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FT-IR Profile and Antiradical Activity of Dehulled Kernels of Apricot, Almond and Pumpkin.

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ABSTRACT

The objective of this study was to evaluate antiradical activities of methanol and 50% aqueous methanol extracts of dehulled kernels of almond, apricot and pumpkin. All the extracts showed 2,2-diphenyl-1-picryhydrazyl, superoxide anion, and nitric oxide radicals scavenging activities. The activities of the methanol extracts were higher than 50% aqueous-methanol extracts. HPLC analysis of the extracts shows the presence caffeic acid and vanillic acid in apricot extracts but not in almond extracts. Vanillic acid was detected in the extracts of pumpkin seed but caffeic acid was not detected. Infrared profiles of dehulled kernel samples showed hydroxyl, aromatic and carboxylic esteric groups.
Keywords: almond, apricot, antiradical, FT-IR, HPLC, pumpkin, extracts

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INTRODUCTION

Antioxidants are substances that can neutralize or inhibit oxidation by decreasing oxygen concentration, intercepting singlet oxygen, preventing chain initiation by scavenging initial radicals such as hydroxyl radicals, binding metal ion catalyst, decomposing primary products to non-radical compounds, and chain-breaking to prevent continued abstraction from substrates [1].

There is growing evidence that eating vegetable and fruits with polyphenols and vitamin may help to prevent a variety of diseases such as cancer, and coronary heart diseases due to their antioxidant properties [2]. The antioxidant properties and stability of extracts from fruits and vegetables depends on the type of solvent used for the extraction. This is because different classes of secondary metabolites have different chemical characteristic. Organic and aqueous solvents including methanol, ethanol, acetone, ethyl acetate, and their combinations are frequently used solvents for extraction of polyphenolic compounds [3]. However, organic solvent and aqueous organic solvent extracts from the same plant material may vary with respect to their antioxidant activities.

Almond (*Amygdalus communis L.),* apricot (*Prunus armeniaca L.)* and pumpkin (*Cucurbita pepo*) fruits contain diverse of phytochemicals including polyphenols, which contribute significantly to their antioxidant properties. Therefore, the aim of this research was to evaluate the effectiveness of extraction solvents, methanol and 50% aqueous-methanol on free radical scavenging activity effect of dehulled kernels of almond, apricot and pumpkin. The study includes qualitative fourier transform infrared (FT-IR) profiling of the samples and high performance liquid chromatography (HPLC) analysis of the extracts.

MATERIALS AND METHODS

Chemicals and reagents

Sodium nitroprusside, sulfanilic acid, gallic acid, caffeic acid, vanillic acid Folin-Ciocalteu's phenol reagent, 2,2-diphenyl-1-picryhydrazyl (DPPH), ,2'-azinobis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS), nitroblue tetrazolium (NBT), Phenazine methosulphate (PMS), β-Nicotinamide adenine dinucleotide (NADH), butylated hydroxyanisole (BHA), and quercetin, were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals and reagents were of analytical or HPLC grade. Water used was of Millipore quality.

Plant materials and preparation of extracts

Dried kernels of almond, apricot and pumpkin were obtained from local supermarket in Kuala Lumpur, Malaysia. The kernels were of natural quality without any additives. Then the dried materials (without the peels) were grounded to powder using blender (SHARP EM-11, Malaysia).

Ten grams (10 g) of dehulled powdered sample were mixed with 150 ml of respective solvents, aqueous methanol (50%) and methanol and placed on water bath at

40 °C for 18 h with stirring/shaking. The extracts were filtered and concentrated by rotary evaporator (BUCHI Rotavapor R-200, Switzerland). The crude extract was freeze-dried. The crude freeze-dried extract was kept in a desiccator filled with silica desiccant until further analysis.

Fourier transform infrared profiling of the samples

The FT-IR spectrum was recorded on Thermo Nicolet Avatar 350 FT-IR spectrometer. Dehulled dr powdered samples were used for FT-IR spectroscopy analysis. The FT-IR analysis recorded in potassium bromide (KBr) disc. One milligram of the sample was mixed with KBr in one to three ratio and ground to fine powder. The mixture was transferred to a die of a hand pressure and compressed to produce a disc which was used for FTIR spectroscopy in the mid-IR range of 4000 – 400 cm^{-1} at resolution of 4 cm^{-1} with 14 scans. The interferometer and the detector chamber were purged with dry nitrogen to remove spectral interference due to atmospheric carbon dioxide and water vapour. The background spectrum was recorded before the experiment was performed.

