

## Solid State Fermentation Effects on Pistachio Hulls Antioxidant Activities

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### Abstract

Pistachio (*Pistacia vera* L.) is a small tree native to mountainous regions of Iran. The seed has a mauvish skin and light green flesh, with a distinctive flavor. The hulls contain high amount of phenolic and flavonoid compounds, which are known as source of antioxidant. Recently, the use of natural additives found in plant material as preservative in food and cosmetic products received considerable attention. On the other hand was know processing method to improve the antioxidant activity of agriculture byproducts and reduce the anti-nutritional metabolites. Therefore, this experiment was carried out to determine the effect of solid state fermentation on pistachio hulls antioxidant activities using five types of fungi namely *White rot fungi* (ATCC 64897), *White rot fungi* (ATCC 90467), *Aspergillus terreus* (ATCC 74135), *Rhizopus oligosporus* and *Aspergillus oryzae*. Pistachio hulls were subjected to fermentation process for the period of 10 days. Freeze-dried samples were extracted with 80% methanol. The result showed that the samples contained varied concentration of phenolic compounds from 0.721 to 2.277 mg gallic acid equivalent/g DM, and total flavonoids varied from 0.249 to 1.204 mg rutin equivalents/g DM. The highest antioxidant activity of 50.39% at a concentration of 300 µg/ml of crude extract was found in crude methanolic extract of control while the lowest antioxidant activity of 31.19% was found in crude methanolic extract of hulls fermented by white rot fungi (ATCC 90467). The result indicated a reduction in the antioxidant activities of pistachio hulls when undergoing solid state fermentation. Therefore, it is not a recommended method to improve the antioxidant activities of pistachio hulls.

**Keywords:** antioxidant activity, Pistachio hulls, solid-state fermentation, flavonoid and phenolic compounds

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## Introduction

Pistachio (*Pistacia vera* L.), a small tree native to mountainous regions, is one of the main agriculture plants of Iran. Iran ranks the world's largest pistachio producer and exporter followed by USA and Turkey ([www.fao.org](http://www.fao.org)) after oil and carpets, with total production of about 280,000 tons in year 2008 (<http://www.iran-daily.com/1387/3286/html/economy.htm>). The seed has a mauvish skin and light green flesh, with a distinctive flavor. At ripening, the shell or hulls colour changes from green to an autumnal yellow/red. The hulls contain high amount of phenolic and flavonoid compounds, which are known as a source of antioxidant.

Recently, the use of natural additives found in plant material in preservation of food and cosmetic products received considerable attention. Antioxidants in food containing lipid also increase the shelf life of food by retarding the lipid peroxidation. Nowadays, using synthetic antioxidant such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) is common although the carcinogenicity of these commercial antioxidants has been reported by Mahdavi and Salunkhe (1995). Hence, a lot of focus has been directed to discovering and investigating antioxidants from natural sources, which are mostly present in plants (Zainol et al., 2003). Furthermore, adding natural sources of antioxidants to foods, due to their potential to scavenge the free radicals (i.e. antioxidant power), has become an interesting subject in the improvement of food safety, security and storage. These radicals not only induce lipid peroxidation that causes deterioration of foods, but also cause oxidative damage by oxidizing biomolecules leading to cell death and tissue damage, as in atherosclerosis, cancer, emphysema, cirrhosis and arthritis (Kehrer, 1993). Having food fortified

by natural antioxidants may also reduce the oxidative damages (Halliwell, 1989).

Microbial sources have been shown to be a potential means of producing natural antioxidants in various fermented products such as Indonesian tempeh produced by *Rhizopus oligosporus* (Sheih et al., 2000), Chinese douchi by *A. oryzae* (Wang et al., 2007), Japanese miso by *A. oryzae* and *Saccharomyces rouxii* (Hirota et al., 2000), and Chinese furu or sufu by *Aspergillus spp.* (Ren et al., 2006). Documentation of such a practice in pistachios, however, is still lacking. Therefore, this research was conducted to evaluate the effect of solid-state fermentation using several fungi on antioxidant activities of pistachio hull.

