Cytotoxicty and Dna Fragmentation in Cancer Cell Lines, Hep–2 and Amn–3, Induced by A Novel Maroon Pigment from Mutant Strain of *Rugamonas rubra* Rr62

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Abstract

The mutant strain of *Rugamonas rubra* RR62 produces a novel maroon pigment after cultivation in casein-enriched medium for 48h at 30ºC**.** The pigment was extracted by acidified methanol and subsequently purified by centrifugation. The partial purified compound was identified using UV– spectrometer. The UV– visible spectrum showed a maximum absorption peak at 500nm. This study was conducted to detect the cytotoxic effects of the maroon pigment on two types of cancer cell lines, Hep–2 and AMN–3, using a cell proliferation assay. The growth of Hep–2 and AMN–3 were significantly ($P \leq 0.05$) blocked at high concentrations of pigment and after increasing time of exposure; the inhibition rate was 90 and 91%, respectively. Whereas, no toxic effects were observed, on both models of cell lines, at low concentrations. In addition, the maroon pigment induced apoptosis. The high concentrations of pigment (125 and 31.25µg/ml) was significantly ($p \leq 0.05$) increased the percentage of fragmented DNA of Hep–2 and AMN–3(78.4% and 75% respectively).

Keywords: *Rugamonas rubra*, pigment, purification, cytotoxicity, Apoptosis, DNA fragmentation.

Introduction

Secondary metabolites of bacterial origin include various enzymes, pigments, antibiotics etc could be of importance to mankind in many ways [1]. Regarding pigments it was found that Prodigiosin group, a red color pigment, produced by both gram negative and gram positive bacteria such as *Serratia marcescens*, *Pseudomonas magneslorubra*, *Vibrio erythrous*, *Rugamonas rubra* and *Streptomyces* sp [2, 3]. Prodigiosin is a typical alkaloid compound that consisting of three pyrrole rings and a pyrrlyol-pyrromethene skeleton with a C-4 methoxy group [4]. Recently, these tripyrrole molecules have received renewed attention owing to reported immunosuppressive and anticancer properties [5, 6].Therefore, in our laboratory and during screening for potential anticancer drugs; we observed a novel pigment with maroon color, synthesized by a mutant strain of *Rugamonas rubra* RR62. The extract of this pigment was used to assess inhibitory activity and induction apoptosis by DNA fragmentation in cancer cell lines.

Materials and Methods

Bacterial strain

The mutant strain of *Rugamonas rubra* RR62, provided kindly by Microbial Ecology Dept/ Ministry of Science and Technology, was used in this study.

Production of the maroon pigment

Bacterial culture

R. rubra RR62 was grown in fermentation broth medium consisted of 20g of casein, 2g of glucose, 10% of glycerol, 1.7g of K_2HPO_4, and 1g of $MgSO_4.7H_2O$ per liter and incubated at 30ºC for 48h.[7].

Purification and identification of pigment

Bacterial culture was harvested by centrifugation at 5000rpm for 15 min at 4ºC. The supernatant was passed through a 0.22 µm filter and this step was repeated three times. The filtrate was extracted with a mixture of methanol/ 1N HCl (24:1 v.v) and precipitated by centrifugation. After centrifugation (5000rpm for 15min), the solvent of the supernatant was evaporated under vacuum [8]. The partial purified extract was re–dissolved in acidified methanol and identified using UV-spectrophotometer (Hitachi, Japan) [9].

Cell Culture

Two types of cancer cell lines, Human larynx epidermoid carcinoma (Hep–2) and mammary adenocarcinoma (AMN–3), were obtained kindly from Iraqi Center for Cancer Researches and Medical Genetics (ICCRMG) and used in this study. Cells were grown at 37ºC in humidified atmosphere containing 5% CO_2 in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), glutamine (2mM), penicillin (100IU/ml), and streptomycin (100µg/ml)[10].