Estimation of total phenolic content

Total phenolic content of the extracts was determined using Folin–Ciocalteu reagent as described by Spanos and Wrolstad [4] with some modifications. Sample was prepared by mixing 0.4 ml extract (10 mg/ml) with 0.4 ml of Folin–Ciocalteu reagent (50% v/v) and 2 ml sodium carbonate (15%). Sample was diluted with deionised water (1.2 ml) prior incubation for 2 h at room temperature. Six concentrations of gallic acid (GA) standard solution ranging from 0.1 to 0.6 mg/ml were prepared to obtain the standard calibration curve. Absorbance of each sample was measured at 760 nm using UV-Vis Spectrophotometer. All tests were performed three times and averaged. Total phenolic content of the extract was expressed as milligrams of gallic acid equivalents (GAE) per 1 g of dry plant extract.

Determination of radical scavenging activities

DPPH radical scavenging assay

DPPH radical scavenging activity was used to measure the free radical scavenging activity of the extracts based on the method by Sharma and Bhat [5]. Two milliliters of methanolic solution of DPPH (0.1 mM) was mixed with 2 ml of extract solution (1 mg/ml) and made up with methanol to a final volume of 3 mL. After 40 min standing at room temperature, the absorbance of the mixture was measured at 517 nm against methanol as blank using spectrophotometer. Quercetin (0.5 mg/ml) was used as positive reference compound. Distilled water was used in place of sample solution as control. The DPPH free radical scavenging activity was calculated. The results were expressed as percentage inhibition of the DPPH free radical.

Superoxide anion (O_2^-) scavenging assay

The superoxide anion radical scavenging activity was measured by the reduction of NBT according to the method described by Beauchamp and Fridovich [6]. Briefly, the

reaction mixture contained 2 ml of phosphate buffer (0.1.M, pH 7.4), 0.5 ml of NADH (500 µM), 0.5 ml of NBT (200 µM), 0.5 ml of PMS (100 µM) and 0.5 ml of sample solution (1 mg/mL). The mixture was incubated in the dark at room temperature for 15 min. The absorbance was measured with spectrophotometer at 560 nm against an appropriate blank to determine the quantity of formazan generated. Quercetin (0.5 mg/ml) was used as positive reference compound. The control sample was the same reaction mixture without the extracts but with an equivalent amount of water. The superoxide anion radical scavenging activity was calculated. The results were expressed as percentage inhibition of the superoxide radical.

Nitric oxide scavenging assay

The scavenging effect of the extracts on nitric oxide radicals was measured according to the method of Rao [7]. Briefly, 0.5 ml of sample solution (1 mg/ml) dissolved in water were mixed with 0.5 ml sodium nitroprusside solution (5 mM) in phosphate-buffered saline (pH 7.4) and incubated at room temperature for 2.5 h. After the incubation period, 0.5 ml of the reaction mixture was added to 1 ml of sulfanilic acid reagent (0.3% sulfanilic acid in 20% glacial acetic acid) and incubated for 5 min. An aliquot of the incubation solution was diluted with 3 ml of Griess reagent, mixed and incubated for 30 min at 25 ^{o}C. The absorbance of the pink chromophore generated was measured with spectrophotometer at 540 nm measured against an appropriate blank. Quercetin (0.5 mg/ml) was used as positive reference compound. The control sample was the same reaction mixture, without the extracts but with an equivalent amount of water. The percentage inhibition of nitric oxide radical generation was calculated.

