Antioxidant, cytotoxicity and phytochemical analysis of 
*Larinus maculates* F. cocoon aqueous extract against lung cancer

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Abstract. Cocoon of larva *Larinus maculates* F. from Curculionidae family (*Echinops* species), locally in Iraq known as Tihan, is one of traditional folk medicine used in the treatment of diversity respiratory system and fever. This study was carried out to assess the bioactive component and the antioxidant capability of aqueous beetle cocoon extract (*Larinus maculates* F.) along with its possible cytotoxic activity against A549 lung cancer cell line. For phytochemical analysis gas chromatography-mass spectrometry (GC-MS) was used, and to detected free scavenging activity 2, 2-diphenyl-1-picrylhydrazyl (DPPH) was used. To determine the cytotoxicity in the cancer cell line 3-(4,5-dimethylthiazol-z-yl)-2,5-diphenyltetrazolium (MTT) was used; peripheral blood monolayer cells (PBMCs) was used as a normal cell. GC-MS analysis identified the presence of 9 phytochemical components. DPPH results suggested a promising antioxidant activity in a dose-dependent, the best antioxidant potential was at 600 µg.ml⁻¹ concentration. Cytotoxic activity results showed that the increase in extract concentration decreases the cell viability, at 50 µg.ml⁻¹ concentration the percentage of viability was (86.76±0.87) µg.ml⁻¹ where at 200 µg.ml⁻¹ the cell viability was (56.44±0.91) µg.ml⁻¹. Taken together, the results showed that larva cocoon of *Larinus maculates* F. extract has an important phyto-molecule with great potent antioxidant and cytotoxicity activity against lung cell line A549.

Keywords. *Larinus maculates* cocoon shell, GC-MS analysis, DPPH, A549 cell line.

1. Introduction

In many cultures, traditional medicine as an alternative medication is still important therapeutic source due to its adequate properties, heritage and popular belief along with the low cost [1]. Therefore, there is growing interest in the study of medicinal therapy in the field of biomedical research. Natural products such as plants, animals and marine organisms contain a large number of active compounds that reflect the potential treatment activity of these organism [2]. It has been suggested that some
alternative drugs might have a greater remedial capacity than those that are owned by manufactured
drugs in the treatment of certain diseases with less side effects that accompany the use of chemical
drugs [3]. One of the interested folk remedy use in Iraq is the cocoon shell produced by salivary
glands of beetle larva of *Larinus maculates* F. from *Curculionidae* family commonly known as
(Tihan). Also known in neighboring countries (Iran and Syria) *Trehala manna* (Shekar tighal). Commonly used in treatment from various diseases including respiratory system diseases, viral or
bacterial infection, antitussive, anti-asthmatic and febrifuge [4]. In our *in vivo* previous study [5].
Tihan exhibited potential role in activated immune system where orally treated mice showed a
significant increase in mitotic index and phagocytosis. Other study by [6] reported *in vitro*
immunostimulatory effects on splenocytes and macrophages by increased PHA- and LPS-stimulated
splenocytes proliferation and IFN-γ production. Additionally, Iranian researcher reported contains
analysis of *Trehala manna* such as water-soluble polysaccharides (e.g. cellulose and trehalose), tannin,
aluminum matter, lipid, and mucilage [7, 8, 9]. As attributable the important role and their biological
activities of *Trehala manna* due to its polysaccharides molecular weights [8]. It is well known that the
unnecessary generation of reactive oxygen species (ROS) causing imbalance in oxidant/antioxidant
status and leading to oxidative stress and consequently oxidative damage induced cell injured. The
implication of ROS as one of the molecular mechanisms involved in carcinogenesis has been reported
[10]) Therefore, antioxidants are proposed as possible applicant for prevention or/treatment of
cancer. Antioxidants play important role in determining the therapeutic effect of plants and other
traditional agent and to make them a good remedy against acute and chronic diseases. These
antioxidants usually found and taken in our dietary from the natural compounds. The current study
aims to investigate the active compounds in comestible Iraqi aquatic cocoon shell of beetle larva of
*Larinus maculates* and their anti-oxidant and cytotoxicity effects on A549 lung cell line. For our
knowledge, this is the first study in this aspect in Iraq.

