 1.45360), 5 mg glacial acetic acid and complete to 1000 ml of 70% ethanol. Hematoxylin solution was coated with deparaffinized slides for 20 min. They were then washed in 70 per cent ethanol for 5 min with distilled water destained by 0.5 per cent HCl.

A drop wise of ammonia has been applied until nuclei come dark against the colorless background. Slides in 70 per cent ethanol were dehydrated. Dehydrated slides were painted with eosin solution for 5 min counter. Surplus stain has been removed.

Slides were dehydrated by serial ethanol dilutions. Clearing of the section was obtained by xylene till visible red tissues with brownish nuclei could observe. Stained sections were permanently mounted by soaking with aqueous Hoyer mounting medium (30g Arabic gum + 200g choloralhydrate +16ml glycerol+50 ml distilled water). A glass cover was stuck and left overnight. Histological examination was done in Histology Department, Faculty of Medicine, Tanta University by fixing a portion of the liver tissues in 10% formalin solution, processed and embedded in paraffin wax. Liver tissues blocks were sectioned at 5 µm thick and stained with Haematoxylin and Eosin (H&E) (Mahmoud *et al.,* 2011). All liver sections were photographed under Carl Zeiss Axiosta light microscope connected with digital Canon camera soft program zoom browser at 10xmags in Central Laboratory, Zoology Department, Faculty of Science, Tanta University, Egypt.

Statistical analysis

The statistical analysis was done using version 6.12 of the SAS programme. The data obtained were statistically analyzed to assess the importance of the various treatments using a one- and two-way variance analysis (ANOVA). Results are presented as mean ± SD (standard deviation) from three readings. P-value was used as a critical value to accept the significance of the tested effect, if P-value is less 0.001 then the significance of the tested factor was accepted (SAS, 1985).

RESULTS

Physical and chemical properties of Mel1 and Mel2

Properties of the extracted Mel pigments were illustrated in Table 1. They were insoluble in water producing dark brown flocculent precipitate, insoluble in hydrochloric acid and organic solvents such as (acetone, chloroform and ethanol) or such solvent mixture, decolorized by oxidizing agents (H_2O_2 and NaOC1), gave positive reactions to polyphenol compounds and produced 1 percent (w/v) flocculent brown precipitates with $FeC1_3$.

Identification of MDR Escherichia coli by 16S rRNA gene sequence

The genotypic identification by 16S rDNA sequence, comparison of the tested nucleotide sequences with members of bacteria clearly showed that the tested *E. coli* revealed 93% similarity with other strains of bacteria strains accessed from GenBank *E. coli* O157:H7 strain Sakai with accession NR074891. Other strains belong to section *Escherichia coli* sp exhibited slightly similarity (91%) as shown in Figure 2.

Effect of Mel1 and Mel2 against *Escherichia coli* infection in Swiss albino mice

Serum chemistries

Determination of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total proteins, urea and creatinine

Effect of injected intraperitoneally of Mel1 and Mel2 on (total proteins, albumin, ALT, AST, urea, and creatinine) in plasma of treated and prevention groups were illustrated in Table 2 compared with -ve control. In +ve control group (infected non – treated mice), albumin content was reduced to 2.30 g/l (normal value of albumin in mice is 2.8-3.0 g/l), while liver enzymes ALT level (128 U/L) and AST level (299.33 U/L) were increased in comparison to immunized mice (treated groups and prevention groups) with microbial melanin (normal values of ALT is 17-77 U/L & AST is 54-289 U/L in mice). Other biochemical parameters including, total proteins, urea and creatinine values were within the normal level. Mel pigments had high productive efficiency against *E. coli* infection.

Hematology

Effect of injected intraperitoneally of the extracted melanin on (RBC 106/mm^3, HGB g/d, HCT %, Platelet $10^5/mm^3$, MCV U^3, MCHC %) in plasma of treated and prevention groups was illustrated in Table 3 compared with -ve control, the results were within the normal limits. The higher values obtained within the immunized groups with Mel pigments than values in +ve control group (infected non-treated).

