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# Evaluation of Antimutagenic and Anticarcinogenic Effects of Phenolic Compounds in Fresh and Dried Kernels of Pistachio, Hazelnut and Walnut.

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**ABSTRACT**

Chemical compounds present in nuts are involved in combating a number of life-threatening diseases such as cancer and cardiovascular. Identifying the antimutagenic compounds from plant sources, and evaluating their beneficial properties is an effective step in exalting the human health. Antimutagenic and anticarcinogenic activities of phenolic compounds (as natural antioxidants) in fresh and dried kernels of pistachios, walnuts and hazelnuts were examined by Ames method, which in it was used from mutant strains of *Salmonella typhimurium* ($TA_{100}$), chemical carcinogen of sodium azid and microsomes of rat liver ($S_9$). The results showed that, the content of total phenol in fresh kernel of walnut was significantly higher than other the kernels. Also, the phenolic compounds of extracted from the kernels were reduced the carcinogenic effects of toxic sodium azide, too. Antimutagenic and anticarcinogenic potential of the fresh and dried walnut and pistachio as well as fresh hazelnut were evaluated strong, while these potential was moderate in dried hazelnut. Due to more activities of phenolic extracts in presence of microsomes, the anticarcinogenic activities were higher than antimutagenic effects in all of the kernels. In the meanwhile, anticarcinogenecity effects in the fresh kernels of the nuts were higher than the dried. This result is likely to be related with high content of total phenol in the fresh kernels. Assessment of correlation results indicated that, there was a positive and significant correlation among content of total phenol and antimutagenic and anticarcinogenic activities in all of the kernels.

**Keywords**: Ames test, Maceration method, Natural antioxidants, Nuts, $S_9$.

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## INTRODUCTION

In the past, the people thought that the diets have any role in death from cancers. But today, scientists have proved that daily diet containing antioxidant compounds can reduce the risk of cancer by about 35 percent [30]. Antioxidants are compounds that hinder oxidative processes and thereby delay or prevent oxidative stress [22]. Oxidative stress is caused by excess of free radicals due to lifestyle and pathological situations and it has been related to cardiovascular disease, cancer and other chronic diseases that account for a major portion of deaths today [39]. Due to the presence of pistachio (*Pistacio vera* L.), walnut (*Juglans regia* L.) and hazelnut (*Corylus avellana*) kernels in the daily diet of most people as well as relatively high levels of antioxidant compounds (such as flavonoids, phenolic compounds and vitamin E) in theirs, it can be stated that these nuts can be important role in reducing the damage caused by free radicals [38]. Several studies indicate that, there was a significant and negative correlation between frequent nut intake and reduction of LDL-cholesterol levels [1, 20]. In a study for assessment effects of phenolic compounds present in daily food basket of adults and older peoples on their health were found that, these compounds were reduced atherosclerosis and cancer diseases (especially prostate and breast) to the extent of 30 to 40 percent [8]. Shun et al., (2006) were also reported, mainly phenolic compounds are including vitamins, pigments and flavonoids, which are responsible for the antimutagenic and anticarcinogenic activities [35]. The antioxidant properties of these compounds is mainly due to their chemical structure and reductional power, which enables them to neutralizing free radicals as well as turning off the triple oxygen molecules [25]. Phenolic compounds, inhibits lipid oxidation reactions by donating electrons to free radicals [17]. Results of past study were indicated, when the phenolic extracts of edibles nuts (as bioactive compounds), were superseded instead chemotherapy drugs, they were prevented the growth of cancer cells by 20% [10]. The key role of phenolic compounds as the eliminator of free radicals, have been reported in several articles. For example, it has been reported that, phenolic compounds can effectively act as a hydrogen donor and therefore have a powerful antioxidant role [2,19, 37]. Besides, in a study for evaluation of free radical scavenging by phenolic compounds in macadamia (*Macadamia ternifolia*) indicated that, there was a positive correlation between phenolic compounds and antioxidant activity [29]. Antioxidant potential of cumin extracts (*Cuminum cyminum* L.) were indicated a significant and positive correlation with present phenolic compounds, too [6]. According to the mentioned results, more attention to presence of the edible kernels in the food basket of the families and recommendation to usage of them by different age groups, can be immense helps to prevention of cancer diseases in human's community (especially children).