Cytotoxic assay

The cytotoxic of soluble pigment was investigated using a cell proliferation assay developed by promega (Cell Titer 96 Aqueous One Solution Cell Proliferation Assay). Briefly, 2×10^4 exponentially growing cells was seeded in 96–well microculture plates with various pigment concentrations (0.122–250µg/ml) in a volume of 100µl. After incubation period (24, 48, and 72h) at 37ºC, 20µl of MTT [3–(4, 5–dimethlthiazol–2–yl–2, 5–diphenyltetrazolium bromide] was added to each well, and the plates were incubated for a further 3h at 37ºC. The absorbance for each concentration was carried out in triplicate including untreated cell control. The inhibitory rate (%IR) of cell proliferation was calculated according to equation:

%IR=A–B/A × 100, while the proliferation rate (%PR) was estimated according to equation:

%PR= B/A× 100. A; represented the absorbance of control, B; represented the absorbance of treatment [11].

DNA fragmentation analysis

Hep–2 and AMN–3 cells at density of 5×10^5 cell/ml treated with various concentrations of soluble pigment (0.122–250 µg/ml) for 48h and then suspended in 0.8ml of 10mM phosphate buffer saline (PBS), pH7.4, and 0.7ml of ice cold lysis buffer. The lysate cells transferred to microfuge tubes and centrifuged at 13000g at 4ºC for 15 min to separate fragmented DNA from high molecular weight DNA. The fragmented DNA was suspended in 1.5 TE buffer, added 10% TCA and incubated for 10min at 25ºC. Centrifugation was done again at 500g for 15 min and the supernatant was discarded. The precipitate was resuspended in 10% TCA and boiled at 100ºC for 15 min. Dimethylamine reagent (1ml) was added to the supernatant, and incubated for 18h at 30ºC. The absorbance was measured at 600nm. The percentage of DNA fragmentation was measured according to formula: $OD_{600}$of the supernatant/ [OD_{600} of supernatant+OD_{600} of pellet] ×100 [12].

Statistical analysis

The differences between treated and untreated groups were determined by Student's *t*- test, and the significance threshold was set to $P \leq 0.05$.

Result and Discussion

Production and identification of maroon pigment

The extracellular maroon pigment was observed in the fermentation broth. It was extracted and partial purified subsequently. The absorbance of maroon pigment was scanned using UV-spectrophotometer, it showed a maximum absorption peak at 500nm (Fig. (1)).

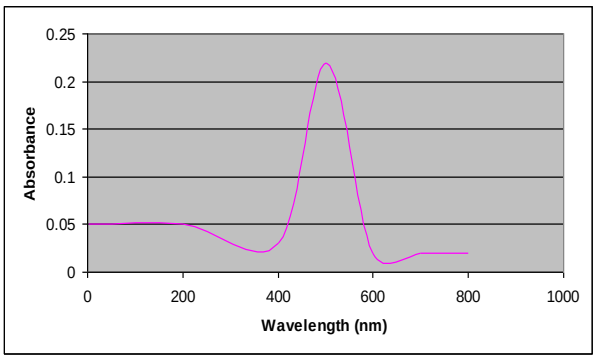

Fig. (1) Absorbance of maroon pigment.

This findings confirmed that the maroon pigment is a novel and it may relate to prodigiosin group that had absorption spectrum ranged between 400–500nm [13].

Cytotoxic effect of maroon pigment on cancer cell lines

Two types of cancer cell lines, Hep–2 and AMN–3, treated with or without tested pigment at various concentrations and incubated at different periods (24, 48, and 72h). The results were evident that this biomolecules inhibited cell growth of cancer in a dose and time-dependent manners. The high concentrations (125 and 250µg/ml) were

significantly ($p \leq 0.05$) inhibited cell growth of both models of cancer cell lines when compared with control (Fig.(2) and Fig.(3)). In contrast, the low concentrations of the pigment ranged from 0.122 to 31.25 µg/ml had no toxic effect on cancer cells. Remarkably, maroon pigment after 24h and 48h of exposure time and at high concentrations were almost completely blocked cell growth of Hep–2 and AMN–3; the inhibition rate was 90% and 91%, respectively.

