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# Comparative study of antioxidant activity of polyphenols isolated from frozen and fresh leaves of *Trachyspermum ammi* (Ajwain)

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**Abstract**
Trachyspermum ammi, Ajwain is a rich in vitamins and minerals, it is also concentrated in health-promoting phytonutrients such as carotenoids (beta-carotene, and lutein) and flavonoids to provide powerful antioxidant protection. The aim of this was to determine the effect on total antioxidant activity in fresh and frozen ajwain leaves. The extract of fresh and frozen ajwain leaves was subjected to in vitro free radical scavenging DPPH assay. The results obtained were analyzed. Antioxidant activity was exhibited significantly higher in frozen ajwain leaves than fresh one. Lutein content was also found higher in frozen Ajwain leaves in comparison with fresh Ajwain. Hence the antioxidant activity of ajwain is highly correlated to the concentration of polyphenols and freezing condition.

**Keywords:** Ajwain, Antioxidant activity, Radical scavenging activity, Polyphenols Lutein. Carotenoids

## 1. Introduction
Leaves of Ajwain, Trachyspermum ammi is used as green vegetable for salad has high nutrition values. The dark green leaves contain many valuable nutrients, especially the antioxidant carotenoids, lutein and zeaxanthin. To get availability of ajwain leaves throughout the year supply of frozen ajwain has become popular now a days. It is most important to check how freezing effects the antioxidant property of ajwain leaves.

Poly phenols mainly lutein is reducing agent and belong to the group of antioxidants (Sies 1997) [13]. The important role of lutein is to neutralize the free radicals or reactive oxygen species such as the hydroxyl radical (·OH), the superoxide anion ($O_2^{\cdot-}$) and others (Bergamini *et al.* 2004; Dean *et al.* 1997; Stoian *et al.* 1996) [2, 4, 15]. Lutein exert the antioxidant actions based on their singlet oxygen quenching properties and ability to trap peroxyl radicals (Paiva and Russell 1999; Stahl and Sies 1996) [10, 14]. The first one depends on the number of conjugated double bonds of the molecule and is influenced to a lesser extent by cyclic or acyclic carotenoid groups (Paiva and Russell 1999; Krinsky 1998) [10, 6]. Also, cyclic substituents as an end group of polyene hydrocarbon chain (in the structure of carotenoids, as well as xanthophylls like lutein), influence the antioxidant activity of the mentioned compounds.

There are several methods for assays have been introduced for the measurement of the total antioxidant activity of pure compounds (Re *et al.* 1999; Rice-Evans *et al.* 1996; Miller and Rice-Evans 1994; Miller *et al.* 1996; Kono *et al.* 1995) [11, 9, 12, 8, 12, 5]. Each method relates to the generation of a different radical, acting through a variety of mechanisms and the measurement of a range of end points at a fixed time point or over a range (Miller *et al*. 1996; Kono *et al*. 1995; Arnao *et al*. 1990) [9, 12, 5, 1] Two types of approach have been taken into consideration. One of them is the inhibition assays; in that, the extent of scavenging by hydrogen or electron donation of a preformed free radical is the marker of antioxidant activity, as well as assays involving the presence of antioxidant system during the generation of the radical (Re *et al*. 1999) [11]. These assays have been based on different principles and experimental conditions. In general, DPPH· (1, 1-diphenyl-2-picrylhydrazyl) and ABTS (2, 2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) radical cations are the basis of the spectrophotometric methods that have been applied to the measurement of the total antioxidant activity of solutions of pure substances. The total antioxidant activity can be expressed by the ferric reducing antioxidant power assay (FRAP), oxygen radical absorbance capacity (ORAC), superoxide radical scavenging activity or Trolox equivalent antioxidant activity (Bertoncelj *et al.* 2007) [3]. The aim of this study was to evaluate the radical scavenging activity of

samples of extracts fresh ajwain and frozen ajwain. The antioxidant activity of extracts from ajwain is highly correlated to the concentration of polyphenols including phenolic acids, flavonoids as well as carotenoids. For that reason, the concentrations of these compounds in analysed extracts were determined.

