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Phenolic profile and antioxidant potential of selected plants of Pakistan

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Summary

Antioxidants play an important role in inhibiting and scavenging radicals, thus providing protection to humans against infectious and degenerative diseases. Literature shows that the antioxidant activity is high in medicinal plants. Realizing the fact that, this investigation was carried out to evaluate the in vitro antioxidant capacity of methanolic extracts of *Acacia leucophloea* (bark), *Albizia lebeck* (bark, flower, seed), *Capparis decidua* (root), *Cicer arietinum* (seeds) and *Grewia asiatica* (leaves). Barks showed the highest phenolic content as compared to seeds, leaves and roots and the order observed was *A. lebeck* bark > *A. leucophloea* bark > *G. asiatica* leaves > *C. decidua* root > *A. lebeck* flowers > *A. lebeck* seeds > *C. arietinum* seeds. Phenolic compounds were identified based on their mass spectral characteristics in each extract. Antioxidant capacity measured by three commonly-benched methods, TEAC, FRAP and TRAP assays indicated that all extracts are a good source of natural antioxidants. Investigated extracts appeared to have potential as a health supplement rich in natural antioxidants and merits further intensive study. The results of this study will promote the reasonable usage of these plants in food and pharmacy industries as well as in alternative medicine and natural therapy.

Introduction

It is well known that reactive oxygen species (ROS) formed *in vivo*, such as superoxide anion, hydroxyl radical and hydrogen peroxide, are highly reactive and potentially damaging transient chemical species. The oxidative damages caused by ROS on lipids, proteins and nucleic acids may trigger various chronic diseases, such as coronary heart disease, atherosclerosis, cancer and aging (UTTARA et al., 2009; BARNHAM et al., 2004). The antioxidants can delay or inhibit the oxidation of lipids and other molecules by inhibiting the initiation or propagation of oxidative chain reactions and they can thus prevent or repair the damage done to the body's cells by ROS (TACHAKITTIRUNGROD et al., 2003). An imbalance between antioxidants and ROS results in oxidative stress, leading to cellular damage. It is possible to reduce the risk of chronic diseases and prevent disease progression with dietary antioxidants (STANNER et al., 2000). Among these, natural antioxidants are regarded as safer than synthetic antioxidants. Therefore, there is a considerable interest in finding new and safe antioxidants from natural sources to replace these synthetic antioxidants (RAHIMI et al., 2005; CHANWITHEESUK et al., 2005).

Plants have many phytochemicals which are a potential source of natural antioxidant, e.g. phenolic diterpenes, flavonoids, alkaloids, tannins and phenolic acids (AMRO et al., 2002; CAI et al., 2004; MOURE et al., 2001). In recent years, considerable attention has been directed towards the identification of plants with antioxidant ability that may be used for human consumption. Pakistan is considered as a paragon of valuable food, medicinal and aromatic plants thanks to its comprehensive latitudinal spread and immense altitudinal range.

Magnificent mountain tops in Northern areas to fertile plain areas irrigated by rivers in Punjab and Sindh, Arid regions of Thal, Thar and Baluchistan to the coastal mangrove forests of the Arabian Sea supports an extensive array of exotic plant species (ZIA-UL-HAQ et al., 2013 a, b). However, few studies have explored the antioxidant capacity of these plants. In this investigation, the total antioxidant capacity (TAC) and the characterization of phenolic compounds of several Pakistan plants, i.e. *Acacia leucophloea* (bark), *Albizia lebeck* (bark, flower, seed), *Capparis decidua* (root), *Cicer arietinum* (seeds) and *Grewia asiatica* (leaves) were explored. As there is no single and widely acceptable assay method for evaluating antioxidant capacity, a battery of assays was used for measuring total antioxidant capacity.

Materials and methods

Plant material

Acacia leucophloea (bark), *Albizia lebeck* (bark, flower, seed), *Capparis decidua* (root), *Cicer arietinum* (seeds) and *Grewia asiatica* (leaves) were procured from Department of Agronomy, Bahauddin Zakariya University, Multan and voucher specimen number (No.18-04-2008) was deposited in herbarium of Department of Botany of same university. The plant material (1 kg for each) was crushed separately to coarse powder separately with help of pestle and mortar and macerated with aqueous methanolic mixture (5 L; 80:20; v/v) at room temperature for fifteen days with occasional shaking. The extracts obtained were filtered through filter paper under vacuum and concentrated under reduced pressure in a rotary evaporator (model Q-344B – Quimis, Brazil) using a warm water bath (model Q-214M2 – Quimis, Brazil) to obtain a thick gummy mass, which was further dried in a desiccator and stored in air-tight vial till further use.