Table 1. Characterization of the extracted melanins**.**

Characters	Mel1	Mel2	Authentic Mel
Color in 1.0 M NaOH	Dark brown	Black	Black
Solubility in water	Not soluble	Not soluble	Soluble
Solubility in organic solvent	Not soluble	Not soluble	Soluble
Solubility in KOH	Soluble	Soluble	Soluble
Precipitation in 3N HCl.	Precipitated	Precipitated	Precipitated
Reaction for polyphenol test ($FeC1_3$ 1 %)	Reddish brown ppt	Reddish brown ppt	Reddish brown ppt
Reaction with H_2O_2	Decolorization	Decolorization	Decolorization

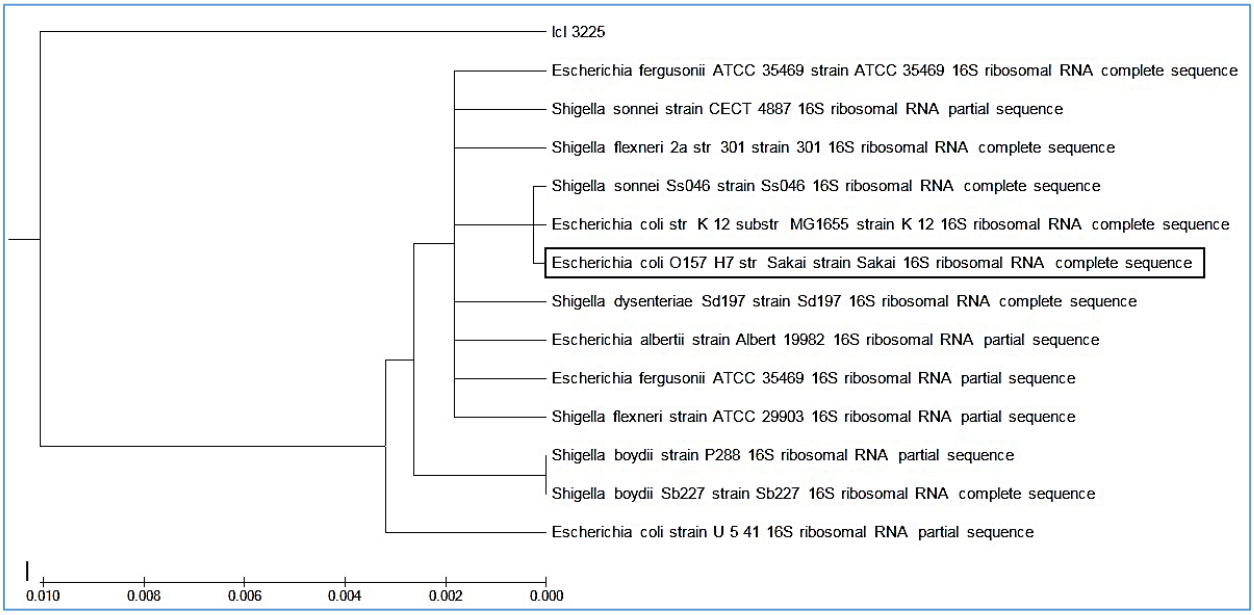

Figure 2. Phylogenetic analysis of *E. coli* based on complete sequencing of 16S rDNA.

Table 2. Effect of Mel1 and Mel2 on total proteins, albumin, ALT, AST, ALK-p, urea and creatinine.

Biochemical measures	+ve control group	Immunized with Mel1		Immunized with Mel2		-ve control	Ref. rang	ANOVA	
		Treated group	Prevention group	Treated group	Prevention group			F-Value	p-Value
AST (U/L)	299.3^{a} ± 34.6	198.3^{b,c} ± 21.5	179.0^{b,c} ± 11.07	147.3^{c} ± 23.5	140.0^{c} ±12.9	240.0^{a,b} ±25.07	54-298	21.03	0.000
ALT (U/L)	128.0 ± 12.5	85.3 ± 13.5	81.7 ± 13.8	75.3 ± 11.4	71.7 ±10.8	77.0 ±13.8	17-77	0.48	0.786
Albumin (g/l)	2.3 ± 0.67	2.60 ± 0.45	2.9 ± 0.3	2.70 ± 0.65	3.0 ±0.50	2.8 ±0.25	2.8- 3.0	0.75	0.601
TPL (g/l)	6.1 ± 1.6	5.37 ± 1.5	5.2 ± 1.1	6.57 ± 1.7	6.8 ±1.30	6.30 ±1.5	3.5-7.2	0.58	0.713
ALK-P (U/L)	95.7 ± 15.1	91.00 ± 10.7	79.3 ± 12.4	96.0 ± 12.7	83.3 ±14.4	96.0 ±15.3	35-96	0.87	0.531
Urea (mg/dl)	33.7 ± 6.8	33.00 ± 7.6	31.97 ± 6.2	32.0 ± 7.6	29.7 ± 5.4	33.0 ±6.5	8-33	0.982	0.982
Creatinine (mg/dl)	0.73^{a} ± 0.16	0.69^{a} ± 0.2	0.50^{a,b} ± 0.18	0.54^{a} ± 0.20	0.49^{a,b} ± 0.18	0.04^{b} ±0.002	0.2-0.9	6.39	0.004