Today, bacteria are being used for the assessment of antimutagenicity activities of different compounds in a short time with excellent results. One of the methods used for assessing the mutation prevention properties of a compound in bacteria is the Ames test. Ames and colleagues assessed the antimutagenic and anticarcinogenic activities of different compounds. In this method, Salmonella strains incapable of synthesizing histidine due to mutations are used [32]. In a comparative study, it was concluded that systems exploiting *Salmonella typhimurium* $TA_{100}$ in the assays are most capable in identifying the mutagenic capacity of different chemicals [16]. This strain carries a specific mutation in its His-operon that makes it histidin auxotroph. This bacterium when in contact with a mutagen will revert and start synthesizing histidin. On the other hand, mouse hepatic homogenate, containing microsomal enzymes including cytochrome P450 has anticancer properties. Therefore, in cases where an antioxidant compound shows a synergistic effect with the anticarcenogenic activity of cytochrome P450, an anticarcinogenic activity can also be assigned to this compound [33].

The aim of this study was to evaluation of antimutagenic and anticarcinogenic effects of extracted phenolic compounds from fresh and dried kernels of pistachio, walnut and hazelnut by using of *Salmonella typhimurium* bacteria and sterile extracts of rat liver microsomal.

## MATERIAL AND METHOD

### Plant material

In this study, pistachio, walnut and hazelnut cultivars were Ouhadi, Franquet and Fertile de Cotard, respectively. Pistachio fruits were collected from the Pistachio Research Center (Rafsanjan, Iran) in late July (the best time for harvesting). Walnut and hazelnut fruits were obtained from Golestan Agricultural Research

Center (Gorgan, Iran) in late of June and early of Agust, respectively. The fresh fruits had normal skin at harvest time. Samples were stored after picking up the skin at 4 ° C.

**Bacterial strains**

*Salmonella typhimurium* strain $TA_{100}$, directly sent to us by professor Ames, was cultured in a nutrient broth. The overnight culture was used for strain identity confirmation.

**Strain $TA_{100}$ identity assays**

**Rfa mutation**

Sensitivity to viole crystal was tested. A 100 µl sample of the overnight bacterial culture was inoculated in 2 ml of melted and cooled top agar and spread over an agar nutrient plate. A disk dipped in crystal viole was later placed on this plate and after a 16-hour period, a bright zone was observed around the disk, an indication of the lack of cell growth due to the Rfa mutation [6].

**R-factor assay**

This assay confirms ampicillin resistance. The absence of zone of growth inhibition around the disk was an indication of $amp^R$ and a proof for the presence of the R-factor in the bacterial strain [6].

**UVrB mutation**

This assay confirms UV sensitivity of the strain. A petri dish containing a dense bacterial lawn of $TA_{100}$ strain was used. One half of the dish was covered with aluminum foil, and the dish was exposed to UV light at a distance of 33cm for 8 seconds. Following an 18 hour heating period, the absence of zone of growth inhibition in the UV-exposed half was an indication of UVrB mutation in the strain [6].

**Chemicals material**

Chemical materials used in different parts of the study were purchased from the companies of Merck and Roche (Germany). The most important chemical materials which used in the study were included; sodium azide (as a carcinogenic compound), amino acids of Histidin and Biotin, potassium hydrogen phosphate, sodium ammonium phosphate, citric acid monohydrate, galic acid, folin ciocaltue reagent, glucose monohydrate.

**Extraction of phenolic compounds**

In the first, kernels of nuts were separated from the shells and were crushed by an electrical mixer (National Blender, Model: NO.MG176NR5430), separately. Blades and glass container machine were disinfected before use with a solution Deconex. In the following, the crushed kernels were poured in dark glass containers which were autoclaved before. In order to extract the phenolic compounds, one gram of sample was poured into a small erlenmeyer flask, then was added 20 ml of 60% methanol and placed in the dark for 90 min. After this period, extraction was carried out using a household microwaves oven devices (full power 700 W, National, Japan). Power of microwaves device and extraction time were adjusted to 10% and 4 min, Respectively [28]. However, in order to prevent heat rise, the samples were withdrawn from microwave device after one minute and placed in the refrigerator to reach the temperature below 30 ◌̊C [9].