Qualitative high performance liquid chromatography

The HPLC analysis of the samples was performed Perkin – Elmer HPLC system equipped with binary pump, autosampler and degasser. The column used LiChrosorb RP-18 column (250 mm x 4.6 mm, 10 µm particle size) (Perkin-Elmer). The separations were done by using isocratic mobile phase consisted of 0.04% (v/v) phosphoric acid in water: methanol (55: 45). The flow rate was 1 ml/min. Injection volume was 10 µl and the UV detector was set at wavelength of 254 nm. The mobile phase was filtered under vacuum through 0.45 µm membrane filter (Merck, Darmstadt, Germany) prior to HPLC analysis. All chromatographic operations were carried out at ambient temperature. The peaks were identified based on comparison of retention times of standard compounds to the peaks in the sample chromatographs**.**

Statistical Analysis

Data were recorded as mean ± standard deviation and analysed by SPSS (version 12 for Windows XP). One-way analysis of variance (ANOVA) and Tukey multiple comparisons were carried out to test for any significant differences between the means. *P*-values of less than 0.05 were considered statistically significant.

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RESULTS AND DISCUSSION

FT-IR profiling

FT-IR spectroscopy is an excellent analytical tool for the analysis of seeds and nuts, with advantage of repetitive analysis at very low cost since no specific reagents are required. The FT-IR spectra of the samples showed similar spectral characteristics (**Fig.1**). The information obtained from the qualitative analysis showed that all the dehulled kernels have aromatic domain bands at frequency range of 1654 – 1651 cm^{-1} due to phenolics present in the sample and weak band at frequency range of 1465 – 1456 cm^{-1} due to carboxylic C-O band of polyphenols (Fig. 1). The sharp absorption peak at frequency range of 1747 – 1746 cm^{-1} was assigned to –C=O stretching vibration in carbonyl compounds which may be characterised by the presence of high content of polyphenols in the samples. The presence of polyphenols were further proven with the hydroxyl (-OH) absorption band at frequency range of 3700 – 3200 cm^{-1}.

