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Original Article

In Vitro Evaluation the Anti-Melanogenic Effects of Various Extracts of Oat Seeds

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Abstract

This study was designed to examine the effects of aqueous and hydroalcoholic extracts of oats *(Avena sativa)*, which are rich in beta-glucan with established anti-pigmentation effects on the melanin biosynthesis rate in melanocyte cell cline, B16F10, as well as its safety effects on skin normal cells. Maceration method was used to extract the seed powder of *Avena sativa* and then the polysaccharide content was determined. The safety of extracts was analyzed using MTT assay on L-929 cells. The anti-melanogenic effects of the extracts were determined using the calculation of mushroom tyrosinase inhibitory effects, the B16F10 treated cells assay for evaluating the extracts tyrosinase inhibitory effects and the melanin contents, in comparison to the PBS as the negative control. Our results showed that the aqueous and hydroalcoholic extracts had no significant toxicity on L-929 normal cells. In comparison to the Kojic acid used at the EC_{50} concentration (3 µg/ml), both aqueous and hydroalcoholic extracts led to mushroom and intracellular tyrosinase inhibition as well as reduction in melanin content of B16F10 treated cells, concentration dependently, especially at the highest concentration, 5 mg/ml. Finally, the total polysaccharide count in water and hydroalcoholic extracts was calculated as about 2.9 and 2.8 µg, respectively, for 5 µg of dried extracts. In conclusion, this study has proven the anti-melanogenic and tyrosinase inhibitory effects of *Avena sativa* extracts without any toxic effects on skin normal cells in the effective concentrations. So, with reliable laboratory effectiveness, attempts to prepare suitable formulation from this herb is therefore suggested.

*Keywords***:** *Avena sativa*, B16F10, Melanin, Safety, Tyrosinase.

1. Introduction

Plants have played a decisive role in the treatment of various disorders, including skin diseases in the last hundred years. However, with the advent of chemically synthesized drugs, their role gradually diminished. However, in recent years, the consumption balance is turning in favor of herbal medicines due to the people re-approaching the use of natural agents, because of their less side effects [1].

Skin lightening is one of the reasons leads to the use of various therapeutic approaches among the people with reducing the content of

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melanin, the main pigment causes the skin color [2]. Melanin synthesis involves a series of enzymatic or non-enzymatic reactions from L-tyrosine as its main precursor to L-DOPA which is catalyzed by tyrosinase enzyme, as the first and speed limiting step [3]. Skin lightening agents such as tretinoin, hydroquinone, azelaic acid, vitamin C, glutathione, and kojic acid, work by reducing the presence of melanin in the skin via several mechanisms [4]. However, due to the side effects of some of these chemical compounds such as kojic acid, for example, allergenic and irritating effects on skin, and also for its low stability in cosmetics, the use of natural agents with anti-melanogenic effects such as herbs seems to be a proper solution in order to diminish the skin hyperpigmentation effects [5].

Avena sativa seeds commonly known as Oat have a high content of carbohydrates and various fatty acids [6]. Furthermore, oat isoflavones also have antioxidant and other effects on the growth and proliferation of the skin cells [7, 8]. *Avena sativa* contains beta-glucan at about 3-7% of total content, with acceptable effects on skin beauty in terms of reducing wrinkles and brightening [9, 10, 11], as well as wound healing effect, antioxidant activity, andmoisturizing effect [12]. Furthermore, the anti-inflammatory effect of oat was evaluated in the treatment of dry skin itching with four oat extracts and it was demonstrated that these extracts reduce the level of inflammatory cytokines, as well as dryness, peeling, redness, and skin irritation in healthy volunteers [13]. The effects of alcoholic and aqueous extracts of oat on wound healing and dermatitis were also established in animal model [14].

However, the anti-pigmentation effects of oat have only been mentioned in a study performed in 2018, which has indicated that the emulsion containing the extract of this plant has no toxic effects on keratinocyte cells and showed suitable effects in reducing the degree of skin pigmentation in clinical studies [15]. On the other hand, one project conducted by Hwang, the oat grain was surveyed for its safety against various cell lines and for *in vitro* anti-melanogenic effects [16].