## 2. Materials and Methods

### 2.1 Materials, Chemicals and Reagents

Fresh and frozen ajwain leaves were obtained from a local marketplace at Lucknow, 1, 1-Diphenyl-2-picrylhydrazyl (DPPH), methanol, acetonitrile, Folin–Ciocalteu reagent, phosphomolybdate, phosphotungstate, gallic acid, lutein, ethyl acetate were all of analytical grade.

### 2.2 Preparation of extracts of Ajwain Leaves

For separation of polyphenols from ajwain leaves, Supercritical fluid extractor SFE 7680 T (Hewlett Packard, Avondale, PA, USA) was applied. Polyphenols and other compounds were extracted from ajwain with supercritical fluid and liquid CO2. The solvent modified with methanol (5%) was applied at the pressures of 100, 175 and 258 bar. Additionally, extraction at 258 bar was made without organic modifier. From every step of extraction, four fractions were obtained (1-mL volume of methanolic extract). Particular supercritical fluid extraction (SFE) conditions include the following: extraction time, 30 min; supercritical fluid, carbon dioxide (purity 99.99%) with organic modifier (5% methanol); fluid temperature, 50 °C; fluid pressures, 100, 175 and 258 bar; temperature of extraction, 39 °C; receiver temperature, 65 °C and trap, a stainless steel tube. For every extraction, 0.2 g of dried ajwain leaves was taken and frozen ajwain leaves was previously kept at room temperature for 3 h.

### 2.3 DPPH Radical-Scavenging Assay of ajwain extracts and Lutein Solutions

The inhibition of free radical scavenging activity of ajwain leaves extracts was measured by spectrophotometer using DPPH [W. Brand-Williams, M. E. Cuvelier and C. Berset 1995] [16]. Three millilitres of DPPH solution (4 mg of pure DPPH dissolved in 10 mL of acetonitrile and 190 mL of methanol) was mixed with 100 mL of extract and stored in the darkness for 30 min. After 30 min, absorbance at 515 nm was measured, and blank was prepared by the use of methanol. Because one of the most important constituents of ajwain leaves extract is lutein, we decided to measure antioxidant activity of this compound. The antioxidant activity of lutein (Roth, Karlsruhe, Germany) solutions in the concentration range of 10 to 100 µg/mL of substance was evaluated. The antioxidant activity was expressed in radical scavenging activity (RSA). RSA was measured by using equation.

$$RSA = \frac{(ADPPH - A)}{ADPPH} X100\%$$

### 2.4 Determination of Total Phenolic Contents in Ajwain leaves extracts

According to references (Wettasinghe and Shahidi 1999; Mélo *et al.* 2006) [17, 7]. The total phenolic content was measured spectrophotometrically at 760 nm using the Folin–Ciocalteu reagent. This reagent is a mixture of phosphomolybdate and phosphotungstate used for the colorimetric assay of polyphenols. Standards of gallic acid in the concentration range of 0.1 to 1.3 mg/mL were also measured. The results were expressed as gallic acid equivalents (GAE) of dry matter.

### 2.5 Quantitative Analysis of Ajwain Extracts and Lutein by HPLC–UV-VIS

The ajwain leaves extracts were analysed using an Agilent model 1100 liquid chromatographic system consisting of a quaternary pump and a UV-VIS detector (Agilent Technologies, Waldbronn, Germany). Compounds were separated on a Discovery C18 column (150×4.6 mm, 5 µm) from Supelco (Bellefonte, PA, USA). Methanol: water (80:20v/v) was used as eluent A, while ethyl acetate was used as eluent B. The mobile phase was delivered at 1.0 mL/min in a gradient mode. For the calibration procedure, standard mixtures of lutein in methanol were prepared. The concentration of lutein was in the range of 5.0–100.0 µg/mL. The linearity of the detector response was determined by the square correlation coefficients of the calibration curves generated by three repeated injections of standard solutions at six concentration levels (5.0–100.0 µg/mL). We defined a signal to noise ratio of 3 as a limit of detection

