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Research Article

Compositional Analysis and Evaluation of Antioxidant Potential of Fruits of Withania somnifera Dunal

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Abstract

The present investigation was carried out on the fruits of Withania somnifera to study its proximate composition and in-vitro antioxidant potential. The fruits were found to have 4.67% crude protein, 2.23% crude fat, 1.16% ash, 6.94% crude fiber and 13.39% total sugar on fresh weight basis. Highest total phenolic content (TPC) and flavonoids were found in methanol extract (42.40; 4.76µg/mg extract) while hexane fraction for TPC (3.07 µg/ml extract) and water fraction for flavonoid (0.39µg/ml) were found unsuitable for extraction. Methanolic extract showed highest hydroxyl radical activity (IC_{50}= 3.48 mg), total reducing power (IC_{50}= 0.46 mg), metal chelation capacity (0.29 mg) and DPPH radical scavenging capacity (0.31 mg).IC_{50} value of hexane extract for DPPH radical and chloroform extract for total reducing power were the highest. In conclusion, the overall highest antioxidant potential of methanolic extract can be attributed to TPC and flavonoids contents present in the extract.

Keywords: Antioxidants, Extraction, Ashwagandha, Free radicals, Herbs

Introduction

In aerobic conditions, cells are always threatened with the insult of reactive oxygen species (ROS) generated during oxidative metabolism which, however, are efficiently taken care of by the highly powerful antioxidant system of the cell without any untoward effect. The enzymatic and non-enzymatic defense mechanisms of antioxidant system act synergistically to cope up the stress (Chae et al. 2004). However, some defense strategies are not perfect to counteract the number of free radicals and consequently cellular macromolecules including DNA, protein and lipid gets oxidatively damaged. Oxidative stress induced by oxygen radicals, is believed to be a primary factor in various degenerative diseases, such as cancer (Muramastu et al. 1995), atherosclerosis (Steinberg et al. 1989), gastic ulcer (Das et al. 1997) and other pathological conditions (Oliver et al. 1987; Smith et al. 1996). Thus, the adequate amount of antioxidants is essential part of food for prevention of these diseases. The synthetic antioxidants (Butylated hydroxy toluene, BHT; Butylatedhydroxy anisole, BHA; Propyl gallate, PG and Tertiary butyl hydroquinone, TBHQ) have always been suspected to cause or promote negative health effects at the genetic and molecular level (Barlow 1990; Branen 1975; Chan 1987; Namiki 1990; Pokorny 1991; Sasaki et al. 2002), yet BHA and BHT are added to food preparations because they are good free radical scavengers. Herbal drugs in the recent years have gained popularity because of their safety, efficacy and cost effectiveness. Research interest has increased considerably in finding naturally occurring antioxidants for use in foods or medicinal materials to replace synthetic antioxidants. In addition, natural antioxidants have the capacity to improve food quality and stability and can also act as nutraceuticals

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thus may provide additional health benefits to consumers. Increasing experimental evidences have suggested that these compounds can affect a wide range of biological functions by virtue of their radical scavenging properties (Aruoma 1998; Lai et al. 2001).

In the search of plants as a source of natural antioxidants, some medicinal plants and fruits have been extensively studied for their antioxidant and radical scavenging activity in the last few decades. Numerous studies carried out on some of these plants resulted in the development of natural antioxidant formulations for food, cosmetic and other applications (Singh et al. 2002). However, scientific information on antioxidant properties of various plants, particularly those which are less widely used in culinary and medicine still remains an interesting and useful task, particularly for finding new sources for natural antioxidants, functional foods and nutraceuticals.

Withania somnifera, also known as ashwagandha, Indian ginseng and winter cherry, has been an important herb in the ayurvedic and indigenous medical systems for over 3000 years. The different parts of plant are used to promote health and longevity by augmenting defence against diseases, arresting the ageing process, revitalizing the body, increasing the capability of the individual to resist adverse environmental factors and by creating a sense of mental well-being (Weiner & Weiner, 1994). It is used for a very long time by all age groups of both sexes and even during pregnancy without any side effects (Pal et al., 2012).

