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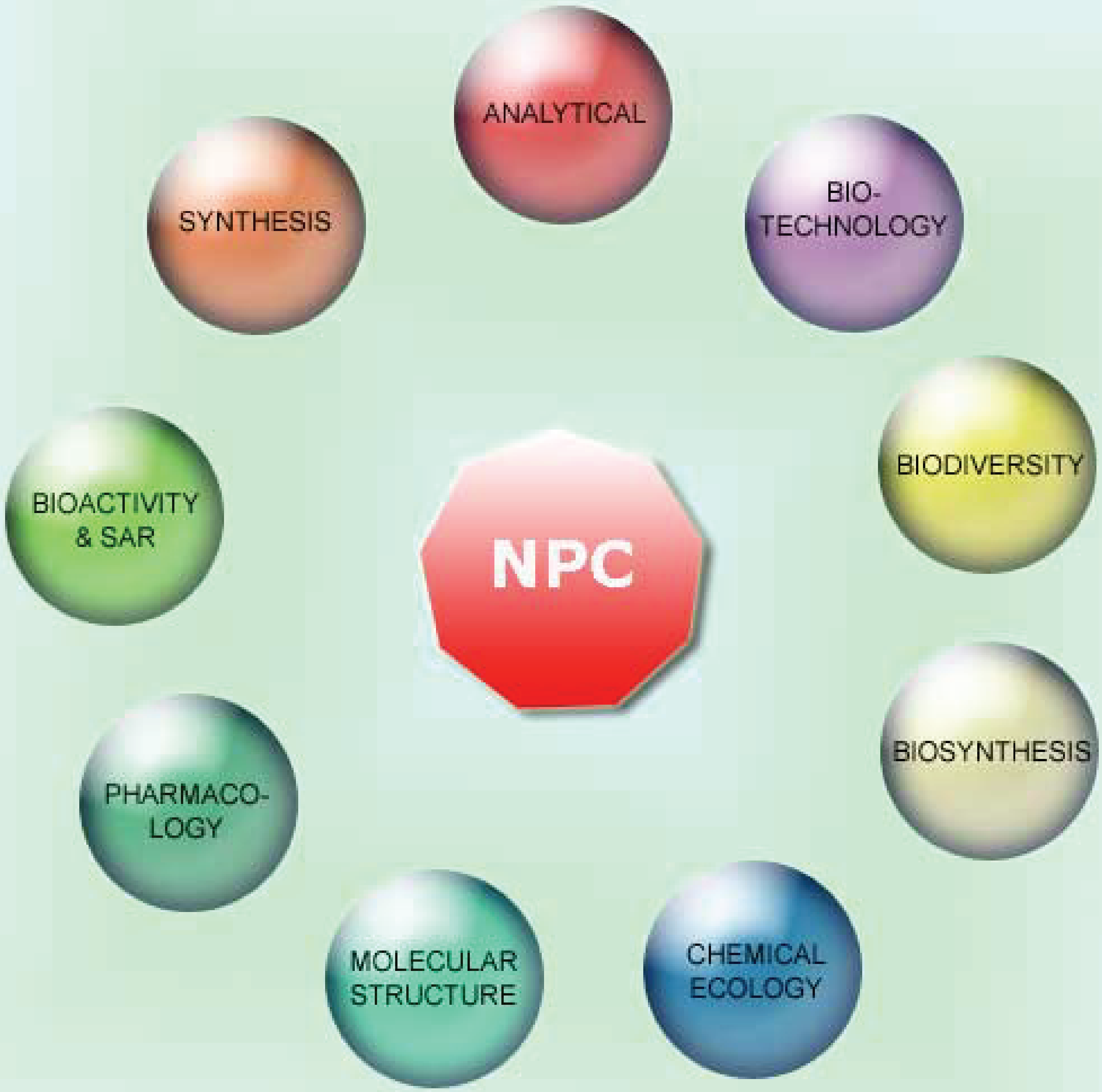