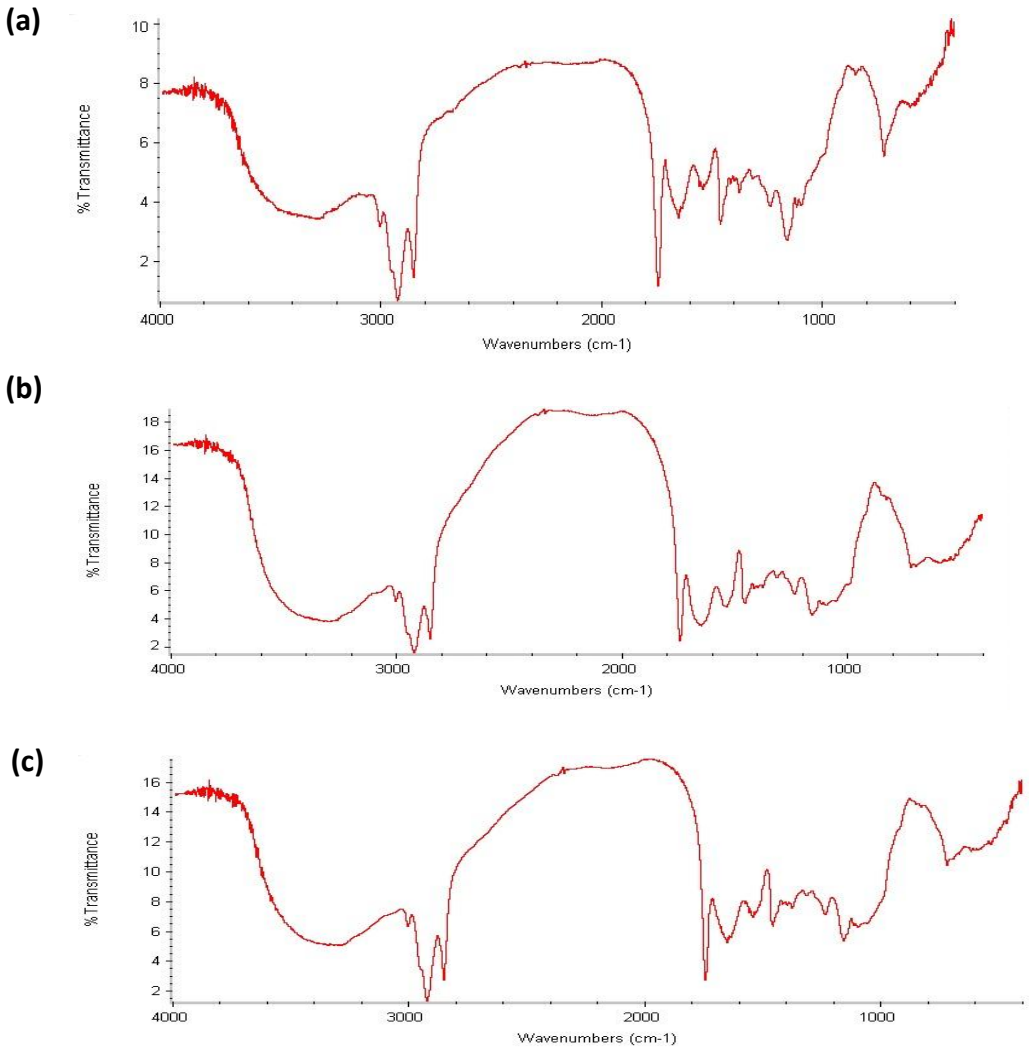

Figure 1: FT-IR spectra of dehulled kernels of (a) almond, (b) apricot, (c) and pumpkin

The FT-IR spectra of the studied seeds and nut showed similar spectral characteristics of the functional group region which indicates similar components of the samples. The only difference in this region is the low intensity of hydroxyl (-OH) absorption band observed in the spectrum of almond kernel. The "fingerprint" characteristics of apricot and pumpkin seeds were similar. Phenolic compounds including hydroxycinnamic acid derivatives which are found in the bound form and in the free form can be commonly found in fruits, seeds, and nuts. Sugar derivatives of hydroxycinnamic acid including esters of *p*-coumaroylglucose and caffeoylglucose have been reported in seed and nuts [8].

Extraction yield and total phenolic content

There are no common extraction procedures for secondary metabolites of plant samples. The best extraction procedure depends on the type of secondary metabolite to be extracted and also whether the purpose of extraction is for qualitative or quantitative evaluations. Extraction yield obtained by extraction of 10 g of sample powder with methanol (150 ml) and 50% aqueous methanol (150 ml) on water bath at 40 °C for 18 h with stirring is shown in Table 1. The yield of methanol and aqueous methanol extracts of apricot were the similar. The yields of the methanol extracts of almond and pumpkin seeds were higher than the yield obtained by using 50% aqueous methanol extract.

The selection of suitable solvents for extraction of polyphenols depends on factors including economy and safety. In this study, methanol and 50 % aqueous methanol were found to be suitable solvents for extraction of phenolics from the dehulled kernels of almond and apricot and pumpkin. Phenolic compounds are polar in nature due to the presence of hydroxyl groups on the cyclic rings which contribute to their polarity.[9] Hence, polar solvents such water; alcoholic or aqueous alcoholic solutions are effective extraction solvents for phenolic compounds from natural source. The FTIR spectra showed the presence of hydroxyl (-OH) functional groups in the chemical components of the sample studied. Polar compounds containing -OH groups are more soluble in polar solvents.

Table 1: Total phenolic content and radical scavenging activity of dehulled kernels of almond, apricot and pumpkin seed extracts

Samples	Solvents	Extraction yield (%)	Total Phenolic content (mg GAE/g)	Radical scavenging activity (%)		
				DPPH assay	Superoxide anion assay	Nitric oxide assay
Almond kernel	Aqueous MeOH	6.30	0.33 ± 0.03c	47.69 ± 1.24e	49.85± 1.34 g	51.22± 1.15 f
	MeOH	8.57	0.27 ± 0.08d	68.96 ± 1.17c	70.13 ± 1.10 d	73.53 ± 1.18 c
Apricot kernel	Aqueous MeOH	10.85	0.71 ± 0.04 a	57.90 ± 3.37 d	56.72± 1.15 f	59.72± 1.04 e
	MeOH	10.76	0.45 ± 0.06 b	87.64 ± 1.48 b	75.36± 1.08 c	75.36± 1.08 c
Pumpkin seed	Aqueous MeOH	13.42	0.72 ± 0.02 a	69.18 ± 1.21 c	66.55± 1.26 e	68.64± 1.35 d
	MeOH	14.15	0.44 ± 0.05b	86.85 ± 1.40 b	85.24± 1.41 b	82.53± 1.44 b
Quercetin	-	-	-	94.23 ± 0.96 a	91.74 ± 1.27 a	90.85 ± 1.13 a

The values are expressed as means ± SD of triplicate tests.
Different letters in columns indicate significantly different values ($p < 0.05$).

