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EVALUATION OF SOME IMMUNOLOGICAL AND CYTOTOXICITY EFFECT OF CRUDE *RADISH SATIVUS* PIGMENT ON RD AND L20B CELL LINES

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ABSTRACT
Medicinal plants have the ability to synthesize different compounds that have important biological functions. These compounds in plants mediate their effect on the human body. This enables the plants to have a beneficial pharmaceutical effect, but also with some side effects, one of these plants is the Red Radish, which produces different compounds such as pigment, that is chemically related to anthocyanin. In this study, the effects of this crude pigment at different concentrations (200, 100, 50, 25, 12.5) µg/200µl on several locally isolated pathogenic bacteria (*Escherichia coli, Klebsiellia pneumoniae, Pseudomonas aeruginosa, and Staphylococcus aureus*) showed, no effect at all. Also, this study aimed to find the counteract proliferation ability of this crude pigment extract on two types of cell lines (RD and L20B). Depending on inhibition rate, the crude pigment exhibited excellent affectivity against cancer cell lines, but with various degrees depending on the usable concentrations. On the other hand, DR cell line was more sensitive than L20B despite the effectiveness of pigment on both cell lines. Whereas, Interleukins (TNF-α, INF-γ, and IL-1β) analysis of all tested concentrations of pigment in both cell lines showed, significant elevation ($P \leq 0.05$) of these cytokines in comparison with the control group. Thus we can concluded, the cytotoxic effects of red radish pigment depends on the type of cell line used, the concentration of the crude pigment extract, and the duration of the exposed time. And this pigment has an excellent effect on cancerous tissues throughout induction of tumor cells to produce pro-inflammatory cytokines.

KEY WORDS: Red Radish, pro-inflammatory cytokines (TNF-α, INF-γ, and IL-1β), RD, L20B

INTRODUCTION

The term "herbs" refer to plants or parts from them, including grasses, flowers, berries, seeds, leaves, nuts, stems, stalks, and roots, which are used for their therapeutic and health-enhancing properties. The proper and judicious uses of plants are often successful in the treatment of illness when other chemical medications are failing (Donga *et al.,* 2011). Herbs demonstrate great variation in the treatment of a broad variety of health needs. This type of plant is the widely used as medicines in the world. About 80% of the world's population employs herbs as a primary medicine (Graz and Malebo 2011; Chintamunnee and Mahomoodally 2012). *Raphanus sativus* (*R. sativus*) radish, usually arises from several types of shape root of several sizes and colors ranged from white to black and fruits that are grown in all seasons (Banihani, 2017). Radish includes raphanin that act as anti-bacterial, fungal, tumors, and reduces the normal p roduction of thyroid hormones. It has been found that anthocyanin (ACN) pigments are responsible for red and purple radishes (Tibor, 2006). It's one of the flavonoid compounds, soluble in water, and the color changes according to pH, they are odorless and may be found in different parts of the plant (Park *et al.*, 2011).Biosynthesis of anthocyanin pigments as all other flavonoids are assembled from two different cascades of chemical raw materials in the cell. More than 5 enzymes are required to synthesize these pigments. Even minor disturbances of genetic or environmental factors of any mechanism of these enzymes would turn off anthocyanin production.

While the biological synthesis of anthocyanin is relatively high, plants benefit significantly from environmental habitation, disease tolerance, and pest tolerance provided by anthocyanin's (Jack, 2009). The major anthocyanin's of radishes is pelargonidin-3-sophoroside-5-glucoside acetylated with malonic acid and either ferulic or p-coumaric acid (Wrolstad *et al.*, 2001). Also, *R. sativus* included 7-glucoside-pelargonidin compound is stable at 60˚C and light, may be used as a food colorant (PuJing *et al*., 2012). Thus the aim of the present study is to investigate some antimicrobial, cytotoxicity, and immunological effects of anthocyanin pigment extracted from *R. sativus* on two types of cell lines (RD and L20B).