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In vitro Shoot Cultures of *Pterospartum tridentatum* as an Alternative to Wild Plants as a Source of Bioactive Compounds

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The aim of this study was to evaluate the composition of aqueous extracts of *in vitro* shoot cultures and wild plants of *Pterospartum tridentatum* in order to promote the use of this plant material as a possible source of bioactive compounds. The extraction yield from wild plants compared with *in vitro* shoot cultures was lower. The total phenolic contents of *in vitro* shoots were significantly lower compared with those of wild plants. The phenolic profiles of *in vitro* shoots were very similar to those of wild plants, regardless of the source. However, taxifolin-6-*C*-glucoside, as well as rutin and isoquercitrin, were not present in extracts of *in vitro* shoots. An interesting result was the higher molar percentages of rhamnose and uronic acids detected in *in vitro* shoots compared with the wild plants, which can make the *in vitro* plant material very useful for obtaining these compounds.

Keywords: *Pterospartum tridentatum*, Prickly broom, Aqueous extracts, *In vitro* cultures, Neutral sugars, Phenolic profile.

Pterospartum tridentatum (L.) Willk., a shrub commonly known as prickly broom, is a European endemic belonging to the Leguminosae family (=Fabaceae), Papilionoideae subfamily. This evergreen shrub, which grows approximately up to 100 cm, is very common in mountainous areas in north and central Portugal, sometimes in low scrub on acid rocks or associated with the undergrowth of *Arbutus unedo*, *Pinus pinaster* and *Eucalyptus* forests, and in abandoned lands with acid soils.

Prickly broom is widely used as a medicinal plant for the treatment of digestive tract diseases and diabetes and for culinary purposes; however, studies on this plant are scarce. The first reported study on the chemical composition and pharmacological properties of *P. tridentatum* in aqueous extract revealed the presence of alkaloids, phenolics (including flavonoids) and terpenoid glycosides. The main identified constituents of the aqueous extracts were three derivatives of genistein (4′,5,7-trihydroxyisoflavone) and the isoflavones, sissotrin, genistin, prunetin and 5,5'-dihydroxy-3'-methoxy-isoflavone-7-*O*-β-glucoside, as well as isoquercitrin [1]. Isoquercitrin prevents endothelial oxidative damage when used at non-toxic concentrations (0.1-1 mM). The flowers may have beneficial effects in patients with diabetes as the aqueous extracts have shown their capacity to prevent oxidative damage to endothelial cells which can prevent or minimize the development of vascular complications [1]. In addition, it was reported that the polysaccharides contained in decoctions of *P. tridentatum* dried inflorescences (e.g. type-I and type-II arabinogalactans, acetylated galactomannans and xyloglucans) have an important role in the bioactive properties frequently attributed by popular tradition to the hot water extracts of *P. tridentatum* dried inflorescences [2].

Chemical variability was verified in the essential oils of the plant [3]. In the leaf extract of *P. tridentatum* subsp. *tridentatum* the isoflavone genistein was identified [4]; this is a phytoestrogen with a wide variety of pharmacological effects in animal cells, including inhibition of tyrosine kinase, antioxidant activity, and chemoprevention of prostate and breast cancers and cardiovascular diseases [5]. Studies on the phenolic content and antioxidant activity of infusions of ten Mediterranean medicinal plants, including prickly broom, have shown antioxidant activity of the extracts and demonstrated that these plants could be used in popular medicine, especially for the prevention and treatment of diseases known to be caused or accelerated by oxidative stress [6].

In some places, there is an intense use of plants with medicinal effects. In order to minimize large-scale harvesting and the consequent destruction of natural habitats, it seems important to develop methodologies for the cultivation of such plants, both *in vivo* and *in vitro.* Today, plant cell culture and micropropagation is of interest, especially for the pharmaceutical, cosmetic and food industries, given their increasing interest in obtaining certain active compounds, for which they rely on raw materials from wild or farmed plants. A difficulty for these industries may be the inability to obtain the plant material in either sufficient quantity or at prices that are economically feasible to meet demand as some plants may be less abundant and even difficult to culture [7]. The potential of *in vitro* propagation is recognized for the production of high quality and pathogen free medicinal plants on a large scale [8a-8f]. Micropropagation of prickly broom was recently reported showing the use of a defined multiplication protocol for obtaining the aerial parts in large quantities [9]. In this context, our present work was designed to evaluate the chemical composition of *in vitro* and wild plant extracts, from three different regions, and in different vegetative states, with respect to extraction yield, total phenolic content, phenolic profile and carbohydrate components.