Recently, publications on exploration and exploitation of bio-resources for natural antioxidant has risen exponentially, principally for three reasons: (1) the overwhelming epidemiological and clinical evidence suggesting an inverse relationship between the consumption of vegetables and fruits and the risk of developing chronic diseases (2) concerns regarding the safety of the chronic consumption of synthetic compounds traditionally used as preservatives in foods and beverages, and (3) the public belief that phytochemicals are inherently safer than synthetic chemicals. Keeping these in view, the investigation was planned and carried out on ashwagandha fruits.

Materials and Methods

Material

Untreated fruit of Ashwagandha were picked from the local nursery of Mysore, India.

Chemicals & Reagents

HPLC grade solvents were used for extraction and evaluation of antioxidant compounds. Other solvents and chemicals used were- petroleum ether (40-600°C), H_2SO_4, NaOH, HCl, H_3BO_3, DPPH (Diphenylpicrylhydrazyl), BHA, gallic acid, Folin-Ciocalteu reagent, $FeCl_2$, ferrozine, potassium ferricyanide, EDTA, ascorbic acid, TCA, $FeCl_3$, Na_2CO_3, catechin, H_2O_2 purchased from Sigma and MP BioMedicals. All the solutions were prepared fresh and utilized on the same day of the assay.

Sequential Extraction of Antioxidant Compounds

Fifty grams of shade dried fruits were taken for extraction procedure. At first, sample was hand crushed and powdered. Then hexane, chloroform, ethyl acetate, acetone, methanol and distilled water were used successively for sequential extraction (from non-polar to polar). Each solvent was treated with sample with frequent shaking until colorless extract or filtrate solution was obtained. Sample and solvent mixture was filtered through four folds of muslin cloth and then filtered through Whatman no. 1 filter paper. The filtrate was flash evaporated using a round bottom flask of known weight. Each of the extract was weighed and total yield was calculated for each solvent system. A known weight of each dried extract was dissolved in Dimethyl sulphoxide (DMSO) to prepare a stock solution of 100mg/ml. All the stock solutions were kept at 4°C until further use.

Estimation of Total Phenolic Content (TPC) and Total Flavonoids

TPC of each extract was determined using Folin-Ciocalteu (FC) reagent (Singleton and Rossi 1965). Briefly, sample in different amounts was mixed with distilled water to make up final volume (3 ml). Then, 0.5 ml FC reagent was mixed and incubated for 10 min at room temperature. Two milliliters of 7% Na_2CO_3 was added and boiled the content in a boiling water bath for one minute. After cooling, absorbance was measured at 650nm. Gallic acid was used as a standard and amount of TPC was expressed as µggallic acid equivalents per mg extract (µg GAE/mg).

The known method of Delcour and Varebeke (1985) was used for the estimation of total flavonoids. Catechin was used as a standard and total flavonoid content was expressed as µg catechin equivalents per mg extract (µg CE/mg). In brief, sample volume was made with methanol to 1 ml. Then, 5 ml of Chromogen reagent (HCl + CH_3OH + Cinnamaldehyde in 1:3:0.004 ratio) was added to each test tube and absorbance was measured at 640 nm.

Evaluation of OH^- scavenging capacity

Deoxyribose degradation assay was performed following the method described by Chung et al. (1997) with slight modifications. Briefly, different concentrations of extracts were mixed with 200mM $FeCl_3$ and 1.04 mM EDTA (0.2 ml, 1:1), 1mM H_2O_2 (0.1 ml), 28 mMdeoxyribose (0.1ml) and 1mM ascorbic acid (0.1ml) and the final volume was made to 1.1 ml with phosphate buffer (0.2 mM, pH = 7.2). The mixture was incubated at 37°C for 1 h. Then, 1 ml of

thiobarbituric acid (1% in 50mM NaOH) and 1 ml of 5% TCA was added followed by boiling in a boiling water-bath for 20min. After cooling, absorbance of the mixture was measured at 532 nm and the percentage inhibition was calculated.