MATERIALS & METHODS

This study was conducted during the period from February-July /2017. Several pathogenic bacterial strains isolated locally were obtained from Biotechnology Department/ College of Science/ University of Baghdad. Whereas, cytotoxicity measurement was carried out in Biotechnology center/ University of Al- Nahrain and all materials were supplemented from the same center.

Pigment preparation

This was accomplished according to (Kumkum and Ranjana, 2014) method with several modifications. One kilogram of local radish was washed with tap water to remove any muddy residue. Then the pigmented peels were taken away carefully and soaked in a suitable amount of PBS buffer (pH 7.2) for 1 hour until the pigment was dissolved, then mixed by the homogenizer for few minutes. The peels residue were squeezed and filtered by

Whatman no.1 filter paper and sterilized by Millipore filter 0.45 mm. Then by using lyophilizer (ALPHA 1-2 LD/Germany), the pigment was concentrated and kept in deep freeze at – 20° C for further experiments.

Antimicrobial activity test

According to Bauer and coworkers (Bauer *et al.,* 1966) method, a serial dilution of (200, 100, 50, 25, and 12.5) µg/200 µl of crude extract was prepared. Several pathogenic bacterial strains isolated locally (*Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumonia*e) were reactivated using brain heart infusion broth (hi media) and incubated overnight at 37°C. These bacterial isolates were cultured by striking on Muller Hinton agar (hi media) plates. Wells on agar were prepared and filled with a serial dilution of crude extracts; the plates were left for several minutes, and then incubated at 37°C for 18 hours.

Cytotoxic activity measurement

Depending on Vida *et al.* (Vida *et al.,* 2016), the toxic effects of lyophilized crude extract pigment of radish on two types of cancer cell lines, which were: rhabdomyosarcoma (RD/ATCC® CCL-136™), its *Homo sapiens*, human/ muscle/ spindle cells/ Large multinucleated cells. And L20B is represented as transformed mice intestinal cells. While, chicken embryo cell line (UMNSAH/DF-1 (ATCC® CRL-12203™) was used as a positive control. All cell lines are supplemented by Biotechnology center/University of Al Nahrain. Cell proliferation kit MTT (Roche applied sciences, Cat. No. 11 465 007 001) was used to evaluate the cytotoxic effect.

Preparation of pigment stock solution

The stock solution was prepared for 48 hours of red radish pigment in a conc. 30 mg/ml, and with a duplicate serial dilution of (15, 7.5, 3.75, 1.9, 0.95, 0.47, 0.234, 0.12, 0.06, and 0.03) mg/ml was also prepared.

Measurement of the cytotoxic effect of crude pigment extract on cell lines

Cell line

According to Marina *et al.* (2014), the RD, L20B, and chicken embryo cell lines were cultured in the tissue culture 96-well plates (Becton, Dickinson, Franklin Lakes, NJ, USA) were used at a density of 7×10^5 cells/mL. These cells were maintained in RPMI1640 Medium ((Sigma-Aldrich, Tauf- kirchen, Germany)). Cell lines were supplemented with 5% calf bovine serum (Sigma-Aldrich, Taufkirchen, Germany). Penicillin (100 U/ml) and streptomycin (100µg/ml) were added as antibacterial agents, and fungizone (100µg/ml) as antifungal (Invitrogen, Grand Island, NY, USA) , and were incubated in a humidified atmosphere of 5% CO_2 in air ,at 37°C. The extracts were dissolved in the tube containing100μL of PBS pH 7.4 in sterile conditions. Each concentration of pigment extract was added to the well in triplicate under strict sterile condition, and then incubated the micro titer plate for 24-72 hours at 37 °C; the plate was monitored during incubation. The cells were harvested when the cells grew in the negative control (RPMI1640 Medium and cell line only) was confluent (±72 hours).