Radical scavenging activity

The free radical scavenging activity of the extracts was evaluated by DPPH assay. All the extracts showed *in vitro* free radical scavenging activity (Table 1). The methanol extracts showed higher activity as compared to the 50% aqueous methanol extracts of all the samples tested. The free radical scavenging activity follows the order: quercetin > methanol > 50% aqueous methanol. It is generally accepted that phytochemicals such as phenolics possess high antioxidant activities. Therefore, seeds and nut which contain natural antioxidants are being used as functional food ingredients and food supplements which are becoming very important in the food industry as an alternative to synthetic antioxidants.

Reactive oxygen species such as superoxide anion can damage cells and DNA resulting in diverse ailments. The superoxide radical scavenging activity of the extracts was evaluated by PMS–NADH–NBT system. Superoxide anion originated from dissolved oxygen by PMS–NADH coupling reaction reduces NBT. The decrease in absorbance at 560 nm with antioxidants signifies the consumption of superoxide anion in the reaction mixture. Table 1 shows the percentage inhibition of superoxide radical generation by the extracts. The inhibitory activity of the methanol extracts was found to be higher than that of the 50% aqueous methanol extracts. The suporoxide anion scavenging activity follows the order: quercetin> methanol > 50% aqueous methanol. The superoxide anion radical inhibition activity of the extracts may be ascribed to the phenolic content including hydroxycinnamic acid derivatives which are reported to inhibit production of reactive oxygen species [10].

Nitric oxide reacts with oxygen to form nitrogen dioxide which in biological systems can initiate auto-oxidation of fatty acids in lipid membranes, resulting in membrane damage due to its ability to abstract hydrogen from unsaturated fatty acids [11]. The extracts exhibited nitric oxide scavenging activity (Table 1). The inhibitory activity of the extracts against the nitric oxide radical decreased in the following order: quercetin > methanol > 50% methanol. The nitric oxide scavenging activities of the extracts could be attributed to the content of hydroxycinnamic acid derivitatives in the extracts. Hydoxycinnamic acid derivatives are reported to inhibit nitric oxide production [12]. In nature *trans*-hydroxycinnamic derivatives serve as important intermediates for the biosynthesis of polyphenols. Polyphenols such as flavonoids are known to suppress nitric oxide production and also scavenge nitric oxide in an acellular system using sodium nitroprusside under physiological conditions at a micromolar range [13].

The radical scavenging properties of phenolics are generally accepted as the basis of their therapeutic effect. A characteristic feature of phenolics is that they have many phenolic hydroxyl groups and as a result have many active points in radical scavenging. Therefore, the presence of phenol structure render compounds effective free radical inhibitors. The FT-IR spectra of all the samples used in the present study also showed a broad peak at frequency range of 3700 – 3200 cm^{-1} due to hydroxyl (-OH) stretching vibration. The hydroxyl moieties have the ability to terminate propagation of chain carrying radicals by acting as H-atom donor.

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Qualitative High performance liquid chromatography

Caffeic acid and vanillic acid were selected as markers for this study since they have been identifies as some of the phenolic compounds in the extracts of fruits, seeds, and nuts [14, 15, 16]. The HPLC chromatograms of the extracts are shown in **Fig. 2**. Vanillic acid and caffeic acid were eluted at 5.5 min, and 7.6 min, respectively. Vanillic acid and caffeic acid were detected in methanol and 50% aqueous methanol extracts of apricot as minor components based on the respective peak areas. Vanillic acid was detected as the major component in the methanol and 50% aqueous methanol extracts of pumpkin seed, however caffeic acid was not detected in the same extracts.