## Materials and Methods

Ferrous chloride,  $\alpha$ -tocopherol, 1,1-diphenyl-2-picryl-hydrazyl (DPPH), rutin, gallic acid, BHT, Folin-Ciocalteu's reagent were purchased from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany). Other chemicals used were of analytical grade and were obtained from Merck. UV-VIS Spectrophotometer UV-3600 shimadzu was used for all colorimetric reaction assays in this research.

### *Plant material*

Pistachio (*Pistacia vera* L.) nuts harvested freshly from the farm, which was located in city of Kashmar, Khorasan Razavi Province, I.R. Iran on October 2008. Hulls were removed and freeze dried, and stored for further experiments.

### *Solid state fermentation (SSF)*

Solid state fermentation was carried out in cotton-plugged 500 ml Erlenmeyer flask on solid pistachio hulls medium, which were wetted with a

salt solution to 66% moisture content. Composition of the salt solution (w/v) was 0.5%  $\text{NH}_4\text{NO}_3$ , 0.5%  $\text{KH}_2\text{PO}_4$ , 0.1%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.1%  $\text{NaCl}$  and 0.1% (v/v) trace element solution at pH 6.0. The composition of the trace element solution (w/v) was of the following: 0.08%  $\text{MnSO}_4$ , 0.17%  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , and 0.25%  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ . The wet SSF media were routinely sterilized in an autoclave at 121 °C in 1.05  $\text{kg}/\text{cm}^2$  pressure for 15, inoculated from fully sporulating petri plate cultures (PDA,  $10^6$  viable spores/g dry matter SSF medium) or by transferring a loopful of mycelium 1% (w/w) into the inoculum medium and incubated at 30°C without shaking for 10 days. Fungal strains were obtained from the fungi culture collection of Faculty of Biotechnology and Biomolecular Sciences in University Putra Malaysia. After fermentation was complete, every flask of fermented substrate was dried in the oven at 60°C for 48 hours. Non inoculated substrate was also performed as a control.

### **Extraction**

Pistachio hulls were extracted using 80% methanol as solvent and the hydrolyze extraction technique was used based on Crozier et al. (1997). Freeze dried sample of 0.5 g was weighed and placed into a 100 ml conical flask. Forty ml of 80% (v/v) methanol was added, and then followed by 10 ml of 6M HCl. The mixture was stirred by using magnetic stirrer. The mixture was placed in a sample flask, attached to reflux for 2 hours at 90°C, after which the mixture was filtered using Whatman No. 1 filter paper (Whatman, England) and taken to dryness by using vacuumed Rotary Evaporator (Buchii, Switzerland) heated at 40°C.

### **Determination of total phenolic compounds**

The amount of total phenolic compounds in the pistachio hulls extract was determined with the Folin-Ciocalteu's reagent (Halicia et al., 2005) using gallic acid as standard. Samples of 100  $\mu\text{l}$  were introduced to test cuvettes, and then 2.5 ml Folin-Ciocalteu's reagent (diluted 1:10, v/v) and 2 ml  $\text{Na}_2\text{CO}_3$  (7.5%) were added. The absorbance of all samples was measured at 765 nm using a UV-visible spectrophotometer after incubation at 30°C for 90 min. Results were expressed as milligrams of gallic acid equivalents (GAE) per gram dry weight.

### **Determination of total flavonoid compounds**

Total flavonoid compound was measured using aluminium chloride colorimetric assay based on Zhishen et al. (1999). An aliquot (0.1 ml) of extracts or standard solution of rutin were added to 0.3 ml of 5%  $\text{NaNO}_2$ . After 5 min, 0.3 ml of 10%  $\text{AlCl}_3$  was added. At 6 min, 2 ml of 1 M NaOH was added and the total volume was made up to 5 ml with distilled water. The solution was mixed well and the absorbance was measured against prepared reagent blank at 510 nm. Total flavonoid compound of extracts were expressed as mg rutin equivalent per gram dry weight.