The extraction yield (g of extract per 100 g dry weight material) for the different harvesting locations of wild plants throughout the growing season are shown in Figure 1, as well as for the *in vitro* plant material. The extraction yield was always relatively high, exceeding 11%. With regard to wild plants, the place of collection and the stage of the growing season did not significantly affect the extraction yield. That of *in vitro* plant material was significantly higher when compared with that of wild plants from all backgrounds, regardless of the stage and season, with the exception of *in vitro* plant material obtained from Malcata (12.2%). Nevertheless, the differences were not significant. The stems and leaves at flowering tended to have a lower extraction yield relative to the flowers at each collecting site, which leads to suppose that the compounds may be channeled to the floral part of the plants.

Though good extraction yields were observed using water, higher values were reported using alcohols as solvents. A yield of 28.6% was achieved with ethanol, compared with 12.0% using water [10]. However, in this work, water was used in order to avoid the application of organic solvents and to implement a greener extraction process. Extraction yields ranging from 26% for stems and leaves and 25% for flowers were obtained using methanol [11]. Similar results were reported in a study with 35 endemic and sub-endemic plants from Galicia, including *P. tridentatum* subsp. *tridentatum*, for which an extraction yield with ethanol and water (1:1) of 30% was obtained for leaves and stems, and 24% for flowers [4].

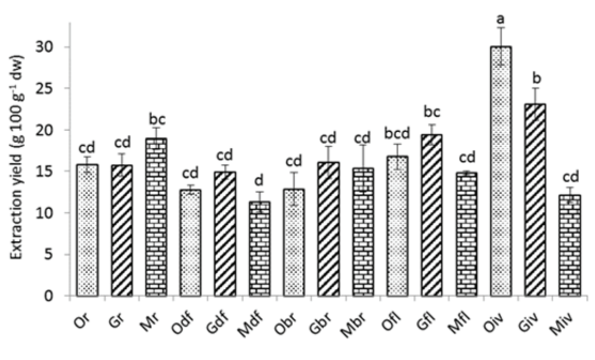

Figure 1: Extraction yield (g extract per 100 g dw) for the plant material with different origins and vegetative phases. (O: Orvalho; G: Gardunha; M: Malcata; r: at rest; df: during flowering; br: beginning of the rest; fl: flowers; iv: *in vitro* plant material). Average values with different letters are significantly different according to the Scheffé's multiple range test at α≤0.05.

The total phenolic content varied significantly with the season and origin of the wild plants, with the highest value observed for the extract of Gardunha plant material at rest (369.2 mg GAE g^{-1}). The extracts of *in vitro* shoots showed significantly lower phenolic content (between 104-121 mg GAE g^{-1}) regardless of the origin (Figure 2). A possible explanation for these differences may be that *in vitro* material has no need to produce these compounds as a defense mechanism against adverse environmental conditions. In a study with *P. tridentatum*, *Erica* spp. and *Cytisus scoparius*, in which aqueous and ethanolic extracts were compared relative to their total phenolic content and antioxidant activity, the authors reported a higher total phenolic content in the aqueous extract from prickly broom (222.7 mg GAE g^{-1} dw) than in that from *Erica* spp. (212.7 mg GAE g^{-1} dw) [10]. The same was observed in ethanolic extracts, 196.6 and 141.5 mg GAE g^{-1} dw for prickly broom and *Erica* spp., respectively [10]. Still, the total phenolic content of both aqueous extracts is lower than that of the wild *P. tridentatum* plant material used in the present work. In addition, the total phenolic content of *C. scoparius* aqueous extract referred to in the same work is also lower (134.7 mg GAE g^{-1} dw) than those of prickly broom presented in Figure 1. In a work with species of Fabaceae, significantly higher levels (523.4 mg of caffeic acid equivalents per gram of extract) of phenolics were observed in *P. tridentatum* when compared with *Cytisus* species [12].