MTT assay

Its act as an indicator of cytotoxic effect for materials, through characterization of dehydrogenase/ reductase amounts produced by cells mitochondria. This assay reflected the number of live cells. Yellowish soluble of MTT pigment is reduced into Formosan salt granules that precipitated in the cytoplasm, then solubilized by dimethyl sulphoxide DMSO (Fluka/ England), therefore the absorbance was measured at 570 nm and the light intensity is concerned with the number of live cells. The evaluation of cytotoxic effect for crude pigment depended on the calculation of inhibitory rate percentages as compared to the control which was concerned as 100% cells growth (Terry, *et al.,* 2016). After incubation period was ended, the content of all wells were denied, then 28 µl of MTT(2mg/ml)was added to each well, and re-incubated at 37 °C for 2 hr. The excess amount of the pigment removed by adding dimethyl sulphoxide (DMSO) in a volume 130 µl/well, while the microtiter plates were left in a micro shaker for 15 min. Staining the cell with MTT pigment, depends on the presence of the life cells with the blue color indicated that the cells are alive, while changing to a yellow color indicated that the cells are died. Hence the results were read by ELISA technique at 570 nm. .The inhibitory rate (IR) of the cell lines was calculated according to the following equation (Sanjay *et al.,* 2009).

$$IR = \frac{A - B}{A} \times 100$$

IR: Inhibitory Rate A: The absorbance of negative control, B: The absorbance of tested dilution.

Quantitative determination of some Cell line cytokines (1B, INFγ and TNF–α)

Removal of pigment color

The pinkish color of radish pigment was removed using activated charcoal (560 mg, 90 veggie caps) as a good bio-sorbent. The chemical nature of activated charcoal combined with a high surface area and porosity made it an ideal medium for the adsorption of organic chemicals (Ravi *et al.,* 2015).

ELISA technique

The enzyme-linked immunosorbent assay (ELISA) kit for the quantitative measurement of interleukin-1beta (IL-1β) (EIA-ab/China), interferon-gamma (INF-γ), and tumor necrosis factor-alpha (TNF-α) (Wuhan/ China) in RD and L20B cell cultures supernatants. This assay employed an antibody specific for human (IL-1 beta, INF-γ, TNF-alpha) coated on triplicate 96 well plates and the procedures were done according to the companies' instructions.

Statistical Analysis

All analytical values represent the means of three replicates. Data of cell proliferation inhibition were analyzed using one-way ANOVA by SPSS 22.0. P value ≤ 0.05 was considered statistically significant

RESULTS & DISCUSSION

Red radish crude pigment

The result of aqueous extraction of 1 Kilogram of red radish roots peels; 276 mg of crude pigments were represented as a lyophilized extraction yield. According to Kottman, (2011) who proved that the red radish pigment is

anthocyanin, thus we suppose that the extracted pigment is anthocyanin. But simultaneously, the pigments yield disagreed with that reported by Giusti (1998), who mentioned that the anthocyanin's pigment spring cultivars in the peels ranging from 39.3 to 185 mg/100g roots. While winter cultivars possessed from 12.2 to 53 mg anthocyanin/100g root. This variation may be due to; the pigment content depending on cultivar, root weight, location, the genetic makeup of the plant, and the extraction procedure used.

Antimicrobial effects of red radish crude pigment

The results of this study revealed that, no effects of crude pigment on selected pathogenic bacteria (*Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*) at the study concentration. Few studies have been reported the influence of anthocyanin's pigment on the growth of bacteria. Mandrik (1952) mentioned that this pigment had no apparent effect on pathogenic bacteria. These data were also approved by Cisowska *et al.* (2011), who investigated the effect of anthocyanin's pigment on pathogenic bacteria and reported that, this pigment had no bactericidal action; these findings were confirmed by the current study. While Marimon *et al.* (1998), elucidated, the bactericidal activity of red wine against helicobacter pylori is very high and greater than ethanol at the same concentration with the same pH *in vitro* study ,and supposed a probable presence of other substances found in the red wine may exert an additional antibacterial activity on these bacteria. As well as to that documented by Rand *et al.* (2012) about the effect of alcoholic extract of red cabbage on several pathogenic bacteria. Therefore we can suggest that anthocyanin's pigment revealed antimicrobial effect on several pathogenic bacteria than other , this may be related with the type of plants that anthocyanin's pigment are extracted , and the rout of pigment extraction.