### **Free radical scavenging activity (DPPH)**

The free radical scavenging activity of extracts were measured by 1, 1-diphenyl-2-picrylhydrazil (DPPH.) using the modified method of Burits and Bucar (2000). Solution of 0.1 mM DPPH in methanol was prepared and 3 ml of this solution was added to 1.0 ml extracts solution in water at different concentrations. The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then the absorbance was measured at 517 nm using a spectrophotometer. Lower absorbance

of the reaction mixture indicated higher free radical scavenging activity. The DPPH radical concentration was calculated using the following equation:

$$\text{Free radical scavenging activity (\%)} = [(A_0 - A_1) / A_0] \times 100\%$$

where  $A_0$  is the absorbance of the control reaction, and  $A_1$  is the absorbance of the sample.

**Statistical analysis**

The data were analysed as a completely randomized design using the ANOVA procedure of SAS 9.1. Means were compared using Duncan test ( $p < 0.05$ ).

**Results**

Data of the total flavonoid compounds (Figure 1) indicated a significant ( $p < 0.05$ ) decrease in the concentration of flavonoid compounds of pistachio hulls with fermentation procedure using all types of fungi in the following descending manner: Non-fermented control > *R. oligosporus*-fermented pistachio hulls (-FPH) > *A. terreus* ATCC-FPH > white ATCC64897 rot fungi-FPH > white rot fungi ATCC90467-FPH = *A. oryzae*-FPH. The reduction in total flavonoid content compared to the control was recorded between 69 and 90%.

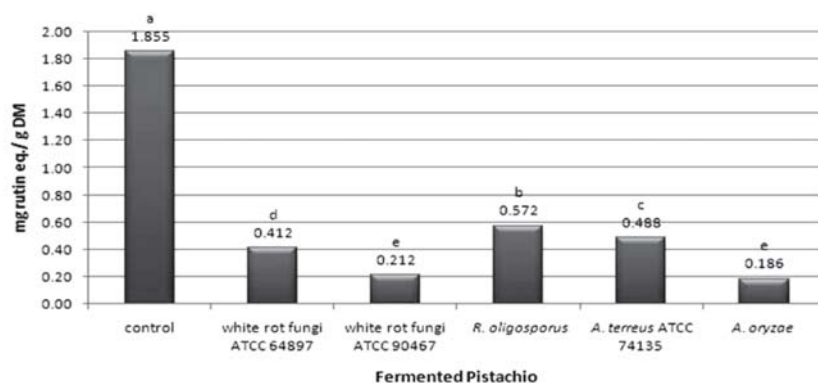


Figure 1. Total flavonoid compounds in pistachio hulls after fermentation

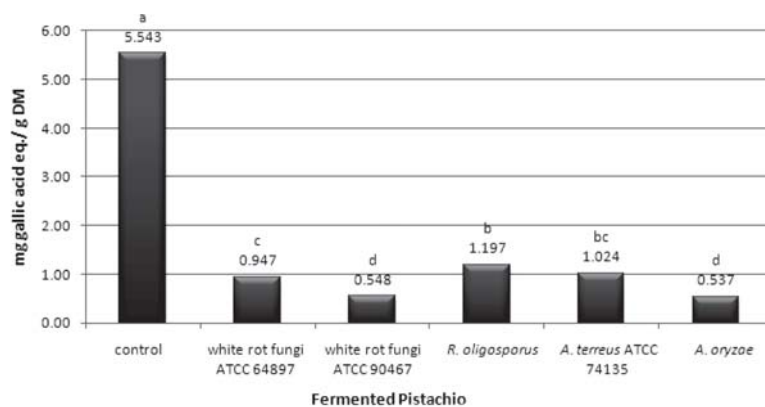


Figure 2. Total phenolic compounds in pistachio hulls after fermentation

Similar trend of significant reduction ( $p \leq 0.05$ ) was observed for total phenolic contents (Figure 2) from microbial-fermented pistachio hull in the following descending manner: Non-fermented control > *R. oligosporus*-fermented pistachio hulls (-FPH) = *A. terreus* ATCC-FPH = white ATCC 64897 rot fungi-FPH > white rot fungi ATCC90467-FPH = *A. oryzae*-FPH. The reduction in total phenolic content compared to the control was between 78 and 90%.