Also, in a study of *Rosmarinus officinalis*, *Thymus vulgaris* and *Lavandula angustifolia*, the total phenolic content (extracted with 50% aqueous ethanol) ranged between 52 and 219 mg GAE g^{-1} for a lyophilized extract [13], values lower than those obtained in this study for prickly broom (226.7 to 369.2 mg GAE g^{-1} dw). Also in a study of 35 wild plants of Galicia, including *P. tridentatum* subsp. *tridentatum* using an ethanol-water (1:1) extraction solution, a total phenolic content between 282 and 292 mg GAE g^{-1} dw was reported for the flowers and leaves, respectively [4]. The values are similar to those obtained in this study for shoots during flowering (299 and 319 mg GAE g^{-1} dw) from wild plants from both Malcata and Gardunha, respectively.

To notice differences between plant extracts from different locations and vegetative phases, as well as wild vs *in vitro* material, a comparative analysis of the respective chromatographic profiles was carried out. Based on these, either the presence or absence of the identified phenolic compounds was determined (Table 1). There were some differences between the phase of the vegetative cycle and the place of harvest. Extracts of plant material harvested during flowering, and flowers, had a more complex phenolic profile than those from material collected during the rest period.

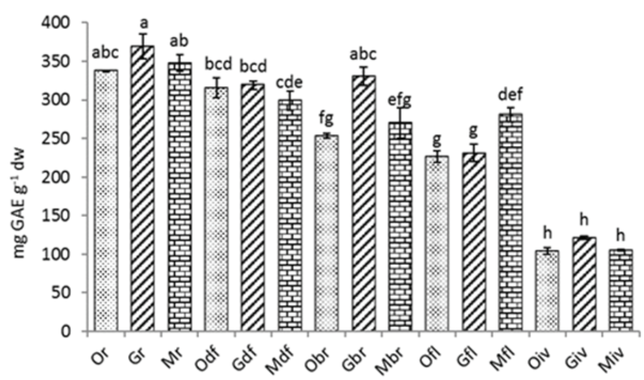

Figure 2: Total phenolic content (mg GAE g^{-1} dw) of the aqueous extracts from plant material with different origins and vegetative phases. (O: Orvalho; G: Gardunha; M: Malcata; r: at rest; df: during flowering; br: beginning of the rest; fl: flowers; iv: *in vitro* plant material). Average values with different letters are significantly different according to the Scheffé's multiple range test at α≤0.05.

The phenolic profile identified in this study for wild plant extracts is in agreement with the studies reported [1, 11, 14a-14d]. In one of these, twenty-one flavonoids were identified in *P. tridentatum*, eight of which are common to this present study, namely myricetin 6-*C*-glucoside (4), genistein 8-*C*-glucoside (5), 5,5' dihydroxy-3'-methoxy-isoflavone-7-*O*-glucoside (6), quercetin 3-*O*-glucoside (isoquercitrin) (8), genistin (10), prunetin (11), 7-*O*-methylorobol (12) and sissotrin (13) [15]. From Table 1 it may be observed that isoquercitrin arises only during flowering, in stems and flowers.

This is the first study reporting the phenolic profile of extracts from *in vitro* shoots of prickly broom. These are more analogous to

Table 1: Phenolic compounds identified in *in vitro* (iv) and wild plant extracts of *P. tridentatum* from Gardunha (G) and Malcata (M) in different vegetative phases (r: at rest; df: during flowering; fl: flowers).