Value is the mean ± SD of three replicates. P-values < 0.05 were considered to be statistically significant. a*, b, c and d letters showed that means do not share letters are significantly different.*

Immunological examinations

The immunological experiment was designed to harvest the total and differential count of blood leukocytes and estimation of total IgM.

The total and differential count of blood leukocytes in mice

Infected mice with *E. coli* injected with different concentration of the extracted melanin to reverse the total and differential count of leukocytes. Table 4 was shown that the total leukocyte count in the first group (positive control group) was $6700x10^6$/ml, second group (treated with Mel1) was $8200x10^6$/m1 and the third group (prevented with Mel1*)* was $10.800x10^6$/ml but the fourth group (treated with Mel2) was $10000x10^6$/ml, and fifth group (prevented with Mel2) was $12.500x10^6$/ml. While. The sixth group (negative control) was $7600x10^6$/m1 (normal value of total leukocyte count in mice is 6 -$15000x10^6$/ml).

In vivo results on the immune system of mice showed a significant change in the differential count of leukocytes (including neutrophils, Eosinophils, lymphocytes and monocytes). Neutrophil values of the first group were 12/100 leukocyte cell, the second group were 36/100 leucocyte cell, the third group were 38/100 leucocyte cell, fourth group were 38/100 leucocyte cell, the fifth group were 40/100 leucocyte cell and the sixth group were 39/100 leucocyte cell, (Normal value of neutrophil in mice 10-40 per 100 leukocytes). Eosinophil values showed a non-difference for the six groups of mice (normal value of eosinophils in mice 0-4 per 100 leucocytes), while the changes in lymphocyte numbers were paralleled to the response of total leukocyte counts in the first group, lymphocyte counts were markedly higher (86 per 100 leucocytes) than that of sixth (negative control) group (57 per 100 leucocytes), the second group were 48 per 100 leucocytes, the third group were 50 per 100 leucocytes, the fourth group were 50 per 100 leucocytes and the fifth group were 58 per 100 leucocytes (normal value of lymphocyte in mice 40-80 per 100 leukocytes). Monocyte values showed no differences in three groups of mice (normal value of monocyte in mice 1-4 per 100 leukocytes). Immunized mice with Mel2 evaluated highest immunological efficiency against *E. coli* infection. This hypothesized that melanin produced by higher fungus *Aspergillus niger* MT355517 immunogenic against bacterial pathogenicity.

Estimation of serum antibodies total IgM

To investigate the role of immunoprotection of serum antibodies in immunized mice by the Mel1 and Mel2 against *E.coli* infection, serum antibodies immunoglobulin M were evaluated in non-treated (reference value), immunized (the treated groups and prevention groups) with Mel pigments and non-immunized (positive infected control) mice after bacterial infection. Serum antibodies IgM in the non-immunized (positive infected control) mice were markedly lower than those observed in immunized mice (the treated groups and prevention groups) before and after being challenged with a large *E. coli* infection when compared with the reference value in Table 5. Mel pigments can stimulate the immune system against the bacterial challenge, and Mel2 had extremely productive function toward *E. coli* pathogenicity.

Determination of bacterial count in infected organ

The obtained data illustrated in Table 6 revealed that bacterial load in the infected control increased with time and reached its maximum value after 15 days especially in the large intestine. Immunization with Mel2 in treated or prevention groups reduced bacterial load in all investigated organs referring to infected control.

All treatments were able to reduce the bacterial load with time, and the lowest bacterial count was detected after 15 days. In 5-day reduction, percent was ranged from zero to 36%, from zero to 100%, from 0 to 38.3 and from 28 to 40% in liver, spleen, small and large intestines, respectively. In 10-day reduction, percent was ranged from 45 to 68%, 100 to 100%, from 18.6 to 56 and from 32 to 60% in the liver, spleen, a small and large intestines, respectively.

Table 3. Effect of Mel1 and Mel2 on complete blood count.