Chemicals

The 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-azinobis (3 ethylben zothiazoline-6-sulfonic acid) diammonium salt (ABTS), and 2,4,6-tripyridyl-s-triazine (TPTZ) were purchased from Sigma-Aldrich (St. Louis, MO, USA). R-Phycoerythrin (R-PE) was purchased from Prozyme (San Leandro, CA, USA); 2,2-azobis (2-amidinopropane) dihydrochloride (ABAP) was purchased from Waco Chemicals (Richmond, VA, USA). All chemicals and solvents used were HPLC-grade and purchased from Carlo Erba (Milan, Italy). Ultrapure water from a MilliQ system (Millipore, Marlborough, MA, USA) was used throughout the experiments.

Determination of total phenolic content (TPC) and TAC

For the determination of TPC and TAC a weighed amount of methanolic extract sample (between 50 and 130 mg depending on the sample) was dissolved in 10 ml of 1 % formic acid methanol solution. The extracts were kept at 4 °C at dark prior to the analysis.

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TPC analysis

The total phenolic content of each extract was determined using the method previously described (ADOM and LIU, 2002). Briefly, the extracts were oxidized with Folin-Ciocalteu reagent and the reaction was neutralized with sodium carbonate. The absorbance of the resulting blue color was measured at 760 nm. Data are expressed as mg catechin equivalents per g plant extract.

TAC analyses

Plant extracts were analyzed for their antioxidant capacity by three different TAC assays: Trolox equivalent antioxidant capacity (TEAC) assay (PELLEGRINI et al., 2003), ferric reducing antioxidant power (FRAP) assay (BENZIE and STRAIN, 1999) and total radical-trapping antioxidant parameter (TRAP) assay (GHISELLI et al., 1995). The TEAC and TRAP values were expressed as micromoles of Trolox per g plant extract, FRAP values were expressed as micromoles of Fe²⁺ equivalents per g plant extract.

HPLC-ESI-MS/MS analysis of phenolic compounds

Phenolic compounds were analysed using a Water 2695 Alliance separation module equipped with a Micromass Quattro Micro Api mass spectrometer fitted with an electrospray interface (ESI) (Waters, Milford, MA, USA). A preliminary investigation on phenolic profiles of selected plants was carried out by means of MS Scan analysis, operating in negative ion mode from 100 to 1000 mass-to-charge ratio (m/z). Then, different Multiple Reaction Monitoring (MRM) methods were developed for all sample types, based on the obtained MS Scan data. Separations were performed using a Waters Atlantis dC18 3 µm (2.1 x 150 mm) reverse phase column (Waters), with the mobile phase, pumped at a flow rate of 0.17 ml/min. The mobile phase was a 30-min linear gradient of 5 to 30 % acetonitrile in 1 % aqueous formic acid. The ESI source worked in negative ionisation mode. Source temperature was 120 °C, desolvation temperature was 350 °C, capillary voltage was 2.8 kV, cone voltage was 35 V, desolvation gas (N₂) 750 l/h, cone gas (N₂) 50 l/h. The collision energy for MS/MS identifications was set at 30 eV, and the collision gas used was argon.

Statistical analysis

To verify the association among the total antioxidant capacity and total phenols method, Pearson correlation analysis was performed using the SPSS statistical software (version 19.0, SPSS Inc., Chicago, IL); *P*-values ≤ 0.01 were considered significant.