Since *Avena sativa* is recognized as a safe herb it has been used as food for many years. Thus, with its previously established skin protective effects, the lack of printed data on the effectiveness of aqueous extract of *A. sativa* - which is rich in polysaccharides and in order to ensure its safety on normal cells, this study was designed to examine the effects of the same extract on the production rate of melanin in mouse melanoma cells (B16F10).

2. Materials and Methods

2.1. Ethical Considerations

The Ethics Committee of Isfahan University of Medical Sciences approved this research with the code IR.MUI.RESEARCH.REC. 1399.237 on 2020-06-09.

2.2. Oat Seeds and Other Reagents

The seeds of *Avena sativa* were prepared from Seed and Plant Research Improvement Institute located in Isfahan, which have been evaluated and approved by the experts of that institute in terms of species and conformed to the Herbarium of Department of

Pharmacognosy, Faculty of Pharmacy, Isfahan University of Medical Sciences, Iran (herbarium number: 1426). Mushroom tyrosinase, melanin, Levodopa and L-tyrosine were purchased from Sigma (Germany). Kojic acid was purchased from Pishgaman Shimi Company (Tehran, Iran) and used as the positive control (with established skin lightening effects). L-929 and B16F10 cells were obtained from National Cell Bank of Iran (Pasteur Institute, Tehran, Iran). Cell culture instruments/consumables? were provided from Biosera, (France). Finally, other used chemicals were prepared from Merck Company (Germany).

2.3. Extraction of Samples from Oat Seeds / Oat Seeds Sample Extraction

The seeds' bran was removed using a burr mill. The seeds were then ground by a hammer mill and passed through a sieve with an appropriate mesh number. The herb seed powder generated was used to prepare aqueous, hydroalcoholic and ethanolic extracts. For the aqueous extract, an amount of 10 grams of the plant powder was mixed with 100 ml of distilled water and extracted by maceration method within 2-3 days. The prepared extract was then filtered and this process was repeated two more times on the remaining powder. The extracts were initially stored at 2-8 °C, and after completing three times of extraction, they were gathered together and dried by a freeze dryer. The above method was also used in preparing the hydroalcoholic and ethanol extracts with water/ethanol solvent (30/70 ratio) and ethanol alone (100%), respectively. The hydroalcoholic and ethanol extracts were eventually dried using rotary evaporator and freezer dryer. All the dried extracts were used to prepare various concentrations for the subsequent biological tests. Phosphate buffered saline (PBS) was used for serial dilutions after the filtration of each stock solution via 0.22 um filters.

2.4. Standardization of Extracts

Phenol sulfuric acid method was used to determine the polysaccharide content of each of the extracts [17]. Briefly, 5 ml of sulfuric acid and 1 ml of phenol 5% was added to about 2 ml of the prepared extract with the final concentration of 5 µg/ml. The mixture was vortex for 10 minutes at room temperature and then centrifuged for 15 minutes at 3000 × g. Finally, the absorbance of the supernatant was determined at 490 nm using an ELISA micro-plate reader. Glucose in various concentrations was used as the standard solution.

2.5. Evaluation of the Safety of Extracts on the Normal Cells

MTT assay was used to determine the safety of three various extracts against L-929 fibroblast cell line. In summary, the cells were cultured in a 96-well plate with final concentration of 5×10^3 cells/ml in 180 µl as the total volume and incubated at 37 ºC in a CO_2 incubator for 24 hrs. Then, 20 µl of six various concentrations (50, 25, 12.5, 6.25, 3.125, and 1.56 mg/ml) of each extracts were added to the respective microplate wells. After 48 hrs of

incubation, 20 µl of MTT solution (5 mg/ml) was added to each of the wells, and the plate was further incubated for 3 hrs. Finally, the formazan crystals were dissolved in 150 µl DMSO, and the plate was subjected to absorbance reading at 570 nm using a micro-plate reader.