Group		Hematological measures						
		RBC (10^6/mm^3)	HGB (g/d)	HCT (%)	Platelet (10^5/mm^3)	MCV (U^3)	MCH (UUG)	MCHC (%)
Positive (Infected) control		9.41 ± 0.61	13.95 ± 0.64	46.6 ± 3.54	8.85 ± 3.46	48 ± 2.83	14.6 ± 0.85	29.15 ± 1.48
Immunized with Mel1	Treated group	9.5 ± 0.71	14.1 ± 1.27	47.05 ± 4.31	10.6 ± 2.26	49 ± 1.41	14.7 ± 0.99	29.6 ± 1.06
	Prevention group	9.77 ± 0.33	14.1 ± 1.41	47.6 ± 4.24	11.65 ± 3.6	49 ± 1.41	14.7 ± 0.71	30.3 ± 2.12
Immunized with Mel2	Treated group	9.88 ± 1.17	14.75 ± 1.20	48.5 ± 5.09	10.95 ± 2.3	48.5 ± 3.54	14.9 ± 0.99	29.8 ± 2.12
	Prevention group	10.3 ± 0.43	15 ± 0.57	49.6 ± 4.24	12.2 ± 2.55	49.5 ± 2.12	15.1 ± 0.71	30.45 ± 1.91
Negative control		10.47 ± 0.75	15.8 ± 0.00	50.7 ± 3.25	11.2 ± 4.24	50 ± 2.83	15 ± 1.13	30.6 ± 1.13
ANOVA	F-Value	0.70	1.05	0.29	0.27	0.16	0.09	0.22
	p-Value	0.645 n.s.	0.466 n.s.	0.904 n.s.	0.917 n.s.	0.968 n.s	0.990 n.s.	0.939 n.s

Table 4. Total and differential count of blood leukocytes in mice.

Group		Total leucocyte counts/ml	Differential leukocytes count %			
			Neutrophils	Eosinophils	Lymphocytes	Monocytes
Positive (Infected) control		6700^{f} ± 0.00	12^{d} ± 0.11	1^{b} ± 0.00	86^{a} ± 5.7	1^{c} ± 0.00
Immunized with Mel1	Treated group	8200^{d} ± 120	36^{a} ± 0.28	1^{b} ± 0.00	48^{b} ± 4.8	3^{b} ± 0.23
	Prevention group	10800^{b} ± 109	38^{b} ± 0.45	1^{b} ± 0.00	50^{b} ± 3.8	3^{b} ± 0.00
Immunized with Mel2	Treated group	10000^{c} ± 117	38^{b} ± 0.23	2^{a} ± 0.11	50^{b} ± 4.3	4^{a} ± 0.17
	Prevention group	12500^{a} ± 137	40 ± 0.43	2^{a} ± 0.00	58^{b} ± 5.4	3^{b} ± 0.37
Negative control		7600^{e} ± 122	39^{a,b} ± 0.57	1^{b} ± 0.00	57^{b} ± 5.7	3^{b} ± 0.00
ANOVA	F-Value	1165.72	2443.61	396.69	24.29	79.56
	p-Value	0.000	0.000	0.000	0.000	0.000

Table 5. Estimation of serum antibodies total IgM.

Days	Groups				
	Immunized with Mel1		Immunized with Mel2		Positive (Infected) control
	Treated group	Prevention group	Treated group	Preventiongroup	
5	135^{d,e} ± 6.3	140^{c,d,e} ± 3.2	140^{c,d,e} ± 5.0	150^{c,d} ± 4.0	120^{e} ± 6.00
10	160^{b,c} ± 5.4	175^{a,b} ± 7.1	175^{a,b} ± 4.0	185^{a} ± 7.0	160^{b,c} ± 10.0
15	155^{b,c,d} ± 4.8	160^{b,c} ± 7.4	160^{b,c} ± 6.0	175^{a,b} ± 9.0	144^{c,d} ± 12.1
Reference value	75	75	75	75	75

In 15-day reduction, percent was ranged from 78.6 to 86%, 100 to 100%, from 49 to 78 and from 56 to 78% in the liver, spleen, a small and large intestines, respectively. No bacteria were observed in kidneys in all groups (Table 7). Observed that the maximum values of bacterial load reduction detected in the prevention groups (15 days). Thus reducing the degree of infection was observed through the reduction of bacterial load and enhancement of digestive system function.