Evaluation of Metal Chelating Capacity

The chelating of ferrous ions by different extracts was estimated (Dinis et al. 1994). The Fe^{2+} was monitored by measuring the formation of ferrous iron-ferrozine complex. The extracts in different concentrations were mixed with 2mM $FeCl_2$ (0.05 ml). After one minute, the reaction was initiated by the addition of 2mM ferrozine (0.2 ml) and the mixture was shaken vigorously and left undisturbed at room temperature. After 10 min, the absorbance was measured at 562 nm. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated by the following equation-

$$\text{Chelating effect } (\%) = \frac{Ao - As}{Ao} \times 100$$

Where A_0 is the absorbance of control and A_s is the absorbance of the test solution.

Determination of Total Reducing Power (TRP)

The TRP of different extracts was determined following the method of Oyaizu (1986). Different amount of extract was mixed with equal volume of phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture followed by centrifugation at 3,000 rpm for 10 min. A 2.5 ml portion of supernatant was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% $FeCl_3$. The absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. The TRP was expressed as $AU_{0.5}$ (amount of extract that produces 0.5 absorbance units). Gallic acid was used as a standard antioxidant.

Evaluation of DPPH Free Radical Scavenging Potential

The antioxidant activity of different extracts was checked on the basis of 1,1 diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity. When an antioxidant reduces DPPH° to DPPH-H, decrease in absorbance occurs. Thus, the degree of discoloration shows the scavenging potential of an antioxidant in terms of hydrogen donating ability (Eberhardt et al. 2000). DPPH (500 µl, 0.5mM in methanol) solution was mixed with different amounts of sample and volume was made to 3.5 ml with methanol. The mixture was incubated in dark for 45 min at room temperature and absorbance was recorded at 515nm. A positive control was prepared by mixing 3 ml methanol and 0.5 ml of DPPH solution. Sample blanks were prepared in methanol without DPPH solution to eliminate the absorbance of crude extracts. Methanol and BHA were used as blank and standard, respectively. The DPPH radical scavenging activity percentage was calculated by using the formula as given below-

$$\text{DPPH}^{\circ} \text{ scavenging activity } (\%) = \frac{Ac - As}{Ac} \times 100$$

where Ac is the absorbance of positive control solution and As is the absorbance of test solution.

Statistical Calculations and Analysis

It was performed by means of Microsoft Office Excel spreadsheet in combination with software 'Statistical Package for Agricultural Scientists' version 9.4. IC_{50} value, the concentration of sample or extract required to scavenge 50% of free radicals in the mixture, was calculated using a linear regression equation derived from the graph of % radical scavenging activity and sample concentration.

Results

Phytochemicals are gifts from nature and antioxidant compounds are typical representative of these botanical gifts. Antioxidants are the substances which can protect the human body from free radicals and retard the progress of many chronic diseases. Apart from their biological functions in plants, these are widely present in food products and agricultural raw materials. As the name antioxidant indicates these compounds participate in oxidation-reduction processes which have complex reaction mechanisms. So, there is no single testing method capable of providing a comprehensive picture of the antioxidant profile of a studied sample. Preliminary studies have confirmed that a multi-method approach is necessary in the study of antioxidant activity. The antioxidant activity of plant extracts has been attributed to various mechanisms including prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxide, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging activity. The results of present investigation on the antioxidant potential of ashwagandha fruits are presented below.

Sequential Extraction of Antioxidant Compounds

The fruit of Withania somnifera selected for evaluation of antioxidant potential, in vitro, was analyzed for its proximate composition (Table 1). Antioxidant compounds from fruits of Withania somnifera were extracted in different solvents of varied polarity. Extraction was carried out in a sequential manner starting from non-polar to polar solvents. The yield of antioxidant compounds in each solvent was calculated and has been shown in Plate 1. Fruits being rich in crude fat (8.148 % dw basis) showed 8.64% yield in hexane

fraction where most of the crude fat was extracted. Among other fractions, water extract gave maximum yield of 8.7% followed by methanol fraction (8.2%) while minimum yield was recorded in ethyl acetate (0.67%).