**Determination of total phenolic contents**

The concentration of phenolics in plant extracts was determined using spectrophotometric method [36]. Methanolic solution of the extract in the concentration of 1 mg/ml was used in the analysis. The reaction mixture was prepared by mixing 0.5 ml of methanolic solution of extract, 2.5 ml of 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 ml 7.5% NaHCO3. Blank was concomitantly prepared**,** containing 0.5 ml methanol, 2.5 ml 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 ml of 7.5% of NaHCO3. The samples were thereafter incubated in a thermostat at 45 ◌̊C for 45 min. The absorbance was determined using

spectrophotometer (JENWAY – 6405 UV/Vis, Belgium) at λmax = 765 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of gallic acid and the calibration line was construed. Based on the measured absorbance, the concentration of phenolics was read (mg/ml) from the calibration line; then the content of phenolics in extracts was expressed in terms of gallic acid equivalent (mg of GA/g of extract).

**Determining the antimutagenic strength of the fresh and dried kernels of Pistachio, walnut and hazelnut using *Salmonella typhimurium* strain $TA_{100}$**

In this assay, each of the test materials (i.e., 0.1 ml of phenolic compounds isolated from the fresh and dried kernels of the pistachio, walnut and hazelnut) were mixed with 0.1 ml of the carcinogen (Sodium azide), present in the positive control, in 3 ml of top agar, 0.1 ml of the overnight culture, and 0.1 ml of histidin and biotin, separately. This mixture was thoroughly spread on a glucose agar plate, and the plate was then overturned and incubated for 24 hours at 37 °C. Each experiment included 3 different plates cultured, simultaneously. Negative control contained 0.5 ml of distilled water instead of sodium azide and shows spontaneous mutation in bacteria, while positive control contains 0.1 ml of the carcinogen. After the heating period, bacterial colonies were counted [3].

**Mouse liver ($S_9$) preparation for carcinogenicity assay**

A broad range of carcinogenic agents require metabolic activation for recognition. In this investigation, 10 male rats, each with an approximate weight of 200 (±5) grams, provided to us by the Pasteur Institute, were used. Rats were starved for 24 hours in order to get the titer of the liver enzymes to their highest levels. Spinal cords of the animals were ceased, livers were surgically removed and washed in a 0.15 M Potassium Chloride solution. Livers were cut into pieces using sterile scissors and smashed prior to a 10 min centrifugation at 9000 g. All the above steps were performed at 4 °C. The supernatant ($S_9$) was stored at -80 °C. The antimutagenic assay was performed in the presence of $S_9$, as mentioned previously. Positive control included 0.1 ml of the O/N culture, 0.1 ml of the mutagen and 0.1 ml of $S_9$, while the test petri dish contained 0.1 ml of the O/N culture, 0.1 ml of the mutagen, 0.1 ml of phenolic compounds isolated from the each kernels and 0.1 ml of S9. The negative control contained 0.5 ml ddH2O, 0.1 ml of the O/N culture and 0.1 ml of S9. It is worth mentioning that histidin and biotin were added to all the above petri dishes and that each experiment was performed in triplicates, simultaneously. Bacterial colonies were counted following the heating cycle [34].

**Calculation of inhibition percentages**

Inhibition percentages were calculated using the Ong and colleagues' formula (1-T/M) x 100, in which T represents the number of revertants in each plate in the presence of the antimutagen, while T stands for the number of revertants in each of the positive control plates [27]. It needs to be mentioned that the number of revertants in the negative control is subtracted from the values of T (the numerator) and M (the denominator). Inhibition of > 40% and 25-40% are indicative of a strong and a medium antimutagenic effect, respectively, while a < 25% inhibition indicates the absence of this effect [24].

**Statistical analyses**

Data such as the number of revertants in the mutagenecity assay were analyzed using the one-way analysis of variance (ANOVA) test in SPSS (ver. 16.00).

## RESULTS

**Mutant confirmation in strain $TA_{100}$**

In accordance with the *Salmonella typhimurium* $TA_{100}$ strain genotype, the reduction in the mutant strain of lipopolysaccharides allowed viole crystal penetration, bacterial death and formation of a zone of about 14 mm, while no such zone was formed in the wild type strain. The experimental strain was ampicillin-resistant due to the presence of the R-factor plasmid. UvrB mutation was con-firmed by the lack of growth in the irradiated section (Table 1).