Results and discussion

Data on TPC (Tab. 1) indicated that barks were the botanical part of plants with the highest amount of phenolic compounds, with *A. lebeck* containing higher amount than *A. leucophloea*. This is in agreement with previous findings that reported that phenolics are present more in leaves, flowering tissues and woody parts, such as stems and barks, and in less amount in seeds (LARSON, 1988; GALLO et al., 2010). Between seeds analysed, *A. lebeck* contained greater amount of phenolics than *C. arietinum*. *A. lebeck* flowers had intermediate phenolic content between bark and seed. The order of TPC was the following: *A. lebeck* bark > *A. leucophloea* bark > *G. asiatica* leaves > *C. decidua* root > *A. lebeck* flowers > *A. lebeck* seeds > *C. arietinum* seeds.

The mass spectral characteristics of phenolic compounds identified in the samples as phenolic acids, flavonoids and condensed tannins were reported in Tab. 2. As example, Fig. 1 and 2 report the chromatographic profile of some polyphenols identified in *A. lebeck*

Tab. 1: Total phenol content (mg catechin equivalents/g). Data are expressed as the mean ± standard deviation (n = 3).

Plant	Total phenol content (mg/g)
<i>Acacia leucophloea</i> bark	218.50 ± 2.95
<i>Albizia lebeck</i> bark	388.51 ± 5.83
<i>Albizia lebeck</i> flowers	8.21 ± 0.05
<i>Albizia lebeck</i> seeds	6.86 ± 0.07
<i>Capparis decidua</i> root	36.26 ± 0.22
<i>Cicer arietinum</i> seeds	1.47 ± 0.03
<i>Grewia asiatica</i> leaves	118.52 ± 0.90

Tab. 2: Tentative identification of phenolic compounds based on their mass spectral characteristics

N°	Compound	[M-H] ⁻ (m/z)	Qualifier ions (m/z)
1	Gallic acid	169	125
2	Galloyl-hexoside	331	169
3	Coumaric acid	163	119
4	Caffeic acid	179	135
5	Coumaric acid-hexosides	325	163, 119
6	Caffeic acid-hexosides	341	179, 135
7	Ferulic acid-hexosides	355	193, 134
8	Syringic acid-hexosides	359	197
9	Vanillic acid-hexosides	329	167
10	Sinapic acid-hexosides	385	223, 149
11	Caffeoylquinic acids	353	191, 179, 173
12	Feruloylquinic acids	367	191
13	Apigenin-hexosides	431	269
14	Kaempferol-rhamnosides	431	285
15	Kaempferol-hexosides	447	285
16	Quercetin-hexosides	463	301
17	Quercetin-glucuronide	477	301
18	Kaempferol-glucuronide	461	285
19	Quercetin-rutinoside	609	301, 447
20	Kaempferol-rutinoside	593	285, 447
21	Quercetin-dirhamnoside-hexoside	755	609, 463, 301
22	Kaempferol-dihexoside	609	447, 285
23	Epicatechin	289	137, 245
24	Epicatechin derivative	-	289, 245, 137
25	Procyanidin dimers B-type	577	125, 287, 289, 407, 425
26	Procyanidin trimers B-type	865	577, 575, 289
27	Procyanidin tetramers B-type	1153	575, 577

flowers. Among phenolic acids the hydroxycinnamate derivatives were present mostly than hydroxybenzoate derivatives. About flavonoids, the flavonols kaempferol and quercetin derivatives were the most representative compounds detected in extracts. The condensed tannins (procyanidins) were identified only in *A. lebeck* bark (Fig. 3). Among these flavonoids, at least three B-type dimers of procyanidins were identified, presenting a [M-H]⁻ at m/z 577 and typical fragment ions at m/z 125, 287, 289, 407, 425, formed by A ring cleavage (m/z 125), interflavanic bond cleavage through the quinone methide mechanism (m/z 289 and 287), retro-Diels Alder