Cytotoxic effect of crud Red radish pigment

The results of cytotoxic effects of crude pigment of red radish on L20B showed, enhanced cytotoxicity effect of cell growth, which depended on the concentration of crude pigment and the exposed time period. The highest IR percentage was recorded at 15 µg/100ml, and then the percentages became decreased according to the concentration of the crude pigment. The cytotoxic effects obviously appeared in the first 24 hours of incubation, but the better effect occurred after 48 hours. The inhibitory rate percentages were (80, 77, 74, 68.5, 65, 47, 33.5, 22, 14.5, 9, and 4.5) % respectively, whereas for the positive control group were (15.7, 15.3, 14.9, 14.4, 13.8, 13.4, 13.09, 12.7, 12.1, 12, 11.6, and 11.2) % respectively, (Figure 2).

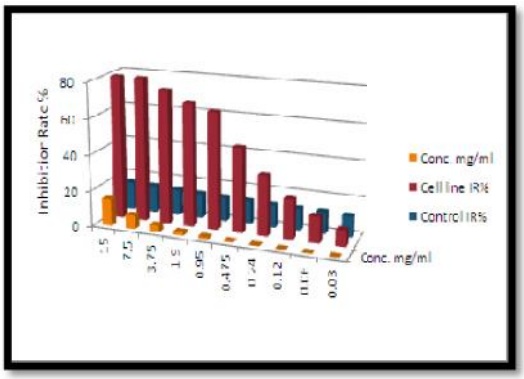

FIGURE 2: Inhibition rate percentage of red radish crude pigment on L20B cell line

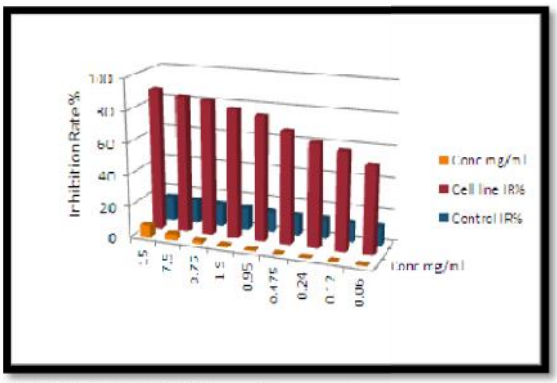

FIGURE 3: Inhibition rate percentage of Red radish on RD cell line

On the other hand, the results of cytotoxic effect of the pigment in RD (muscular sarcoma) cell line showed, sensitivity of cell line to the crude pigment extract more than in L20B, with cell growth inhibition rate (90, 89.5, 86, 84.75, 81, 78.5, 70.5, 65, 61.25, 54.5, 49.25, and 40.5) %, in comparison with the positive control. As well as the best effect appeared after 48 hours (figure 3).

These findings agreed with that of Faria et al.,(2010), who demonstrated, the blueberry anthocyanin's has anticancer properties by inhibiting cancer cell proliferation and invasion as well as acting chemo-inhibitors. Also, it has been improved by (Willig J.A. 2009) and (Ferrazzano et al., 2011) reported that, anthocyanin which was extracted and purified using acetone, chloroform, methanol, and water were used as anti-carcinogenic.

Evaluation of some pro-inflammatory cytokine levels

IL-1β is one of IL-1 member, and it possess a strong pro-inflammatory effect, produced by a various cell types including peripheral blood monocytes, macrophages, B cells, helper T lymphocytes, NK cells and muscle cells (Dinarello , 2011). In this study, the results of IL-1β showed a significant elevation (P≤ 0.05) in comparison with the control group. Simultaneously, tumor necrosis factor-α is a cytokine as the cell associated with acute and chronic inflammation. It is produced chiefly by activated macrophages, although it can be produced by many other cell types such as CD4+ lymphocytes, NK cells, neutrophils, mast cells, eosinophil's, and muscle cells (Tripuwabhrut ,2014). The results of this cytokine showed a gradual elevation also according to the concentrations of radish crude pigment extract. Thus, the present study suggests, the pigment is very important in restriction of tumors and act as a good pro-inflammatory stimulator agent. Moreover the crude pigment may be used for the treatment of diseases associated with imbalanced cytokine production and for enhancing cancer and other immune - therapies. Interferon-γ is synthesized mostly by Th1 lymphocytes, after their activation with immune and