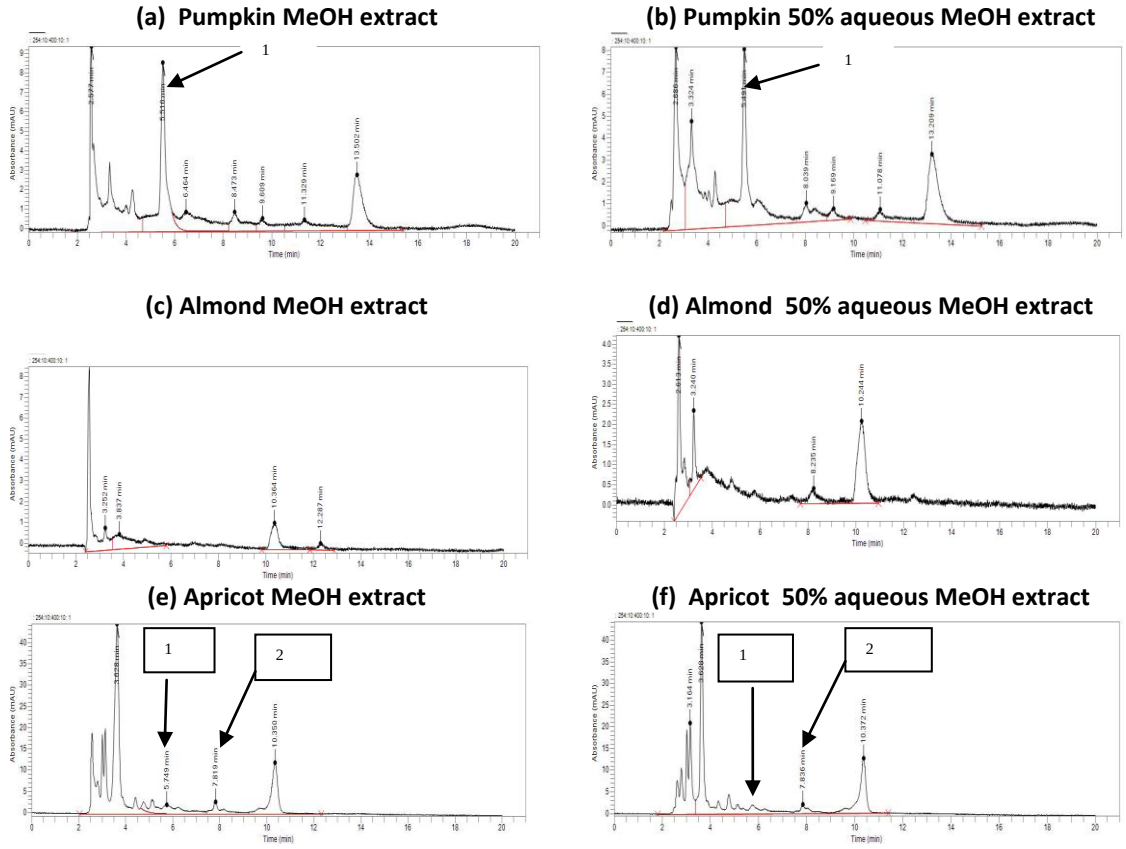

Figure 2: The HPLC chromatograms of (a) methanol extract of pumpkin; (b) aqueous methanol extract of pumpkin; (c) methanol extract of almond; (d) aqueous methanol extract of almond; (e) methanol extract of apricot; (f) aqueous methanol extract of apricot. Markers identification: 1, Vanillic acid, 2, caffeic acid.

CONCLUSION

In conclusion, the infrared spectra of dehulled kernels of almond, apricot and pumpkin showed great intensity of hydroxyl (-OH) bands, aromatic domain bands, and carboxylic esteric bands. Similarities in the FT-IR spectra were observed which may be assigned to the similar components of the samples. The information obtained from the preliminary qualitative analysis by FTIR provides entire chemical composition of the sample hence serves as a suitable method for quality control of botanicals including seeds and nuts. The methanol and 50% aqueous methanol extracts of the samples were found to contain phenolic compounds and also exhibited radical scavenging activities comparable to that of

the positive control drug, quercetin which has been reported to exhibit potent radical scavenging activity. Hence, methanol and aqueous methanol extracts of the dehulled kernels can be used as source of natural antioxidants. The proposed HPLC method can be used in routine analytical purposes. HPLC-MS characterization of all components observed in the HPLC chromatogram is in progress.

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