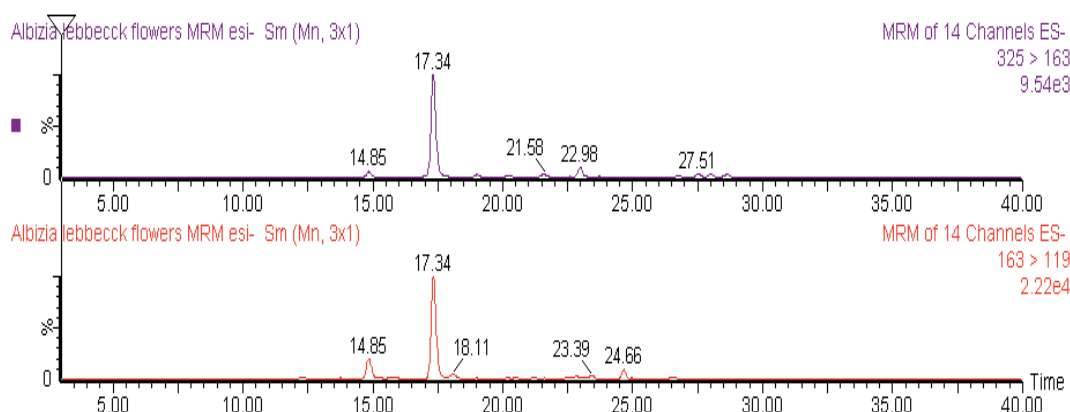


Fig. 1: MRM chromatograms of coumaric acid-hexosides. It is possible to note at least two isomers (14.85 and 17.34), identified through the loss of hexose moiety 325>163 and further fragmentation of coumaric acid 163>119.

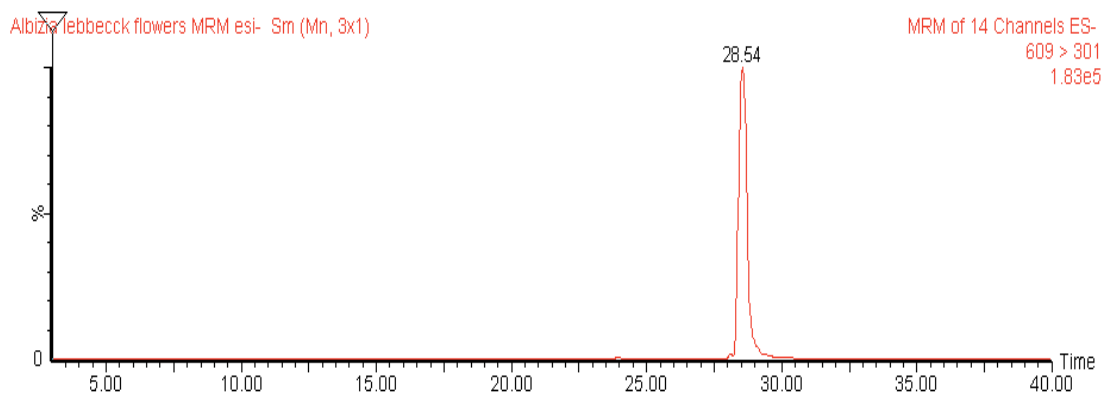


Fig. 2: MRM chromatogram of quercetin-rutinoside (609>301), identified through the loss of rutinosyl moiety (308 amu) and subsequent ionization of formed quercetin.

(RDA) cleavage and loss of a water molecule (m/z 425 and 407). Instead, five procyanidin trimers were identified, characterized by a $[M-H]^-$ at m/z 865 besides typical fragment ions at m/z 577, 575, 289 formed through the same fragmentation pattern observed for procyanidin dimers. Moreover, the bark of *A. lebeck* contained several tetramers, identified through their $[M-H]^-$ at m/z 1153, besides fragmentation forming ions at m/z 577 (dimers) and its quinone counterpart at m/z 575 (Li et al., 2007; GU et al., 2003). *G. asiatica* leaves had the highest number of phenolic compounds (Tab. 3), associated to a high TPC, followed by *C. decidua* root, while few phenolic compounds were identified in *C. arietinum* seed and *A. leucophloea* bark. As the LC-MS/MS instrument allows to identify up to fourth degree of polymerization, the high TPC found in the two barks analysed was probably owed to procyanidins with a higher molecular weight than tetramers, which also justified the high values of TAC measured by TEAC and FRAP assays (Tab. 4). The presence of tannins in methanolic extracts of *A. leucophloea* bark has been previously demonstrated (ANJANEYULU et al., 2010). Several phenolic compounds were identified by HPLC-ESI-MS/MS analysis in other botanic parts of *A. lebeck* (i.e., flowers and seed), even though the TPC values were lower than those in bark as well as their TAC values (Tab. 4).