2. Materials and Methods

2.1. Chemical and reagent

Cell culture media and reagents were obtained from GIBCO (Rockville, MD, USA), all other
chemicals and reagents were obtained from Sigma Aldrich (Louis, USA).

2.2. Beetle cocoon collection and extract preparation

Tihan cocoon shells were obtained from local shelf market (Iraq/Baghdad), identified by expert in
Iraqi Natural History Museum Figure (1). Cocoons aquatic extract were prepared as the following (5)
firstly emptied from inside insect, grounded using a laboratory electric grinder. 10 g from the powder
dissolved in 100 ml of distal water using heating stirrer for two hour. Followed by centrifuging at 4000
rpm for 30 min and then the filtered supernatant dried using a rotary evaporator under reduced
pressure and kept at 4°C until uses in the experiment.

Figure 1. Tihan cocoon shells produced by of *Larinus maculates* F from Curculionidae family.
2.3. Gas chromatography-mass spectrometry (GC-MS) analysis and identification of phytochemical components

The GC-MS analysis for aqueous beetle cocoon extract was performed as described previously [11] using a Clarus 500/580 Perkin Elmer GC (Connecticut, USA). The unknown components mass spectrum of the unknown components was compared with the spectrum of the components stored in the NIST library [12].

2.4. Anti-oxidant activity measurement

The antioxidant activity was determined according to [13] using 2, 2-diphenyl-1-picrylhydrazyl (DPPH). Briefly, 1 ml of the samples (different concentration 100, 200, 400 and 600 µg.ml\(^{-1}\)) was mixed 1:1 with DPPH solution (60 µM, 1 ml). After incubation in darkness at 37°C for 30 min, the absorbance was read at 517 nm spectrophotometrically (Perkin-Elmer Lambda 25, Germany). Vitamin C considers as a positive control, and measurements were carried out in triplicate. The IC50 value was calculated according to the absorbance and the inhibition percentage of was calculated using the following equation:

\[
\text{Scavenging activity(\%)} = \frac{(A_c - A_s)}{A_c} \times 100
\]

Where, \(A_c\) = absorbance of control and \(A_s\) = absorbance of samples

2.5. A549 Cell line culture

The lung cell line A549 was provided by Center of Biotechnology at AL-Nahrain University. A549 cell line is usually used as a representative of type two alveolar pulmonary epithelial cell derived from pulmonary adenocarcinoma of 58-years old man [14] Dulbecco’s Minimal Essential Medium (DMEM) was used for cell culture and maintenance and provided with 10% fetal bovine serum (FBS) and antibiotic penicillin & streptomycin (1%). Cells were incubated at 37°C in humidified 5% CO\(_2\) and cultured at 2 \(\times\)10\(^5\) cells ml\(^{-1}\) concentration.

2.6. Human Peripheral blood isolation and culture

In order to evaluate Tihan extraction effect on normal cells Human peripheral blood samples, blood samples were collected from healthy volunteers in heparinized tubes after taken their permission to participate. The peripheral blood mononuclear cells were isolated by using Ficoll-Paque after centrifuged in a falcon tube for 30 min at 3000 rpm. Cells were cultured in 96-well plates in RPMI medium and incubated for 4 h in 5% CO\(_2\) at 37 °C. Concentration of 50, 100, 150 and 200 µg.ml\(^{-1}\), the same concentration used with A549 cell, was add, lymphocytes in culture media without extract was considered as control and the cell viability calculated using MTT assay after 24 h incubation. Cisplatin was used a positive control (stock solution 1000µg/ml).