Histopathological examination of mice's liver tissues in response to *Escherichia coli* infection

Livers of 4 mice groups were histopathological examined, first positive infected group, second group (prevention group immunized with Mel2), third group (treated group immunized with Mel2), and negative control group. Histopathological studies on healthy mice liver tissues showed normal hepatic cells, while histological section in liver tissues of the positive infected group (untreated) showed the presence of inflammatory reaction and vacuolar or hydropic degeneration as early infiltration. Histopathological studies on prevention and treated groups immunized with Mel2 revealed that liver tissues with normal hepatic cells as shown in Figure 3.

DISCUSSION

Interacellular brown Mel1 of *Streptomyces longisporoflavus* NR043926 and black Mel2 of *Aspergillus niger* MT355517 were precipitated when their solutions were acidified by concentrated HC1 to pH 2.0, completely soluble after 2 hours in KOH at 100°C, were insoluble in water and organic solvents, were decolorized by an oxidizing agent and gave polyphenols a positive reaction and formed flocculent brown precipitates with $FeC1_3$ 1% (w/v).

Table 6. Determination of *E. coli* load in infected organs.

Day	Liver	Spleen	Small intestine	Large intestine
No. of bacteria (CFU/organ) x10^4				
Positive (Infected) control				
5	$55^{a} \pm 6.6$	$8^{a} \pm 0.96$	$30^{a} \pm 3.6$	$60^{a,b} \pm 7.2$
10	$55^{a} \pm 6.2$	$8^{a} \pm 0.96$	$30^{a} \pm 3.4$	$60^{a,b} \pm 6.8$
15	$60^{a} \pm 7.1$	$8^{a} \pm 1.02$	$30^{a} \pm 3.3$	$65^{a} \pm 7.8$
Treated group immunized with Mel2				
5	$50^{a} \pm 6.1$	$6^{b} \pm 0.72$	$28^{a} \pm 3.36$	$50^{b} \pm 5.8$
10	$30^{b} \pm 3.6$	0	$20^{b} \pm 2.4$	$34^{c} \pm 4.08$
15	$10^{d} \pm 1.2$	0	$11.2^{c,d} \pm 1.34$	$20^{c,d} \pm 2.4$
Prevented group immunized with Mel2				
5	$24^{b,c} \pm 2.7$	0	$12.7^{b,c} \pm 1.52$	$30^{c} \pm 3.6$
10	$14^{c,d} \pm 1.7$	0	10 ± 1.2	$20^{c,d} \pm 2.4$
15	$8^{d} \pm 1.03$	0	$5^{d} \pm 0.6$	$10.2^{d} \pm 1.21$

Table 7. Effect of Mel2 on *Escherichia coli* reduction in infected organs.

Day	Liver	Spleen	Small intestine	Large intestine
	Reduction of *E. coli* (%)			
	Treated group immunized with Mel2			
5	0	0	0	28
10	45	100	18.6	32
15	78.3	100	49	56
Prevented group immunized with Mel2				
5	36	100	38.8	40
10	68	100	56	60
15	86	100	78	78

These results are almost similar to the results reported by Krysciak (1985) who found that the chemical properties of dark-colored Mel pigments were insoluble in the most solvents, bleaching by oxidizing agents (H_2O_2), and soluble in alkali and phenols. Butler and Day (1998) confirmed the same results by described chemical properties of Mel pigments as amorphous, insoluble in aqueous or organic fluids, resistant to concentrated acids and susceptible to bleaching by oxidizing agents. ferricyanide. Melanin pigments are polyphenol compounds that are insoluble in water. Natural melanin's metal ion chelating property is exploited to remove heavy metals from the contaminated water. We optimized marine bacterial melanin biosynthesis using growing growth media, medium components and operating conditions (Harsha *et al.,* 2018). The Immunological efficiency of Mel1 and Mel2 was investigated on mice infected with *Escherichia coli* which identification molecularly by 16S rRNA gene sequence revealed 93 % a similarity to *E. coli* O157:H7 strain Sakai with accession NR074891.