No	Rt (min)	$[M-H]^-$ (*m/z*)	Main MS^2 fragments (*m/z*) (%)	Possible identification	Type of plant material							
					Giv	Miv	Gr	Mr	Gdf	Mdf	Gfl	Mfl
1	11.55	191	111(90), 87(100), 85(52)	Quinic acid	x	x	x	x	x	x	x	x
2	13.30	191	111(100), 87(57), 85(41)	Citric acid	x	x	x	-	x	x	x	x
3	29.94	465	345(100), 327(68), 317(54), 167(85)	Taxifolin 6-*C*-glucoside	-	-	x	x	x	x	x	x
4	43.27	479	359(100), 479(43)	Myricetin 6-*C*-glucoside	x	x	x	x	x	x	x	x
5	65.66	431	311(100), 431(46)	Genistein 8-*C*-glucoside	x	x	x	x	x	x	x	x
6	69.92	461	341(100), 326(32), 298(49)	5,5'-Dihydroxy-3'-methoxy-isoflavone 7-*O*-β-glucoside	x	x	x	x	x	x	x	x
7	77.09	609	300(100), 301(73)	Rutin	-	-	x	x	x	x	x	x
8	77.37	463	300(100), 301(4)	Quercetin 3-*O*-glucoside (isoquercitrin)	-	-	-	-	x	x	x	x
9	79.57	463	300(100), 301(45)	Quercetin 3-*O*-galactoside	x	x	-	x	x	-	-	x
10	80.42	431	268(100), 269(43)	Genistein	-	x	-	x	x	-	x	x
11	87.61	431	268(100), 431(18)	7-O-Methylgenistein (prunetin)	-	-	-	-	x	-	-	-
12	96.45	283	268(100), 283(98), 240(66)	7-*O*-Methylorobol	-	x	x	-	x	-	-	x
13	105.00	445	283(100)	Sissotrin	-	-	x	-	x	-	x	x

extracts from wild plants at the time of rest regardless of the geographic source. However, taxifolin-6-*C*-glucoside (3), one of the most representative substances in wild plants, as well as rutin (7) and isoquercitrin (8), were not present in extracts of *in vitro* shoots. This can be explained since plant tissue culture techniques may allow changes in the chemical profile of the plants by manipulating the physical and chemical environment, for example, by changing the *in vitro* culture conditions. For example, in the case of *Thymus lotocephalus*, the conditions used *in vitro* favored the accumulation of high levels of rosmarinic acid, suggesting that this micropropagated species could be used as a source of natural antioxidant compounds, thereby helping to preserve the natural populations [16].

The total mass of monosaccharides of the plant extracts was more dependent on the vegetative status of the plant than on the harvesting location (Table 2). A slightly higher monosaccharides content was observed in extracts of stems during flowering (between 287 and 289 mg g^{-1}) when compared with those of flowers (between 270 and 276 mg g^{-1}). The extracts of *in vitro* plant material presented lower values, which can be correlated to the low photosynthetic rate of the shoots.

In *P. tridentatum* extracts, glucose and uronic acids were the major components; these are the levels that are most distinctive between wild and *in vitro* plants (Table 2). In wild plant extracts glucose is the predominant sugar, regardless of the location and vegetative phase of collection, but in *in vitro* plant extracts uronic acids are the major components (Table 2).

Table 2: Monosaccharide total mass of extracts (mg g-1) and respective molar percentage (in parenthesis), from extract samples of wild plants of *P. tridentatum* from Malcata (M) and Gardunha (G) at rest (r), during flowering (df), from flowers (fl) and from *in vitro* plants (iv).