2.6. Evaluation the Inhibitory Effects of Extracts on Mushroom Tyrosinase Enzyme

In order to determine the inhibitory effects of extract with high amounts of polysaccharide content and without toxicity against normal skin cells, the study of Baurin et al was used as the reference with slight changes [18]. Briefly, 20 µl of L-dopa at a concentration of 0.9 mg/ml and 70 µl of tyrosine (300 µg/ml) were added to 90 µl of PBS followed by addition of 10 µl of each concentration of the aqueous and hydroalcoholic extracts (with the final concentrations as 5, 2.5, 1.25, 0.625, 0.31, and 0.156 mg/ml), were used in order to measure the inhibitory effects of each of the extracts on the mushroom tyrosinase activity. Finally, 10 µl of a tyrosinase enzyme sample with a known final activity of 500 IU/ml was added to (each of)? the above mentioned concentration mixtures and stirred thoroughly. The mixtures were then incubated at 37 °C for 30 minutes. After the incubation, the amount of dopachrome produced in the reaction mixtures were measured by spectrophotometry at 475 nm [19] followed by calculating the inhibition percentage for each sample concentration, using this equation:

$$1 - \frac{\text{sample absorbance}}{\text{negative control absorbance}} \times 100$$

2.7. Measurement the Melanin Content of Cells

The B16F10 cells were cultured in 24-well plates with the final concentration of 5×10^4 cells/ml in 900 µl as the total volume in each well and incubated at 37 ºC in a CO_2 incubator for 24 hrs. After that, 100 µl of six concentrations (50, 25, 12.5, 6.25, 3.125, and 1.56 mg/ml) of each of the extracts was added to the wells, as appropriate. Following 48 hrs of treatments, culture media were aspirated and thecells were separated from the plate using trypsin and washed twice with PBS. The cells were then lysed at 100 °C for 30 minutes by adding 100 µl of 2 M NaOH. The cell lysate was then centrifuged at 16,000 × g for 20 minutes [19]. The supernatant was added to DMSO with equal volume in order to dissolve the melanin content, the absorbance was read at a wavelength of 405 nm and its concentration in each sample, was measured against various concentrations of melanin purchased from Sigma.

2.8. Evaluation the Inhibitory Effects of Extracts on Cellular Tyrosinase Enzyme

Similarly, the B16F10 cells were cultured in 24-cell plates as mentioned in the previous section and treated with the same concentrations of the aqueous and hydroalcoholic extracts as above. Following 48 hrs of the treatment, the trypsin treated cells were twice washed with PBS and lysed by freeze/thaw method with the addition of Triton X-100 (1%). Finally, each of the lysed solution was centrifuged at 16000 × g for 20 minutes, and then L-dopa at the final concentration of 2

mM was added to 100 µl of the centrifuged supernatant and the absorbance was read at 492 nm [20]. Again, the cellular tyrosinase inhibition percentage was calculated using this equation:

$$1 - \frac{\text{sample absorbance}}{\text{negative control absorbance}} \times 100.$$

2.9. Statistical Analysis

SPSS 25 software was used for statistical analysis. Analysis of variance (ANOVA) followed by a suitable post hoc test, according to the result of homogeneity test, was used to distinguish the differences between groups. The significance was assumed as $p < 0.05$. For all the experiments, kojic acid and PBS were used as the positive and negative controls, respectively.

3. Results and Discussion

3.1. Polysaccharide Content of Various Extracts

According to the results from the phenol sulfuric acid standardization method (section 2.4), the polysaccharide content of the aqueous extract was calculated as 2.9 µg/ml from the extract having a final concentration of 5 µg/ml. Similarly for the hydroalcoholic extract, the polysaccharide content value was determined to be 2.8 µg/ml in the extract with the same final concentration of the hydroalcoholic extract. This means, the standardized amounts were both found to contain about 60% of the total extract volumes (2.9/5 and 2.8/5 ug/mL) of plysaccharides, for the aqueous and hydroalcoholic extracts, respectively.