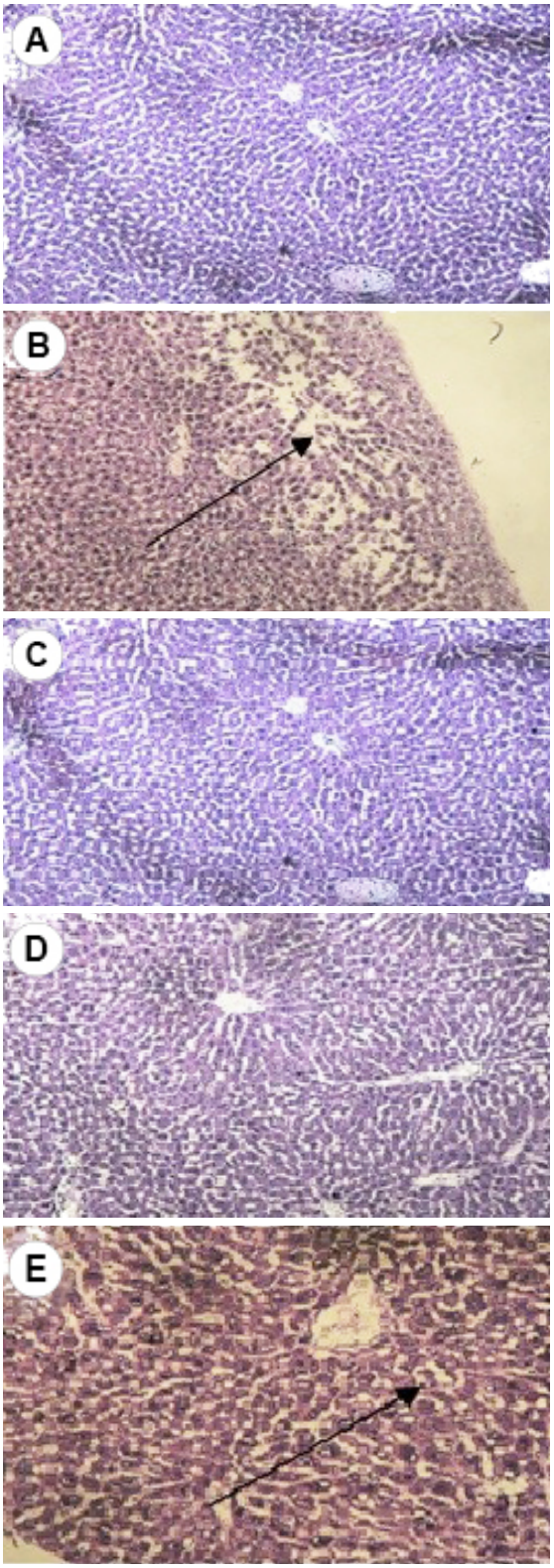

Figure 3. T.S in mice liver tissues infected by *E. coli* magnification 100x. A. Negative control showed normal hepatic cells. B. Positive control (infected non – treated) showed the presence of inflammatory reaction and vacuolar or hydropic degeneration. C.D. Prevention group (immunized with Mel2) showed normal hepatic cells. E. Treated group (immunized with Mel2) showed normal hepatic cell.

Shrishailnath *et al.* (2010) also studied chemical characterization of the Mel pigment particles produced by *Klebsiella* sp. showed that melanin pigment was acid resistant, alkali soluble, insoluble in most of the organic solvents and water, got bleached when subjected to the action of oxidants as well as reductions, and was precipitated with ferric chloride and potassium

Albumin makes up over half of the total protein in serum. It is the main protein that provides the essential colloid osmotic pressure that controls the movement of water through the capillaries and diffusible solutes. Albumin is used in conveying bilirubin, hormones, metals, vitamins, medicines. Binding fatty acids and maintaining them in a soluble state in the plasma plays a significant role in fat metabolism. Albumin content showed decreased in In positive control group to 2.30 g/l (normal value of albumin in mice is 2.8-3.0 g/l) this agree with Berne *et al.,* (1983) they illustrated that the consequence of serum albumin decrease in *S. Aureus* infected mice is a fluid change from the intravascular to the interstitial space resulting in loss of the intravascular volume and edema development.