Antioxidant activity of pistachio hulls (Figure 3) was compared with butylated

hydroxytoluene (BHT) and vitamin E as standards. Values of both antioxidant activities of fermented and non-fermented pistachio hulls were lower than the standards (Table 1) by 31.2 to 50.4 % depending on type of fungi used in the fermentation process. Values of fermented hulls, however, had consistently recorded lower antioxidant activity than non-fermented pistachio hulls implying a reduction in the antioxidant activity of pistachio hulls with conduction of fermentation process.

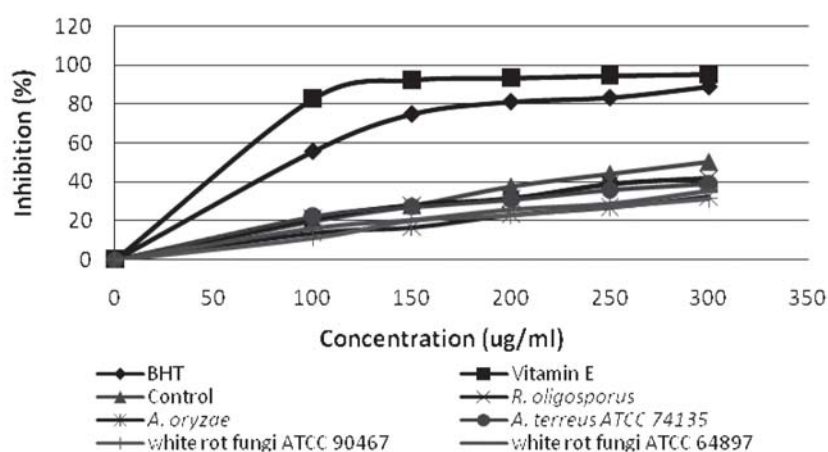


Figure 3. Free radical scavenging activity in fermented pistachio hulls

Table 1. Antioxidant activity of the standards, and fermented and non-fermented pistachio hulls extracts

Sample	% Inhibition of free radicals in 300 $\mu$ g concentration	% Difference from BHT/Vitamin E
BHT	99.2	-
Vitamin E	99.7	-
Pistachio hulls	50.4	-
<i>R. oligosporus</i>	41.8	58
<i>A. terreus</i> ATCC 74135	39.2	61
white rot fungi ATCC 64897	35.6	64
<i>A. oryzae</i>	32.5	67
white rot fungi ATCC 90467	31.2	69

## Discussion

It has been observed that solid state fermentation was unable to improve the phenolic and flavonoid contents of pistachio hulls as well as its antioxidant activity although these results were in contrast with those of Sheih et al. (2000), Hirota et al. (2000), Ren et al. (2006), Wang et al. (2007) and Moktan et al. (2008) who indicated that solid state fermentation can improve the antioxidant activity. The contradictory results obtained from the present work could be attributed to either the sample concentration of phenolic and flavonoid compound, or the duration of the fermentation process.

Basically, the improvement in antioxidant activity occurs while the fermentation is taking place where microorganisms start breaking down the linkage of phenolic and flavonoid compounds, which free the compounds to actively play the role of antioxidants Moktan et al. (2008) and Ren et al. (2006). Increasing the fermentation time might allow the microorganism to use those available compounds as substrate for their growth, thus reducing the sample concentration. Therefore, the fermentation period is critical to ensure optimal breaking down of the compound linkages but not to allow compounds to be substrates for microbial growth. Present research of fermentation process using different microbial types is not able to improve the antioxidant activities of pistachio hulls, thus it is not recommended. Since pistachio hull, an agricultural waste, is high in phenolic and flavonoids contents, and its antioxidant activity (54.2%), improved fermentation duration should be investigated further to tap this inexpensive source of antioxidant with the possibility of being converted into functional food, and at the same time help to reduce its potential as an agriculture waste in the environment.

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