The five plant species considered in this study were subjected to antioxidant capacity screening using different testing methods, i.e. TEAC, FRAP and TRAP assays, and the results were present in Tab. 4. TEAC and FRAP values were in agreement, with *A. lebeck* bark and *A. leucophloea* bark having the highest values followed

by *C. decidua* root and *G. asiatica* leaves. Conversely, these latter plants exhibited the highest TRAP values followed by *A. leucophloea* bark and *A. lebeck* seeds. This discrepancy could be related to the different sensitivity of TAC assays toward different phenolic compounds. In the case of TRAP assay, its low sensitivity of high polymerised polyphenol rich matrices could be owed to the putative interaction of phycoerythrin (used as a target molecule in the TRAP assay) with high molecular weight polyphenols, such as proanthocyanidins and gallotannins (HAGERMAN and BUTLER, 1981). Conversely, high polymerized phenolic compounds exhibit higher TEAC values than simple ones (HAGERMAN et al., 1998). Finally, due to the low TPC and a low number of phenolic compounds identified by HPLC-ESI-MS/MS analysis, *C. arietinum* seeds showed the lowest TAC value regardless of the assay applied. Similar results have been obtained analysing the TAC of chickpea by TEAC assay (HAN and BAIK, 2008; PELLEGRINI et al., 2006). Pearson correlation analysis was performed to corroborate relationships between TEAC, FRAP, TRAP values and TPC of plants. Strong positive and significant correlations between total antioxidant capacity measured by FRAP and TEAC assays and total phenols were observed. In particular, the best correlation was observed between FRAP and TPC ($r = 0.964$, p value ≤ 0.01), whereas the TEAC and TPC values were less correlated ($r = 0.833$, p value ≤ 0.01). Conversely, no correlation was found between TRAP and total phenolic content. These positive correlations observed between FRAP and TEAC assays and TPC are consistent with previous findings (SRIVASTAVA et al., 2012) and support the hypothesis that phenolic