Liver enzymes as ALT level (128 U/L) and AST level (299.33 U/L) were increased in + ve (infected – non treated) mice in comparison to – ve control and immunized mice with Mel pigments which were detected within normal limits. The liver is damaged or certain enzymes are poured into the bloodstream in cases of liver cell death arising from other causes, such as shock or drug toxicity. The liver cells spill the enzymes into the blood, increasing the levels of enzymes that cause liver damage and necrosis (Iweala and Obidoa, 2010). Onu *et al.,* (2013) studied the effect of aqueous stem bark extract of *Khaya senegalensis* on some biochemical, hematological and histopathological parameters of rats. The data showed that twenty-eight days of administering to *Khaya senegalensis* at the experimental dose resulted in substantial rises in levels of urea and creatinine. The extract also increased the serum production of ALT, AST, and ALP considerably. Protein and albumin levels have improved dramatically when compared with control values. In the current study, the level of ALk-p, urea and creatinine showed no changes in all groups. Navratil *et al.* (1998) and Iweala and Obidoa (2010) indicated that the changes in biochemical parameters in male rats fed with *Ocimum gratissimum* supplemented diet showed no changes in the activities of ALT and AST enzymes. Taha (2013) indicated that after 8 and 10 days of treatment, with non-toxic doses the biochemical and histopathological analyses revealed that at high doses the aqueous extract of *Euphorbia prostrata Aiton* which induced liver damage, as illustrated by a rise of serum transaminases levels in male and female rats, respectively.

Effect of injected intraperitoneally of the extracted melanin on (RBC 106/mm^3, HGB g/d, HCT %, Platelet 10^5/mm^3, MCV U^3, MCHC %) in plasma of treated and prevention groups with extracted melanin compared with -ve control, the result was within the normal limits. The higher values obtained within the prevention groups and treated groups than values in + ve control group. Leukocytes count in the immune response against pathogenic bacteria showed in Table 4 which evaluated the highest values in immunized prevention group with Mel2 in comparison to other groups.

Differential leukocytes count divided into neutrophil, eosinophil, lymphocyte, and monocyte. Eosinophil values showed a non-difference for the six groups, while the changes in neutrophil and lymphocyte numbers were paralleled to the response of total leukocyte counts. Finally, monocyte values showed no differences in the three groups of mice. Microbial Melanin can stimulate the immune system against the bacterial challenge, and Mel2 had high immunoprotection defense against *E. coli* infection. Similar result found by Iweala and Obidoa (2010) who studies the changes in some biochemical parameter in male rats fed with *Ocimum gratissimum* supplemented diet for six months, there was a significant increase in increase in white blood count. The role of leukocytes in the immune response against pathogenic bacteria was reported by many investigations. The absence of leukocytosis in serious bacterial pathogenicity is considered as a prognostic sign in *S. aureus* septicemia which serious disease with high mortality (Al Awar, 2009)

The investigation of the role of antibodies immunoprotection M (total IgM) against *E.coli* infection was determined. Serum IgM antibodies in the non-immunized (positive infected control) mice were markedly lower than those observed in immunized (the prevention groups and treated groups) with extracted melanin before and after being challenged with a large bacterial infection when compared with the reference value. Observed that microbial melanin enhancing the immune system against the bacterial challenge. The obtained results were comparable with those of another study investigating that melanin developed by the higher fungus, *Bjerkandera adusta*, was immunogenic against fungal infection, melanin was isolated from the melanized fungal hymenium layer and used in mice immunization, the radioimmunodiffusion diffco-plate response of IgM was evaluated for accurate quantitative immunoglobulin measurements in biological fluids. After the challenge candidasis was identified in mice target organs, and the degree of kidney damage was measured by urea. The results show that melanin can be immunogenic; this is indicated by IgM follow-up, and its development may suggest that this amorphous insoluble polymer can stimulate the immune system against the latter challenge, thereby reducing the degree of infection. This was noticed by reducing candidiasis and improving function in the kidneys (Allam and Abd El-Zaher, 2012).

Another study we investigated that murine monoclonal antibodies (MAbs) to melanin were recently generated and used to study melanization of *C. neoformans in vivo*, whether MAbs to melanin are protective against *C. neoformans* infections in mice. These findings suggest that MAbs to melanin will extend the survival of mortally infected mice and reduce the fungal burden. Nosanchuk *et al.* (1998) hypothesized that the fungus developed melanin, *C. neoformans* showed immunogenicity. *C. neoformans* melanin was isolated from melanized fungal cells and used to immunize BALB / c mice, C57BL/6, BALB / c and T cell deficient (nude). The response including IgM and IgG isotypes showed that melanin can be immunogenic, and the humoral immune response can be T independent cell.