The cosmetic effects of *Avena sativa* investigated in various projects have been attributed to the presence of considerable amounts of beta-glucan, especially in its seed with just 3-7% of total content, which is much less than the 60% total content found in this study(?). For example, in one study which investigated the anti-pigmentation effects of oat as an emulsion containing the *Avena sativa* extract, it was reported that reducing the degree of skin pigmentation in healthy volunteers was established as a clinical study, with 1.7 mg of beta-glucan per gram of emulsion containing oat seed extract as the active ingredient [15].

3.2. Safety Profile of Various Extracts on Normal Cells

The MTT assay results showed that ethanol extract of *Avena sativa* seeds led to reduce the survival percent of L-929 cells concentration dependently, in comparison to the PBS treated cells. Actually, in three concentrations of 1.25, 2.5, and 5 mg/ml, this extract (which of the extracts is being referred to here please? ethanolic or hydroalcoholic ??) decreased the survival percent of cells (p value < 0.05). For the aqueous extract, on the other hand, the results showed that increasing the extract concentration, led to the increasing in the cell survival (which of the 6 concentrations please?) and the higher concentration (5 mg/ml) revealed the most proliferative effects in comparison to the negative control (p value = 0.003). Finally, for the cells treated with the same concentration of hydroalcoholic extract (which of them? 5 mg/ml ??), the results

showed that increasing the extract concentration led to the reduced survival of the cells but not significant in comparison to the negative control (p value = 0.07). Figure 1 showed detailed MTT assay results.

In the study of Parzonko, et al., the pro-apoptotic effects of the beta-glucan extracted from this herb was investigated against human melanoma HTB-140 cells [19]. Their results established that this compound led to the cell toxicity with an IC_{50} of 200 µg/ml after 24 hrs of treatment. However, in our study, after 48 hrs of incubation with 5 mg/ml of water extract, it has not shown any toxic effects and even the cell viability was found to increase in this concentration. Furthermore, in the study of Hwang et al., the cytotoxicity of *Avena sativa* grain and germinated oat was surveyed against Human dermal fibroblast, RAW264.7, and B16F10 cells in the final concentration as 500 µg/ml. Their results showed that after 24 hrs of treatment, oat led to significant reduction in cell survival for three investigated cell lines [19]. However, in our study, the aqueous and hydroalcoholic extracts used in higher concentration of 5 mg/ml showed no significant cytotoxicity against L929 normal cell. The most probable reason here may be the fact that cytotoxicity of oat is due to it's water insoluble ingredients.

3.3. Inhibitory Effects of Various Extracts on Mushroom Tyrosinase Enzyme

Comparison the inhibitory effects of two various extracts of *Avena sativa* seeds on the mushroom tyrosinase was explained in the figure 2. According to the calculated data, it was shown that the inhibitory effects of both extracts on tyrosinase activity have increased dose-dependently when compared to the PBS as the negative control.

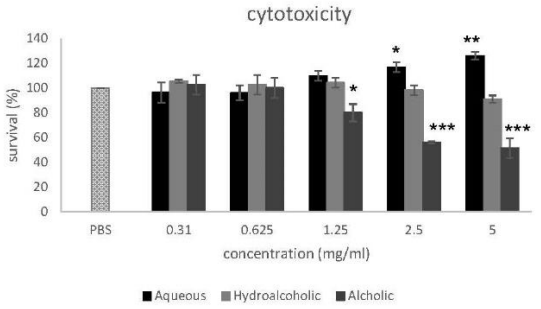

Figure 1. Cytotoxicity evaluation of three different *Avena sativa* extracts against L-929 normal cell line, n =3, Error bar represents mean ± SD. Stars showed the significant differences of cell survival in comparison to the negative control. * P< 0.05, ** P< 0.01 and *** P< 0.001.