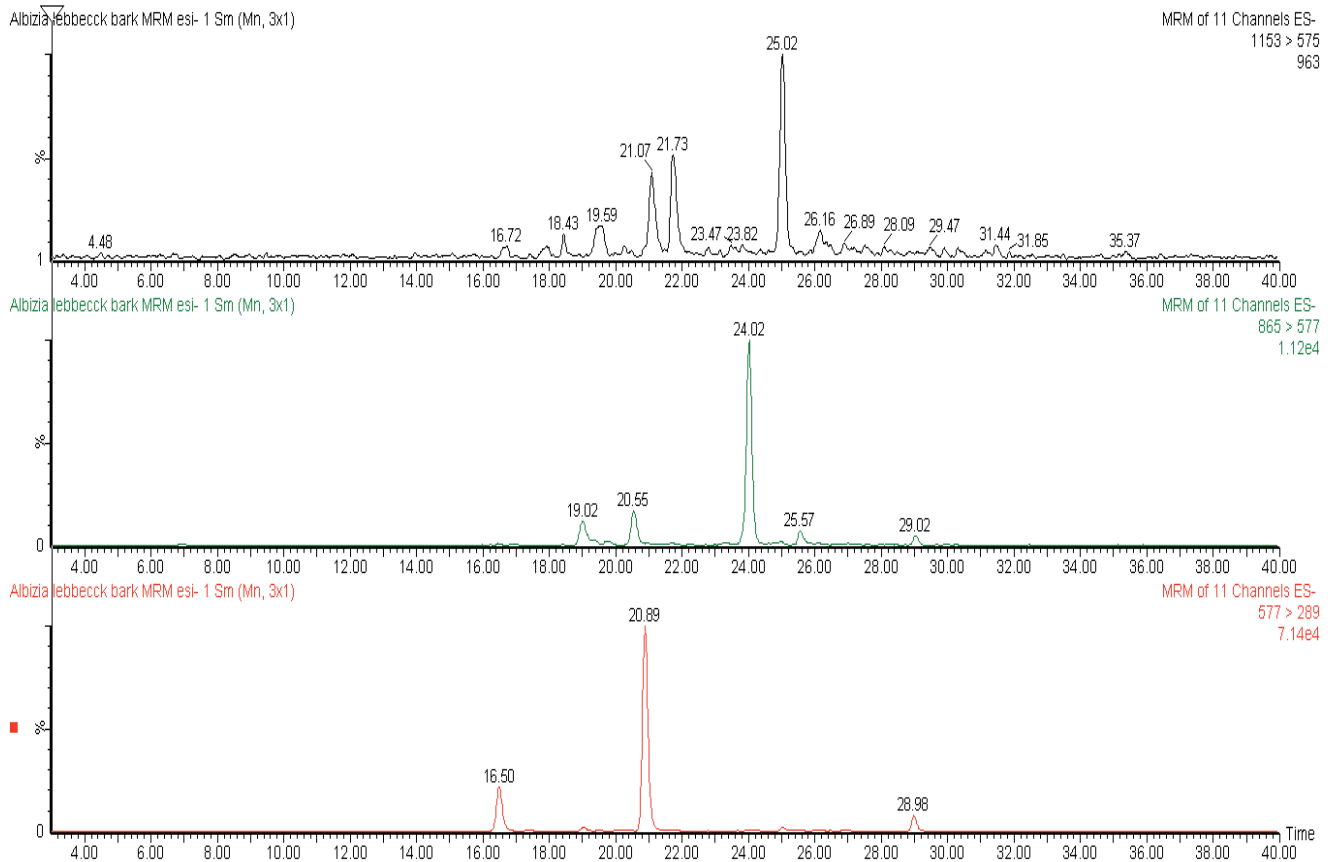


Fig. 3: MRM chromatograms of B-type procyanidins. In detail dimers identified through the interflavanic bond cleavage (577>289), as well as for trimers (865>577) and tetramers (1153>575). In the tetramers it is possible to note the quinone dimers formation (m/z 575).

compounds contribute significantly to the total antioxidant capacity of medicinal plants. The lack of correlation between TRAP assay and TPC is probably due to the before mentioned low sensitivity of this assay to high polymerised phenolic compounds.

Conclusions

Investigated extracts appeared to have potential as a health supplement rich in natural antioxidants and merits further intensive study. The results of this study will promote the reasonable usage of these plants in food and pharmacy industries as well as in alternative medicine and natural therapy.

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Tab. 3: Phenolic profile of plant extracts

Phenolic compound	<i>A. lebbbeck</i> bark	<i>A. lebbbeck</i> flowers	<i>A. lebbbeck</i> seeds	<i>A. leucophloea</i> bark	<i>C. arietinum</i> seeds	<i>C. decidua</i> root	<i>G. asiatica</i> leaves
1		+		+*		+*	
2							+
3							+
4							+
5		+	+*			+	+
6		+				+	+
7		+	+*		+	+	+
8						+	
9				+*		+	+
10					+	+	+
11		+*	+*			+	+
12						+	
13		+		+		+	
14							+
15						+	
16		+	+*				+
17						+	
18						+	
19		+	+				+
20		+	+				
21		+					
22							+
23	+*						
24	+						
25	+						
26	+						
27	+						

+: present; *: trace (present at limit of detection)

Tab. 4: TEAC, FRAP and TRAP values of plant extract analysed. Data are expressed as the mean \pm standard deviation (n =3).

Plant	TEAC $\mu\text{mol/g}$	FRAP $\mu\text{mol/g}$	TRAP $\mu\text{mol/g}$
<i>Albizia lebbbeck</i> flowers	33.11 \pm 0.99	176.55 \pm 8.15	53.65 \pm 2.90
<i>Albizia lebbbeck</i> bark	502.56 \pm 2.37	2561.50 \pm 46.74	43.66 \pm 1.13
<i>Albizia lebbbeck</i> seeds	41.38 \pm 1.56	48.20 \pm 2.29	62.08 \pm 3.07
<i>Acacia leucophloea</i> bark	682.95 \pm 3.06	2234.43 \pm 83.55	90.91 \pm 3.84
<i>Cicer arietinum</i> seeds	3.34 \pm 0.01	23.71 \pm 1.06	3.72 \pm 0.00
<i>Capparis decidua</i> root	384.91 \pm 3.07	338.68 \pm 12.57	202.21 \pm 0.00
<i>Grewia asiatica</i> leaves	72.47 \pm 1.62	939.89 \pm 46.96	353.63 \pm 10.72

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