Bacterial load in the infected control increased with time and reached its maximum value after 15 days especially in the large intestine. With time, all treatments were able to reduce the bacterial load, and after 15 days, the lowest bacterial count was detected. The maximum values of bacterial load reduction detected in the prevention groups (15 days). No bacteria were observed in kidneys in all groups**,** the degree of infection was observed through the reduction of bacterial load and enhancement of digestive system function. Histopathological studies on healthy mice liver tissues showed normal hepatic cells while histological section in liver tissues of +ve group (infected non-treated) showed the presence of inflammatory reaction and vacuolar or hydropic degeneration as early infiltration. This result was supported by Morris-Jones *et al.* (2005) who confirmed the presence of melanin particles in the target organ pathogenesis (heart, lungs, liver, spleen, and kidneys). Digestion of infected murine kidneys led to the isolation of melanin particles that reacted with monoclonal antibodies (MAb) to antimelanin. In vivo evidence reveals the compounds that inhibit melanization, and that can reduce *C. neoformans* virulence.

The administration of monoclonal antibodies to melanin or glyphosate, which inhibits *C. neoformans* melanization prolongs the survival of infected mice lethally *C. neoformans* (Nosanchuk *et al.,* 2001; Rosas *et al.,* 2001). The production of medicinal products that interfere with melanin polymerization or rearrangement can be seen as useful therapeutic compounds for the treatment of these melanin-producing melanin fungi and other pathogens (Nosanchuk *et al.,* 2003). Vilanova *et al.* (2004) found that the immunization with native aspartic proteinase 2 and alum could trigger a highly specific humoral response with a 20-fold decrease in *C. albicans* load in the kidney when compared with unimmunized animals. This investigation was in agreement with Cunha *et al.* (2010), nitrite production (Bocca *et al.,* 2006). Phagocytosis blocking has been observed by melanized *Cryptococcus neoformans* and *pencillium brasiliensis* (Wang *et al.,* 1995; da Silva *et al.,* 2006; Volling *et al.,* 2011). Melanin can improve immune function in other ways. In experimental mouse infections, cryptococcal

melanin amends cytokine levels due to infection and activates the complement system (Rosas *et al.,* 2000a). The melanin pigment immunogenicity is promising due to their implication in the pathogenesis of several diseases, including some fungal infections, malignant melanoma, Parkinson's disease, traumatic anterior chamber uveitis, and vitiligo (Wasserman and Walt, 1973; Hill, 1991; Broekhuse *et al.,* 1992; Enochs *et al.,* 1994; Wheeler and Bell, 1998). For a number of pathogenic microbes, the synthesis of melanin was associated with virulence. Melanin is thought to contribute to microbial virulence by reducing the susceptibility of a pathogen to killing by host antimicrobial mechanisms and by influencing the immune response of the host to the infection. Melanin synthetic pathways are thus potential targets for the discovery of antimicrobial drugs. Interestingly, both host and microbial melanin's drug-binding properties may influence the outcome of antimicrobial therapy (Nosanchuk and Casadevall, 2006).

CONCLUSION

Mel1 and Mel2 were insoluble in water, decolorized by an oxidizing agent, and gave a positive reaction for polyphenols. In vivo, Mel2 had high immunoprotection defense against *E. coli* infection in comparison to Mel1. The degree of bacterial infection was observed through the reduction of bacterial load and enhancement of the digestive system function in immunized mice. Histopathological studies on healthy mice liver tissues showed normal hepatic cells, while histological section in liver tissues of +ve group ((infected non-treated) showed the presence of inflammatory reaction and vacuolar or hydropic degeneration as early infiltration.

Conflicts of interest

All authors have approved this article and declare no conflict of interest.

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Egyptian Association for Cancer Research (EACR)
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EACR is an NGO society that was declared by the Ministry of Social Solidarity (Egypt) No. 1938 in 19/11/2014 based on the initiative of Prof. Mohamed Labib Salem, the current Chairman of EACR. EACR aims primarily to assist researchers, in particular young researchers in the field of cancer research through workshops, seminars and conferences. Its first international annual conference entitled "Anti-Cancer Drug Discovery" was successfully organized in April 2019 (http://acdd.tanta.edu.eg). Additionally, EACR aims to raise the awareness of the society about the importance of scientific research in the field of cancer research in prediction, early diagnosis and treatment of cancer. EACR is also keen to outreach the scientific community with periodicals and news on cancer research including peer-reviewed scientific journals for the publication of cutting-edge research. The official scientific journal of EACR is "International Journal of Cancer and biomedical Research (IJCBR: https://jcbr.journals.ekb.eg) was successfully issued in 2017 and has been sponsored by the Egyptian Knowledge Bank (EKB: www.ekb.eg).

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