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**Table 1: Confirmation tests of mutagenicity in *Salmonella typhimurium* $TA_{100}$**

| Experimental strain | rfa mutation | UvrB mutation | R-factor plasmid |
|---|---|---|---|
| *Salmonella typhimurium* $TA_{100}$ | + | + | + |

**Total phenol content**

Analysis of variance for total phenol content indicated significant differences (P<0.01) among fresh and dried kernels of pistachio, walnut and hazelnut (Table 2). Among kernels of the nuts, the highest and lowest total content of phenol were observed in kernels of fresh walnut and dried hazelnut, respectively (Fig 1.). Although, differences among kernels of dried pistachio, fresh hazelnut and pistachio and dry walnut were not significant. Assessment of correlation tables indicated that, there was a positive and significant correlation between inhibition percentages of antimutagenicity and anticarcinogenicity with total content of phenol in all kernels of the nuts (Table 3).

**Table 2: The mean squares of ANOVA for content of total phenol and average percentage of inhibition in the absence and presence of $S_9$ (antimutagenic and anticarcinogenic effects, respectively) in fresh and dried kernels of the nuts**

| Features | df | Mean Square | | |
|---|---|---|---|---|
| | | content of total phenol | average percentage of inhibition in the absence of $S_9$ (antimutagenic effects) | average percentage of inhibition in the presence of $S_9$ (anticarcinogenic effects) |
| Between Groups | 7 | ** | ** | ** |
| Within Groups | 16 | ns | ns | ns |

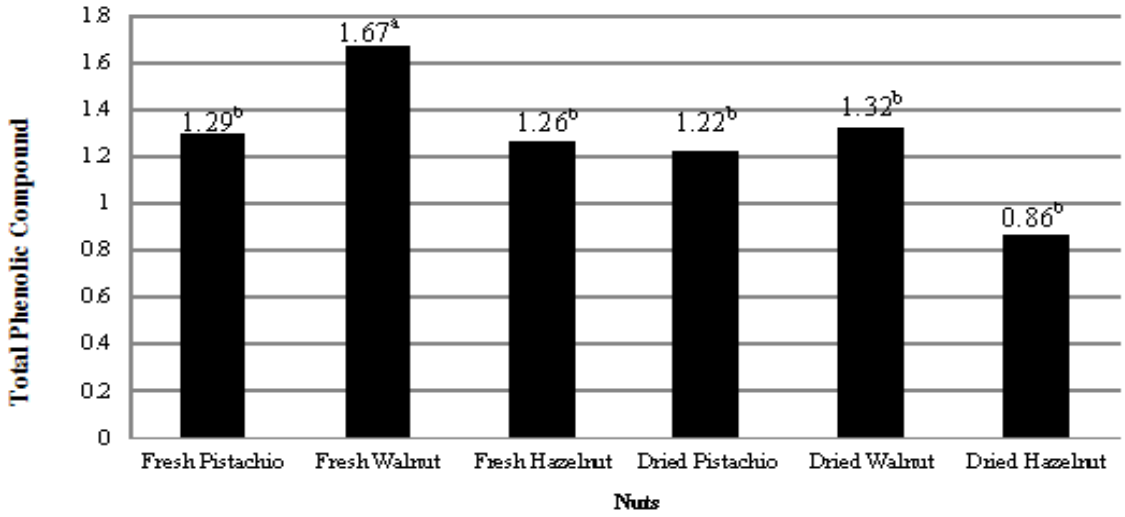


**Figure 1: Average content of total phenol in fresh and dried kernels of pistachio, walnut and hazelnut**

**Antimutagenicity effects of extracted phenolic compounds**

Assessment the results of ANOVA table were distinguished that, a significant change (P<0.01) occurred in antimutagenic effects of fresh and dried kernels of the nuts (Table 2). The mean number of reverted colonies and also percent inhibition of fresh and dried kernels of pistachio, walnut and hazelnut in the absence of $S_9$ were shown in table 4. Sodium azide was used as the mutagen in the positive controls. A reduction in the number of reverted colonies was observed in the negative control. This reduction was confirmed the antimutagenic effect of sodium azide. According to the results of Duncan's test, fresh walnut was demonstrated high antimutagenic effects and ranked alone at the top group. In the other grouping, kernels of fresh hazelnut, fresh pistachio and dried walnut was ranked in a group. The lowest percentage of inhibition in the absence of $S_9$ was belonged to dried kernel of hazelnut (Table 4).