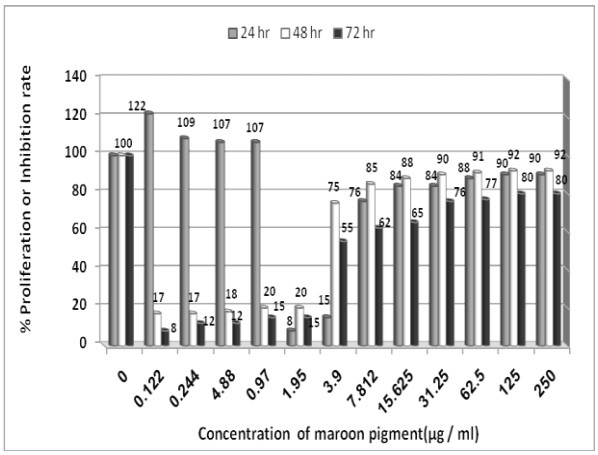

Fig. (2) Inhibition activity of various concentrations of maroon pigment on cellular growth of Hep-2 at different exposure time.

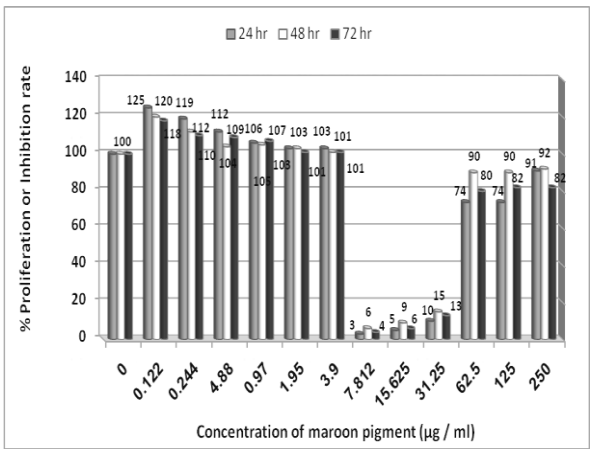

Fig. (3) Inhibition activity of various concentrations of maroon pigment on cellular growth of AMN-3 at different exposure time.

However, this finding illustrated that this pigment had a cytotoxic properties and antitumor activity. The molecules of this compound may act as a proton sequestering agent that destroys the intercellular pH gradient, and propose that its main toxic effect could be related to its action on mitochondria, where it exerts an uncoupling effect on the electronic chain transport of protons to mitochondrial ATP synthase. This mechanism of action differs from those induced by conventional chemotherapeutic drugs, suggesting a possible role for this pigment to enhance the effect of antitumor agents in the treatment of cancer cells [14]. On the other hand, Montaner and his colleagues (2000)[15] demonstrated that the molecules of such pigments may induce tumor suppressor gene, p38, by blocking the phosphorylation and decrease the expression of oncogenes, c-Jun and c-fos, and this action may inhibit cancer cell growth.

Maroon pigment induces apoptosis on cancer cell lines

Apoptosis was expressed by measuring the percentage of fragmented DNA. Therefore, the ability of maroon pigment to induce apoptosis in Hep–2 and AMN–3 cells treated with or without pigment at various concentrations and different time of exposure was studied. Fig.(4) and Fig.(5) showed that this pigment was induced apoptosis in both models of cancer cell lines in a concentration-dependent fashion.