Table 1.Proximate composition of fruits/berries

Component	Amount in g/100g sample
Moisture	71.61
Crude protein	4.67
Crude fat	2.23
Ash	1.16
Crude fiber	6.94
Carbohydrates	13.39

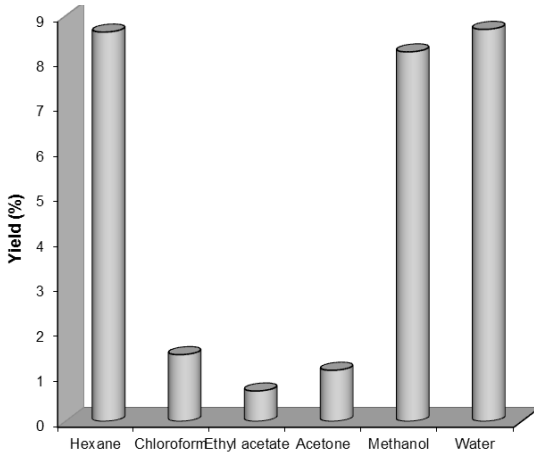

Plate 1.Relative yield in different solvents

Estimation of TPC and Flavonoids Content

TPC were partitioned into six fractions namely hexane, chloroform, ethyl acetate, acetone, methanol and water fractions. Plant phenolics constitute one of the major groups of compounds acting as primary antioxidants or free radical terminators. Therefore, it is necessary to estimate their total amount in the sample chosen for extraction (Fig. 1). Highest TPC was found in methanol extract (42.40 µg/mg), followed by ethyl acetate (23.62 µg/mg) and acetone (22.81 µg/mg), as shown in Table 2. Hexane fraction was found to have minimum amount of TPC (3.07 µg/mg). The free radical scavenging activity in different assays can be linked to the presence of phenolic compounds in sample extract because these compounds exhibit important antioxidant activities.

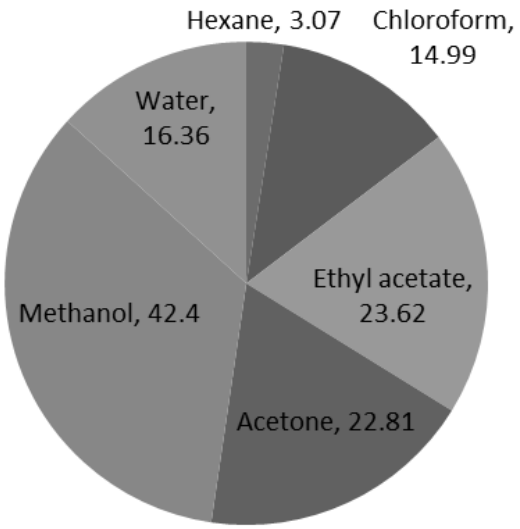

Figure 2.Total flavonoid content in different extracts

Flavonoids are a class of secondary plant phenolics with powerful antioxidant properties (Pietta, 2000). Therefore, it would be valuable to determine the total flavonoids content of the extracts under study (Fig. 2). Maximum and minimum amount of flavonoids was found in methanol (4.76µg/mg) and water extracts (0.39µg/mg), respectively. Studies have shown that many flavonoids contribute significantly to the total antioxidant activity of many plants (Luo et al., 2002).