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**Table 3: Correlation coefficient between average percentage of inhibition in the absence of $S_9$ (antimutagenic effects) and presence of $S_9$ (anticarcinogenic effects) with content of total phenol**

| | Features | Content of total phenol | Antimutagenic effects | Anticarcinogenic effects |
|---|---|---|---|---|
| Content of total phenol | Pearson Correlation | 1 | 0.930** | 0.968** |
| | Sig. (2-tailed) | | 0.000 | .000 |
| | N | 18 | 18 | 18 |
| | | | | |
| percentage of inhibition in the absence of $S_9$ (antimutagenic effects) | Pearson Correlation | 0.930** | 1 | 0.969** |
| | Sig. (2-tailed) | 0.000 | | 0.000 |
| | N | 18 | 18 | 18 |
| | | | | |
| percentage of inhibition in the presence of $S_9$ (anticarcinogenic effects) | Pearson Correlation | 0.968** | 0.969** | 1 |
| | Sig. (2-tailed) | 0.000 | 0.000 | |
| | N | 18 | 18 | 18 |
| **. Correlation is significant at the 0.01 level (2-tailed). | | | | |

**Table 4: The mean number of reverted colonies and average percentages of inhibition in the presence and absence of mouse liver microsome ($S_9$) in fresh and dried kernels of pistachio, walnut and hazelnut**

| | | Absence of mouse liver microsome (-S9) | | | Presence of mouse liver microsome (+S9) | | |
|---|---|---|---|---|---|---|---|
| | | The mean number of reverted colonies | Std. Deviation | Average percentage of inhibition | The mean number of reverted colonies | Std. Deviation | Average percentage of inhibition |
| Con + | | 314.67 | 13.20 | 0[e] | 291.00 | 12.12 | 0[e] |
| Fresh kernels | Pistachio | 173.00 | 7.21 | 45.02[c] | 159.00 | 6.24 | 45.36[c] |
| | Walnut | 145.00 | 5.00 | 53.92[b] | 128.00 | 2 | 56.01[b] |
| | Hazelnut | 165.33 | 3.79 | 47.46[c] | 150.00 | 2.64 | 48.45[c] |
| Dried Kernels | Pistachio | 188.00 | 5.00 | 40.25[c] | 161.00 | 9.54 | 44.67[c] |
| | Walnut | 181.00 | 7.94 | 42.48[c] | 154.00 | 4.36 | 47.08[c] |
| | Hazelnut | 210.00 | 3.61 | 33.26[d] | 182.00 | 3.61 | 37.46[d] |
| Con- | | 8.67 | 0.57 | 97.25[a] | 6.67 | 0.58 | 97.71[a] |
| Total | | 173.20 | 80.61 | | | 74.22 | |

- For a given means within each column of each section followed by the same letter are not significantly different ($P < 0.05$).

**Anticarcinogenic effects of extracted phenolic compounds**

In this part of research, the mean number of reverted colonies and also percent inhibition to be calculated in the presence of mouse liver microsome ($+S_9$). Based on the results, a Significant differences ($P<0.01$) were revealed in anticarcinogenic effects of fresh and died kernels of the nuts (Table 2). Also, the number of reverted colonies was reduced in this condition ($+S_9$) in comparison with absence of $S_9$ in all of the nuts and so positive and negative control (Table 4). Among kernels of the nuts, the highest and lowest reverted colonies obtained from fresh walnut and dried hazelnut. Increase in percent inhibition was also observed in all kernels of the nuts under in this condition ($+S_9$). Among of all of the nuts, fresh kernel of walnut indicated the highest anticarcinogenic effects (56.01%). Also, Similar to condition of absence microsome ($-S_9$), dried kernel of hazelnut was allotted the lowest percent inhibition to itself. These nuts were placed on the separated groups. Other the nuts had no significant differences together and thus were classified on a group (Table 4). Classification was calculated by Duncan's test.