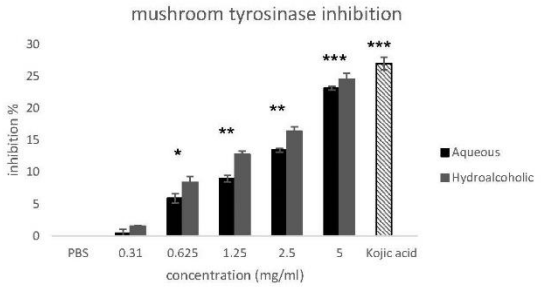

Figure 2. Comparison of the mushroom tyrosinase inhibitory effects of aqueous and hydroalcoholic extracts in various concentrations, n =3, Error bar represents mean ± SD. Stars showed the significant differences of mushroom tyrosinase inhibitory effects in comparison to the negative control. * P< 0.05, ** P< 0.01 and *** P< 0.001.

However, in similar concentrations, there were no significant differences between two extracts in this regard (p value = 0.072). Finally, in comparison to the Kojic acid, the tyrosinase inhibitory effects used as the positive control in a constant concentration (3 µg/ml) as its EC_{50},

according to a previous study, there was no significant differences between the effectiveness of extracts in the higher concentration (5 mg/ml), whereas Kojic acid at the same concentration was shown to induce only 27 % of tyrosinase inhibitory effects [20].

3.4. Melanin Content of Cells Treated with Various Extracts

Figure 3 compares the melanin content in the supernatant of cells treated with various concentrations of aqueous and hydroalcoholic extracts as well as cells treated with PBS or Kojic acid as the negative and positive control, respectively.

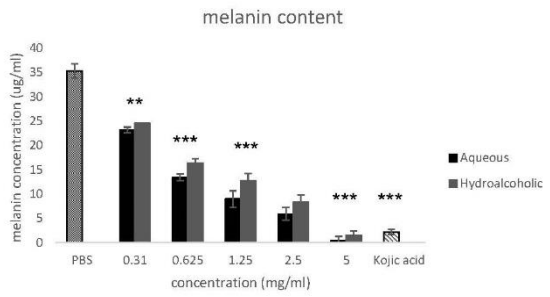

Figure 3. Comparison of the melanin content of the supernatant B16F10 cells treated with various concentrations of aqueous and hydroalcoholic extracts, n =3, Error bar represents mean ± SD. Stars showed the significant differences of melanin content in comparison to the negative control. * P< 0.05, ** P< 0.01 and *** P< 0.001.

According to the analyzed data, it was shown again, that there was no significant differences between the melanin content in the supernatants of cell treated with the same concentrations of the two different extracts (p value = 0.37). Furthermore, cells treated with about 15 µg/ml of Kojic acid as the positive control, according to the Uchida et al. study [21], showed about 2.2 µg/ml of melanin recovered in their supernatant in comparison to the cells treated with the highest concentration of hydroalcoholic extract, which recovered 1.5 µg/ml of melanin (without statistically significant differences, p value = 0.068).

For evaluating the *in vitro* anti-melanogenic effects of any type of chemical or herbal agents, a combination of tests used in our study was routinely performed. For example, in the study of Chang et al., the mushroom tyrosinase inhibitory effects of ethanol extract of *Morus alba* roots and branches was established at about 97% (of what?, in total cells/total melanin/tyrosine contents?) with method used in our study [22]. In another study in this area, five tropical plants have been studied for their inhibitory effects on tyrosinase, including *Stryphnodendron barbatimao*, *Portulaca pilosa*, *Cariniana brasiliensis*, *Entada africana*, and *Prosopis africana*. They have been evaluated in terms of inhibitory effects on mushroom tyrosinase using *Morus alba* mentioned above, with a significant tyrosinase inhibitory effect, as a positive control [22]. In the same study, it was discussed that although *Cariniana brasiliensis* showed significant skin lightening effects, its tyrosinase inhibitory effects was negligible and its effects on skin lightening was attributed to its hyaloronidase inhibitory effects. However, in our study, the presence of beta-glucan was established in *Avena sativa* seeds and its tyrosinase inhibitory effects was also as proven elsewhere [18].