2.7. Measurement of cell viability

The 3-(4,5-dimethylthiazol-z-yl)-2,5-diphenyltetrazolium (MTT) assay was used as described by [15] for cell viability detection. Extract concentrations of 50, 100, 150 and 200 µg ml\(^{-1}\) was used. Cells were seeded at a concentration of 1 \(\times\)10\(^5\) cell ml\(^{-1}\) and 100 µl of extracts was. After the incubation period, 10 µl of (5 mg ml\(^{-1}\)) MTT solution was added to each well and incubated 4 h at 37°C. Then 50 µl of dimethyl sulfoxide (DMSO) was added and left for 10 min. A549 cells without aqueous beetle cocoon extract represent control group. The absorbance was read at 570 nm using spectrophotometer
reader (VersaMaxTM, Molecular Devices, Sunnyvale, CA). Based on the inhibition concentration the IC\textsubscript{50} value calculated using GraphPad Prism 6 software.

2.8. Statistical analysis

SPSS software version 16.0 was used for Statistical analysis. The results are accessible as means ± standard error. Analysis of variance (ANOVA) was used and Fisher Least Significant Difference (LSD) test.

3. Results and Discussion

3.1. GC-MS analysis

The GC-MS analysis of the cocoon extract showed the presence of 9 compounds (Table 1). The major components were identified as benzoic acid, (19.89%); 2-butanol, (17.86%); followed by hexamethy1-3 (16.41%) and purin-6-1 (15.68%) as the major constituents cocoon extract. These elements have been reported for their biological and therapeutic properties including the antioxidant for example, Hydroxy benzoic acids and its derivatives showed antioxidant and anti-inflammatory properties against free radicals especially superoxide radical [16, 15, 17] where it decrease overproduction of reactive species. Also, it has been reported that antioxidant activity of butanol fraction was higher than α-tocopherol and butyrate hydroxytoluene (BHT) [18].

Table 1. Phytochemical analysis of Larinus maculates F. cocoon extract by GC-MS.

| NO  | RT    | Phytochemical compound                                      | Peak area % | CAS NO.       |
|-----|-------|------------------------------------------------------------|-------------|---------------|
| 1   | 4.316 | Performic acid, trimethysilyl derivative                   | 7.92        | 1000368-52-2-25 |
| 2   | 4.536 | Glyceraldehyde                                            | 2.93        | 000056-82-6   |
| 3   | 4.961 | Cyclotrisiloxane, hexamethyl-                             | 2.19        | 000541-05-9   |
| 4   | 5.136 | Tricyclo [4, 3, 1, 1 (3, 8)] undecane-3-carboxylic acid, methyl ester | 6.06        | 031061-61-7   |
| 5   | 5.477 | Acetophenone, 4'-methoxy-, oxime                           | 11.06       | 002475-92-5   |
| 6   | 5.675 | 6H-Purin-6-one, 2-amino-1, 7-dihydro-1-methyl-             | 15.675      | 000938-85-2   |
| 7   | 6.069 | 2-Butanol, 3-methyl-                                       | 17.86       | 000598-75-4   |
| 8   | 7.268 | Benzoic acid, 3-methyl-2-trimethyl silyloxy-, trimethylsilyl ester | 19.89       | 1000153-57-1  |
| 9   | 8.088 | 7, 7, 9, 9, 11, 11-Hexamethyl-3, 6, 8, 10, 12, 15-hexaopa-7, 9, 11-trisilaheptadecane | 16.41       | 1000375-89-1  |