## DISCUSSION

Cancer is considered as one of the main causes of mortality throughout the industrial world in the present century. To this date, a wide range of chemical mutagens and carcinogens have been identified. Scientists believe that damage to the genetic material, changes in DNA sequence and continuity, mutation in genes and other genetic changes in chromosomal structures play important roles in carcinogenesis. The Ames test is a common methodology for screening and identifying both mutagens and antimutagens. In this method, using mutant strains of *Salmonella typhimurium*, a number of plant-derived compounds have been introduced as both antimutagens and anticarcinogens [31]. Results of the past studies were indicated that, strains of $TA_{92}$, $TA_{97}$, $TA_{98}$ and $TA_{100}$ have plasmids of PKM101 and R-factor. These strains can uses for identification of a very weak carcinogen [12, 26]. In present study was used a mutant strain of $TA_{100}$.

The data obtained from this study indicated that, there were different contents of total phenol in kernels of the nuts. Of course, these contents were higher in all of the fresh than dried kernels. Also, results were demonstrated that, increase in content of total phenol resulted to increase in antioxidants properties. Review in past studies showed that, high levels of phenolic compounds is the main reason for the high antioxidant activity of some plant extracts. Based on the results, there was a positive and significant difference between content of phenolic compounds and their antioxidants properties [5, 10, 11, 18]. Generally, the most important role of a phenolic compound is elimination of free radicals as antioxidant. This role was clearly stated in different scientific report. It has been reported that, the phenolic compounds inhibits lipid peroxidation by donating electrons to free radicals [19, 2, 14]. Of course, Lagouri and Boskou, (2008) were emphasized that, high capacity of phenolic compounds for scavenging of free radicals is related to its high molecular weight [21].

Our results on the antimutagenecity and anticarcinogenicity effects of fresh and dried kernels of the nuts with respect to the positive control (sodium azide) indicated strong (in walnut and pistachio) and moderate (in hazelnut) of these effects. However, the inhibition mechanism of phenolic extracts on the sodium azid was not very clearly reported. But researchers were supposed that, the main reason of these effects can be related to controlling effects of the phenolic compounds on cancerogens and their precursors, inhibition of act in the bacteria which convert precarcinogens to carcinogens, and or activation of safety systems in consumers, Probably [23]. They also were stated that, phenolic compounds can neutralize sodium azide mutagenicity property through binding to the mutagenic agent. Bo et al., (2011) in their study were demonstrated, because of phenolic compounds act as electron donors, therefore, they can neutralize the undesired reactions caused by free radicals in the body. Therefore, they have high anticarcinogenic effects [7].

The anticarcinogenecity of the phenolic extracts were increased by inclusion of the microsomes. This is because in the presence of antioxidants and antimutagens, cytochrome P450 enhances this effect, and hence the phenolic extracts are called anticarcinogens [24]. It is also possible that, there are other compounds in those extracts which can be activated in the presence of cytochrome P450 [4]. A review on the molecular mechanisms of the effects of dietary lipids, including nuts and oil olive, on cancer concluded that phenolics and MUFAs (monounsaturated fatty acids), are responsible for lowering the incidence of cancer [15]. Finding of Devore et al., (2012) has been shown that, flavonoids by scavenging free radicals and a lower tendency to interact with oxygen, play an important role in reducing DNA damage, too [13]. In another study for

assessment of beneficial effects of edible nut on certain cancer types including breast, prostate, colon, bladder and stomach demonstrated that phenolics in the edible nuts are important in scavenging DNA damaging reactive oxygen species (ROS) [40].

## CONCLUSION

According to the confirmation of antimutagenecity and anticarcinogenecity effects in fresh and dried kernels of the nuts, it is an emphatic recommended that consumption of these nuts should be placed in daily diet of people. If only fresh kernels of the nuts are available, it is better that, walnut to be used as first priority and pistachio or hazelnut as secondary priority. The results were demonstrated, if only dried kernels of the nuts are available, priority of consumption will be included walnut or pistachio (as first priority) and hazelnut (as secondary priority), respectively. Finally, when fresh and dried kernels of the nuts are available, it is more correct that, priority of consumption to be as followed;

Fresh kernel of walnut (as first priority), kernels of dried walnut or fresh hazelnut or fresh pistachio or dried pistachio (as secondary priority) and dried kernel of hazelnut (as third priority).

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