**Table 1:** Calibration data used for the determination of lutein in ajwain extracts by HPLC–UV-VIS

| Method estimated | Concentration range (µg/mL, injected) | Retention time $t_R$ (min) | Calibration curve | $R^2$ (µg/mL, injected) | LOD (µg/mL, injected) | LOQ (µg/mL, injected) | RSD For peak areas (%) |
|---|---|---|---|---|---|---|---|
| HPLC–UV-Vis | 5.0–100.0 | 8.208 | y040.189x−18.907 | 0.9992 | 0.2 | 0.6 | 5.5 |

(LOD) and a signal to noise ratio of 9 as a limit of quantification (LOQ). All calibration data for lutein, including retention time (tR), calibration equations using peak area, linearity with correlation coefficient (R2) of the calibration curves, LOD and LOQ; precision data as a relative standard deviation (RSD) estimated for peak areas are presented in Table 1. Standard solutions and plant extracts were analysed in triplicate.

## 3. Results and Discussion

In the presented study it was found that we obtained higher results of RSA for frozen ajwain leaves than for fresh ajwain leaves. The RSA value obtained for frozen ajwain leaves were 30.3%. Meanwhile, the RSA obtained for fresh ajwain did not exceed 19.8 %. Particular results obtained during experiments concerning the determination of RSA for fresh and frozen ajwain leaves are presented in Table 2.

Lutein, which is a xanthophyll and belongs to the carotenoid group. Radical scavenging activity was evaluated for lutein solutions in the concentration value range of 10 to 100 µg/mL of substance. The lower concentration of lutein in the solution gave a RSA value of 6.2%, but for ten times higher concentration value of lutein (100 µg/mL), obtained values of RSA did not exceed 10.0%. Obtained results show that lutein

as a component of ajwain leaves extracts takes an insignificant part in the total antioxidant activity of these plant extracts. The total content of polyphenols in ajwain samples varied from 1.823 to 4.946 mg/g of dry matter.

For the determination of lutein, the quantitative analysis of ajwain leaves extracts was performed by the use of the external standard method. Data obtained for the standards in concentration values that range from 5.0 to 100.0 µg/mL gave a calibration curve, which was used for the determination of lutein in real samples (extracts from fresh and frozen ajwain leaves). Regression coefficients of the calibration curves 0.999 for UV-VIS detection were obtained. LOD and LOQ for UV-VIS detection were 0.2 µg/mL and 0.6 µg/mL, respectively. Since the values for LOD and LOQ are low in relation to the usual content of lutein in ajwain leaves extracts, we concluded that this method can be used for quantitative analysis. RSD values estimated for the peak areas of the lutein standards did not exceed 5.5%.

The results after analysis of extracts from fresh and sample of frozen ajwain leaves shown the highest concentration values of lutein 0.88 mg/g for frozen ajwain and 0.83 mg/g for fresh ajwain. Polyphenol, and lutein concentrations in samples of green ajwain leaves (calculated for dry matter of sample, mean of triplicate analysis)

| Sample Ajwain leaves | Concentration of polyphenolic acids (mg GAE/g dry matter) | Concentration of lutein (mg/g dry matter) |
|---|---|---|
| Fresh | 1.791±0.040 | 0.722±0.047 |
| Frozen | 4.312±0.098 | 0.871±0.049 |

The detection of the amount of polyphenols and carotenoids must be related to their radical scavenging activity values, as this assay shows the potential health benefit of these compounds, which is very often independent of their absolute quantity. The RSA value is useful in the choice of the plant genotype and also provides information on the optimal preparation way, which is the most important step to be checked before freezing the vegetables to maintain the antioxidant activity of polyphenols and carotenoids.

## 4. Conclusions

This study confirms that the freezing of ajwain leaves is not the only way of food storage, but freezing also affects the release of carotenoids from plants. The low temperature influences the destruction of the walls of the plant cells. Antioxidant activity of extracts from ajwain leaves is correlated to the concentration of carotenoids and polyphenols. The high level of the content of polyphenol acids and flavonoids in ajwain leaves influences the high antioxidant activity.

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