3.2. Antioxidant activity

The aqueous extract was tested for its antioxidant activity with DPPH at 100,200, 400, and 600 µg.ml\textsuperscript{-1} concentration. The results showed a significant higher radical scavenging activity as compared with the positive control (L-ascorbic acid) in a concentration-dependent inhibition. Higher activity (P ≤ 0.01) was shown at 600 µg.ml\textsuperscript{-1} concentration, while the lower one was with 100 µg.ml\textsuperscript{-1} concentration (P ≤ 0.05) compare with ascorbic acid control (Figure 2). Many different compounds found in the extract of the present study can be contribute to free radical scavenging activity, such as the phenolic...
compounds with its multiple hydroxyl groups showed higher ability to quench DPPH [19]. Moreover, [20] reported that the number of hydroxyl groups attached to the aromatic ring is associated with the phenolic acids anti-radical activity, though some other compounds may also be involved. Present study finding support this suggestion by which Butanoic acid, Oleic acid, Pimaric acid, Phenol and Benzoic acid found in the extracts might be responsible to the antioxidant activity. As a little-known of functional food sources of the cocoon extract, therefore, the isolation and purification of these phyto-compounds may be useful in the research of new treatment drug. The antioxidant activity performance in two levels, the first one at the cellular membrane level by breaking peroxyl radical chain reactions followed by the second level by reacting with intracellular ROS [21].

![Figure 2. DPPH free radical scavenging activity. The inhibition percentage of different concentrations of extract observed at 517 nm. Data are means ± SE (n = 3). a significantly different at P≤ 0.05 as compared to L-ascorbic acid (positive control). * significantly different at P≤ 0.05 as compared to 100 µg.ml⁻¹.](image)

3.3. Cell cytotoxicity

The cytotoxicity effect of different concentrations of the aqueous Tihan extract was evaluated using two Human cell line, A549 lung cancer cell line and PBMCs as a normal cell. The results showed significant cytotoxic effect in a dose dependent manner. The treatment of A549 cells with concentrations of (50, 100, 150 and 200 µg.ml⁻¹) decreased significantly the % viability. Where the highest inhibition was at higher doses, however, a moderated inhibition (not significant) was found at the lower doses (Table 2). However, there was no potent cytotoxic effect on normal PBMCs cell (Table 2). Cisplatin effect on the percentage viability of PBMC cell was dose dependent by which the viability decreased significantly as the dose 8 and 12 µg.ml⁻¹ compared to the control. However, at the dose 2 and 4 µg.ml⁻¹ there was no significant difference.

![Table 2. Cell percentage of viability on A549 and PBMCs cell line using MTT assay and treated with different concentrations of cocoon extract for 24 h. Data are means ± SE in triplicate. A significant at p≤0.05.](image)
This is because the cocoon extract of Tihan contains polysaccharide [22] and when the concentration of the extract is increased the ratio of polysaccharide increased that stimulate the production of IL-2, which plays a role in the activation of NK cells [23] as well as its role in T cell division. This demonstrates the role of polysaccharide in the development of immunity and its potential for use in therapeutic applications such as bacterial infections and cancer [24]. Study by [5] have indicated the role of these polysaccharide in increasing phagocytic index (PI). The reason for this increase is the effect of the active ingredient in the extract in stimulating phagocytosis by stimulating the release of certain cellular attractions specifically TNF and IL-1, which stimulates macrophage cells, including neutrophils to leave the blood stream to the peritoneal cavity [25]. But the inhibition was decreased with reducing the concentration at 100 μg.ml⁻¹ concentration. The suppression of proliferation was concentration-dependant. Additionally, elevated ROS level has been associated with cancer, one of the causes is the abnormal mitochondrial oxidative metabolism which is responsible for the initiation and progression of cancer.

4. Conclusion

In conclusion, the finding of the present study identified that Larinus maculates F. cocoon aqueous extract has several phyto-molecule possessing anti-cytotoxicity and anti-oxidant therapeutic activities. The results of antioxidant activity suggested that ROS may be a potential mechanism involved in cancer cell death and the antioxidant capacity of the extract can decrease cell proliferation. For our knowledge this is the first study that recorded the in vitro toxicity for the cocoon extract. However, further research on cyto-toxicology still needed in order to insure the safety especially in vivo.

5. References

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