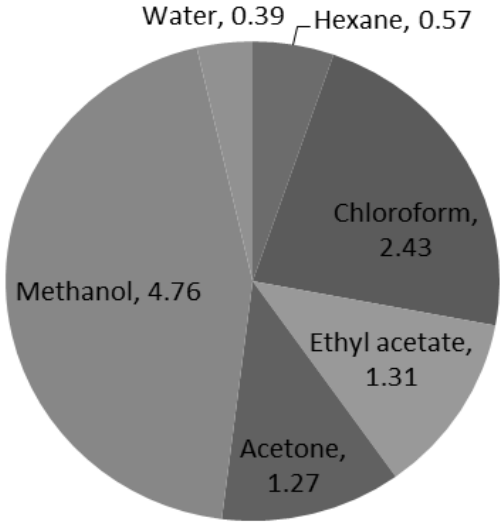

Figure 2.Total flavonoid content in different extracts

Determination of OH^- scavenging Activity

Hydroxyl radicals are most reactive ROS capable of attacking most of the biological substrates. The prevention of such deleterious effect is very necessary in terms of both human health and the self-life of food, cosmetics and pharmaceuticals. So, it was considered important to assess the protective ability of the sample extract against OH radicals. Three sample extracts (acetone, methanol and water) were taken for estimation of OH radicals scavenging activity. Water and acetone extracts were prepared in their respective solvents as DMSO, methanol and ethanol interfere in this assay while methanol extract was dissolved in distilled water to make sample solution. IC_{50} values were calculated using linear regression equations. Methanol extract showed highest activity (IC_{50}= 3.48 mg) followed by water (IC_{50} = 4.59 mg) and acetone extract (IC_{50} = 6.49 mg) (Table 2). Table 2.

Table 2.Regression equations, coefficient of determinations and IC50 values of different extracts of ashwagandha fruits for hydroxyl radical scavenging activity

Extract	Regression equation	Coefficient of determination	IC_{50} (mg)
Hexane	Not determined	-	-
Chloroform	Not determined	-	-
Ethyl acetate	Not determined	-	-
Acetone	y = 7.118x + 3.752	0.963	6.49
Methanol	y = 11.11x + 11.35	0.952	3.48
Water	y = 7.987x + 13.34	0.920	4.59

Evaluation of Metal Chelating Capacity

Ferrous ion (Fe^{2+}) is the most powerful lipid pro-oxidant among the various species of metal ions via its Fenton reaction mechanism. Ferric ion (Fe^{3+}) also produces radicals from peroxides although the rate is ten folds less than that of Fe^{2+} ion (Miller 1996). Ferrozine can quantitatively form complexes with Fe^{2+} but in the presence of chelating agents, the complex formation is disrupted, which results in a decrease in pink color. Increase in the discoloration or lower absorbance indicates increase in metal chelating activity of the chelator. Methanol extract showed highest chelating effect as reflected by its lowest IC_{50} value of 298.72 µg. The lowest chelating effect was recorded for acetone fraction (IC_{50} = 16.97 mg) (Table 3). Hexane extract was excluded from this assay based on its lowest free radical scavenging activity recorded earlier. The highest metal chelation activity of methanol fraction may be because of polyphenols and flavonoids present in it.

Table 3.Regression equations, coefficient of determinations and IC50 values of different extracts of ashwagandha fruits for metal chelation activity

Extract	Regression equation	Coefficient of determination	IC_{50} (mg)
Hexane	Not determined	-	-
Chloroform	y = 9.39x + 2.726	0.969	5.03
Ethyl acetate	Not determined	-	-
Acetone	y = 2.170x + 13.16	0.938	16.97
Methanol	y = 0.156x + 3.34	0.960	0.29
Water	y = 0.074x + 11.53	0.902	0.52

Determination of TRP

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Hsu et al. 2006). The presence of reductants such as antioxidants in the sample causes the reduction of ferricyanide complex to the ferrous form. Therefore, Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700nm (Chung et al. 2002). In this assay, yellow color of the test solution changes to various shades of green and blue depending on the reducing power of antioxidant samples. Table 4 shows the reductive capability of different sample extracts. The reducing power $AU_{0.5}$ (the amount of sample in µg that produces 0.5 absorbance unit at 700nm) was calculated from regression equation.

Methanol extract was found to be most potent with $AU_{0.5}$ = 460µg. Other extracts exhibited comparatively lesser reducing potential. The reducing potential of methanol extract can be credited to the presence of polyphenols and flavonoids in the extract.