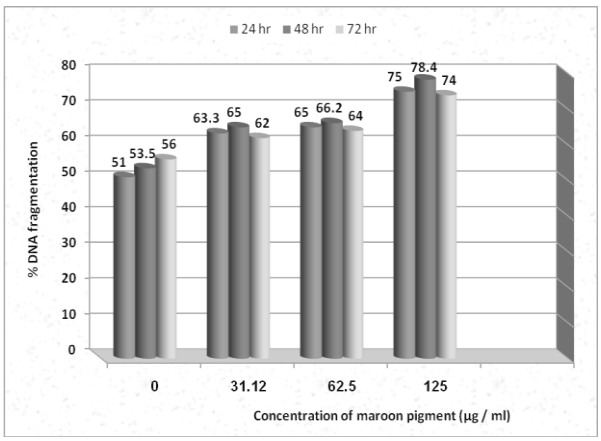

Fig. (4) DNA fragmentation of Hep-2 cells after exposure to different concentrations of maroon pigment.

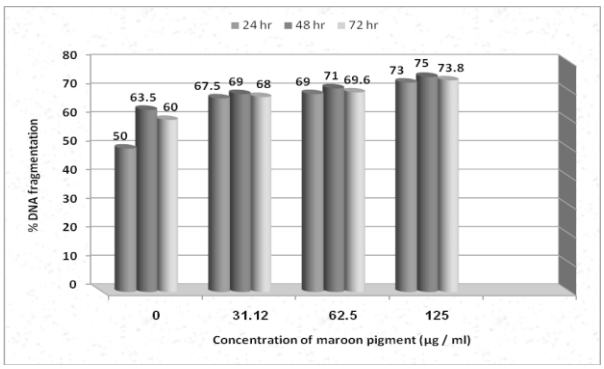

Fig.(5) DNA fragmentation of AMN-3 cells after exposure to different concentrations of maroon pigment.

Obviously, Maroon Pigment at high concentrations (125 and 31.25µg/ml) was significantly ($p \leq 0.05$) increased the percentage of fragmented DNA of Hep–2 and AMN–3; the percentage was 78.4% and 75% respectively. In this experiment, the activity of pigment did not depend on the time of exposure. Indeed, the mechanism of maroon pigment on DNA of cancer cells is not completely known. However, It may induce DNA breaks by cleavage poly(ADP-ribose) polymerase (PARP) which in turn activate caspases such as –9,–8, and –3 and this action in directly induces apoptosis[16].

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الخلاصة

أنتجت السلالة الطافرة *Rugamonas rubra* RR62، بعد تنميتها في وسط غني بالكازائين لمدة ٢٤ ساعة و بدرجة حضانة ٣٠مْ، صبغة غير مألوفة مارونية اللون. أستخلصت الصبغة بالميثانول الحامضي ونقيت بشكل جزيئي مرات عديدة بأستخدام الترسيب. شخصت الصبغة بأستخدام مقياس الاشعة فوق البنفسجية. وقد أظهرت الصبغة أعلى قمة أمتصاص عند الطول الموجي ٥٠٠ نانوميتر. كما شملت هذه الدراسة التحري عن التاثيرات السمية الخلوية للصبغة في نوعين من الخلايا السرطانية (AMN–3 و Hep–2) بأستخدام أختبار تضاعف الخلايا. لوحظ أن التراكيز العالية من الصبغة وزيادة فترة التعريض أدت الى أيقاف نمو الخلايا السرطانية وبشكل معنوي (بمستوى ٠.٠٥)؛ إذ بلغت نسبة التثبيط ٩٠،٩١% على التوالي. وعلى العكس يلاحظ أي تأثير سمي على الخلايا المعاملة في التراكيز الواطئة. فضلا عن ذلك تمكنت الصبغة من حث الموت المبرمج للخلايا فقد أدت التراكيز العالية (١٢٥ و٣١.٢٥ مايكروغرام/ مل) من تجزئة دنا الخلايا السرطانية المعاملة(Hep–2 و AMN–3) و بشكل معنويا حيث بلغت النسبة المئوية للدنا المجزء ٧٨.٤ و ٧٥ % على التوالي.