The anti-melanogenic effects of the fruits of *Antrodia camphorate* have been assessed based on method used in our study [23]. The beta-glucan content of ethanolic extract when calculated was nearly same with the total beta-glucan found in our investigated extract. Furthermore, the extract concentrations examined were similar to those used in our study. In similarity to our results, the effects of ethanolic extract at 5 mg/ml concentration, which led to 60 % of tyrosinase inhibitory effects in comparison to the B16F10 cells treated with Kojic acid in the same concentration, therefore confirms the fact that the tyrosinase inhibitory effects of both herbs are related to the beta-glucan content.

In the other *in vitro* study, when the oat grain extract was used in B16F10 cells in the 200 and 300 µg/ml final concentrations, only about 20% of melanin content was found to decrease in comparison to the negative control and the higher concentration (?? mg/ml?) was not evaluated in this regard [19]. However, in this project, we were able to calculate the EC_{50} of two various extracts with melanin inhibitory effects of 490 and 550 µg/ml, for the water and hydroalcohol extracts, respectively.

3.5. Inhibitory Effects of Various Extracts on Tyrosinase Enzyme

In order to evaluate the inhibitory effects of Aqueous and hydroalcholic extracts on cellular tyrosinase, as shown in figure 4, the EC_{50} value for each extract was determined. The EC_{50} values for the aqueous and hydroalcoholic extracts were determined as 1.62 and 1.78 mg/ml, respectively, without any significant difference according to the ANOVA test (p value = 0.071).

In our study, the melanin content of water and water-alcoholic extracts of *A. sativa* seeds was also investigated. As mentioned above, in comparison to the outcomes of Kojic acid used as a standard anti-pigmentation agent, two types of extracts with acceptable amounts of polysaccharide, especially beta-glucan, have led to decreases in the melanin content of treated cells. The study of Cizauskaite et al., where the aqueous extract of *A. sativa* seeds with total count of 44% melanin-contents (?)(in addition two other plant extracts including ethanol rosemary and Linseed extract), the extract was actually formulated as a multiple emulsion and used for *in vivo* assay. The beta-glucan content of the same emulsion was calculated as 1.7 mg per each grams of the formulation, as totally related to the oat extract. Their results showed that after seven days of treatment, the pigmentation percentage was statistically decreased to 16.94% in comparison to the control emulsions in 42 healthy volunteers. However, this reduction was further calculated as 5% for cases that encountered exposure to UV light in order to stimulate the hyperpigmentation. Reducing the effects of the emulsion in pigmentation inhibition has been attributed to the effect of increasing dose of UV light [15]. In comparison to our in vitro study where we observed about 95 % of reduction in the melanin content of cell treated with the highest concentration of Aqueous extract of oat, the decrease observed in the above mentioned clinical trial was only 17 %, thereby proving a fact which highlights the necessity of conducting more *in vivo* studies in this regard.

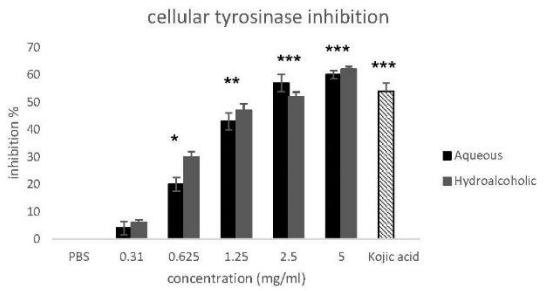

Figure 4. Comparison of the tyrosinase inhibitory effects of various concentrations of aqueous and hydroalcoholic extracts on B16F10 treated cells, n =3, Error bar represents mean ± SD. Stars showed the significant differences of tyrosinase inhibitory effects in comparison to the negative control. * P< 0.05, ** P< 0.01 and *** P< 0.001.

4. Conclusion

Our results on the anti-pigmentation of various extracts of *A. sativa* showed that these extracts determined with similar contents of polysaccharides, have caused *in vitro* anti-pigmentation effects, with no toxic effects on the survival percentage of skin normal cells and are therefore safe for usage in the lightening cosmetic formulations. As such, attempts to prepare suitable formulation from this herb with effective concentrations are suggested.

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