Table 4.Regression equations, coefficient of determinations and AU0.5 values of different extracts of ashwagandha fruits for total reducing power

Extract	Regression equation	Coefficient of determination	$AU_{0.5}$(mg)
Hexane	Not determined	-	-
Chloroform	y = 0.183x + 0.010	0.980	2.68
Ethyl acetate	y = 0.188x + 0.017	0.979	2.57
Acetone	y = 0.198x + 0.021	0.975	2.42
Methanol	y = 0.001x + 0.037	0.986	0.46
Water	y = 0.214x-0.005	0.992	2.36

Evaluation of DPPH Free Radical Scavenging Potential

The antioxidant activity of different extracts was calculated as their capacity to scavenge free radicals of DPPH which has been widely used to evaluate the antioxidant activity of natural products from plant and microbial sources (Ko et al., 1998; Chang et al., 2001; Wang et al., 2002). Although radical scavenging activity should not be considered as being synonyms with antioxidant activity, it is a fact that almost all of the powerful natural antioxidants, such as tocopherol, carnosal and ascorbic acid are also strong scavengers of the DPPH radical. Thus, good activity in this test is also an indication of the presence of possible antioxidants. All the six extracts were used to check DPPH activity and IC_{50} values were calculated using regression equations (Table 5). IC_{50} value of methanol extract was minimum (311.46 µg) followed by acetone (324.43µg), chloroform (449.06 µg), ethyl acetate (471.59 µg), distilled water (2229.87 µg) and hexane (7.025 mg). The IC_{50} values clearly indicate that methanol fraction has highest free radical scavenging activity while hexane fraction is least potent. The highest free radical scavenging activity of methanol extract can be attributed to the presence of polyphenols and flavonoids as this fraction contains maximum amount of these secondary metabolites.

Table 5.Regression equations, coefficient of determinations and IC50 values of different extracts of Ashwagandha fruits for DPPH assay

Extract	Regression equation	Coefficient of determination	IC_{50} (mg)
Hexane	y = 8.043x-6.502	0.978	7.02
Chloroform	y = 0.096x + 6.688	0.963	0.45
Ethyl acetate	y = 0.069x + 17.40	0.851	0.47
Acetone	y = 0.118x + 12.14	0.932	0.32
Methanol	y = 0.144x + 5.621	0.952	0.31
Water	y = 0.020x + 5.485	0.978	2.22

Discussion

The active principles of Withania somnifera have been tested for antioxidant activity using the various assays (Bhattacharya et al., 1997). Its root extract has been evaluated for its effect on stress-induced lipid peroxidation (LPO) in mice and rabbits. Simultaneous oral administration of ashwagandha extract prevented an increase in LPO (Dhuley, 1998). Iron overload induced marked increase in hepatic LPO and serum level of the enzymes was attenuated by withaferin-A and sitoindosides VII-X in a dose-related manner and by silymarin (Bhattacharya et al., 2000). In a rat model of chronic stress, ashwagandha and Panax ginseng extracts were compared for their ability to attenuate some effects of chronic stress. Both botanicals were able to decrease the number and severity of CS- induced immunosuppression, but only the ashwagandha extract increased peritoneal macrophage activity in the rats. The activity of the Withania extract was approximately equal to the activity of Panax ginseng extract. Ashwagandha, however, has an advantage over Panax ginseng that it does not appear to result in ginseng-abuse syndrome, a condition characterized by insomnia (Bhattacharya & Muruganandam, 2003). In another study, ashwagandha methanolic extract for 15 days significantly reduced the ulcer index, volume of gastric secretion, free acidity and total acidity. Study also indicated an increase in antioxidant defense, that is, SOD, catalase and ascorbic acid increased significantly, whereas a significant decrease in lipid peroxidation was observed. Ashwagandha inhibited stress- induced gastric ulcer more effectively as compared to the standard drug rantidine (Bhatanagar et al., 2005). EuMil, a polyherbal formulation consisting of ashwagandha as one of its ingredients for 14 days treatment normalized the perturbed regional noradrenaline, dopamine and 5-hydroxy-serotonin concentrations induced by chronic stress.

Conclusion

At present, consumers demand for natural functional foods is increasing and the present study was aimed at evaluating the potential of bioresource for pharmaceutical or nutraceutical purposes. The present suggests that Withania fruit can positively influence cell physiology under conditions of excessive oxidative stress. The antioxidant capacity lies in various phytochemicals like polyphenolics and it was an attempt to describe the potential embedded in the fruit through extracting the active ingredients in different

solvents. Although a lot of pharmacological investigations have been carried out based on the ingredients presents but a lot more can still be explored, exploited and utilized.

Conflict of Interest: None

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