inflammatory stimuli, rather than viral infection (Frucht,2001) Interestingly, natural killer cells can furthermore function as adaptive effectors once activated by T cell-induced IFN-γ or when IgG eventually elicits antibody-dependent cell cytotoxicity (Flores , 2011) and (Gao et al., 2014). It's essential for the development of an immune response that prolongs the life of an infected animal (Arko-Mensah, 2008).The current results of this cytokine showed a significance elevation occurred among all concentrations in comparison with the control except of 8th concentration, (P≤ 0.05). Thus, present data suggests that radish crude pigment can stimulate Th1 which is responsible for the synthesis of IFN-γ, actively stimulated cell-mediated immunity, and reflected the counteracting of the humoral response. On the other word, the increase of INF-γ led to activating cytotoxic T lymphocytes (CTL), (table 1, 2).

TABLE 1: Titers of some pro-inflammatory cytokines (Mean ± SE) in the supernatant of L20B cell line

Conc. of extracted pigment(mg/ml)	TNF-α (pg/ml)	INF-γ (pg/ml)	IL-1β (pg/ml)
15.0	72.0±0.44*	68.0±0.123*	27.0±0.31*
7.50	88.0±0.25*	70.0±0.32*	30.0±0.52*
3.75	66.0±0.153*	74.0±0.34*	44.0±0.31*
1.875	70.0±0.32*	76.0±0.23*	60.0±0.43*
0.937	94.0±0.191*	77.0±0.23*	84.0±0.15*
0.469	66.0±0.26*	46.0±0.43*	66.0±0.34*
0.234	40.0±0.42*	33.0±0.36*	40.0±0.21*
0.117	33.0±0.31*	10.0±0.31	22.0±0.43*
Negative Control	20.0±0.51	6.0±0.41	2.0±0.32
Positive Control	24±0.32	9.1±0.51	5.2±0.63

*indicated to a significant variation at P≤0.05.

TABLE 2: Titers of some pro-inflammatory cytokines (Mean ± SE) in the supernatant of RD cell line

Conc. of extracted pigment (mg/ml)	TNF-α (pg/ml)	INF-γ (pg/ml)	IL-1β (pg/ml)
15.0	94.0±0.32*	70.0±0.43*	30.0±0.08*
7.50	80.0±0.43*	75.0±0.11*	36.0±0.55*
3.75	73.0±0.11*	78.0±0.90*	48.0±0.67*
1.875	69.0±0.87*	80.0±0.56*	69.0±0.83*
0.937	93.0±0.54*	84.0±0.89*	90.0±0.23*
0.469	76.0±0.49*	61.0±0.65*	73.0±0.67*
0.234	55.0±0.09*	43.0±0.76*	61.0±0.11*
0.117	48.0±0.66*	21.0±0.32	32.0±0.56*
Negative Control	20.0±0.51	6.0±0.41	2.0±0.32
Positive Control	24±0.32	9.1±0.51	5.2±0.63

*indicated to a significant variation at P≤0.05.

Therefore, it can be concluded that radish pigment treatment; mostly led to elevation of these cytokine types. The concentrations of the radish pigment reflected a critical concentration led to obviously increasing of some pro-inflammatory cytokines represented with the 5^{th} concentration. An overproduction of pro-inflammatory cytokines especially TNF-α mediates the damaging squeal of inflammation and cancer in pathologic conditions as Shin *et al*., (2015) as well as Kang *et al.*, (2011) reported about the biological effects of radish which one of them was anticancer. In a conclusion, the cytotoxicity effects of the pigment depended on the type of the cell line, the concentration of crude pigment extract, and the exposure period

RECOMMENDATIONS

More studies must be done to improve red radish pigment effects on other cell lines as well as to investigate the effect *in vivo*.

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