Origin	Monosaccharides mass (mg g^{-1}) and molar percentage (in parenthesis)						
	Rhamnose	Arabinose	Xylose	Mannose	Galactose	Glucose	Uronic acids
Mr	4.8(1.9)	5.6(2.4)	1.1(0.5)	1.5(0.6)	9.5(3.5)	192.6(70.2)	61.8(21.0)
Mdf	2.8(1.1)	7.0(2.9)	1.9(0.8)	6.0(2.1)	6.9(2.4)	213.2(74.1)	51.1(16.6)
Mfl	1.9(0.8)	6.1(2.7)	3.1(1.4)	15.9(5.8)	16.2(5.9)	172.0(62.8)	61.1(20.7)
Gdf	3.5(1.3)	8.2(3.4)	1.9(0.8)	4.7(1.7)	7.5(2.6)	208.0(72.7)	53.6(17.4)
Gfl	1.8(0.7)	5.8(2.6)	2.7(1.2)	17.1(6.4)	10.9(4.1)	162.0(60.7)	69.8(24.3)
Miv	10.8(4.9)	5.4(2.7)	1.9(0.9)	1.6(0.7)	15.8(6.5)	79.3(32.8)	135.4(51.5)
Giv	7.5(3.2)	6.7(3.2)	1.8(0.8)	5.0(2.0)	16.6(6.5)	92.2(36.4)	131.0(47.9)

Besides uronic acids, the extracts of *in vitro* Gardunha and Malcata plants also present a content of rhamnose superior to that of wild plants. Thus, the glucidic analysis shows that the *in vitro* plant material presents a composition that can elicit more interest than the wild plants. Rhamnose and uronic acids are rare sugars, which are referenced as showing interesting properties, such as anti-inflammatory, antioxidant and antiviral activities, with application in the pharmaceutical and cosmetic industries [17]. Some of these can be produced by specialized enzymes or reactions using glucose as the main precursor, but the processes are quite costly [18a, 18b]. Their scarcity makes these sugars highly valuable. Consequently, the presence of these compounds in *in vitro* plants is an additional factor that can justify the investment in their production. To increase biomass production capacity in order to make the production process more profitable, one of the most promising ways is the use of temporary immersion bioreactors as has been done with other species [18c-18e].

In conclusion, this is the first experimental work that characterizes the aqueous extracts of *in vitro* plant material of *P. tridentatum* and the results obtained open new possibilities to ensure that this material has enough potential for its use as a replacement of wild plants with a view to preserving them. They have higher extraction yields, are richer in rare sugars with well-known pharmaceutical and cosmetic properties, and present similar phenolic contents and profile as wild plants.

Experimental

Plant material: For this study the *in vitro* plant material was obtained as described previously [9], using the three wild ecotypes of the respective wild plants, Gardunha, Malcata and Orvalho. For the wild plants, we used the aerial parts of the plant, stems and leaves together, collected randomly in 3 georeferenced locations in the center of Portugal, Gardunha (40º06'27.822N; 7º28'52.241W), Malcata (40º14'05.942N; 7º06'52.804W) and Orvalho (40º01'39.92N; 7º46'33.624W), in three different vegetative phases: the beginning of the rest period (October), at rest (February) and during flowering (May). The species were identified and voucher specimens were prepared and deposited at the herbarium of the Escola Superior Agrária de Castelo Branco. The identification numbers of the voucher specimens are: *P. tridentatum* Gardunha, Malcata, and Orvalho ESACBPTG01 and ESACBPTG02, ESACBPTM01 and ESACBPTM02, ESACBPTO01 and ESACBPTO02, respectively.

Total phenolic and Phenolic profile determination: Aqueous extracts of *P. tridentatum* were obtained by boiling under reflux (modified Clevenger apparatus). The total phenolic content of the extracts was quantified by spectrophotometry according to the method of Ribéreau-Gayon [19], by measuring the absorbance at 280 ηm in a quartz cell of 1 cm optical path (UNICAM UV/Vis Spectrometer UV4). The results were expressed in mg of gallic acid equivalents per g dry weight extract (mg g^{-1}), based on a calibration curve derived by linear regression, established from concentrations of 0 to 30 mg L^{-1} of gallic acid. Identification of the phenolic compounds present in the prickly broom extract was carried out by high-performance liquid chromatography with diode array detection (HPLC-DAD) [20a]. For a putative identification of compounds, HPLC-DAD-MS/MS analyses were also performed using a Waters® Alliance 2695 HPLC equipment fitted with a diode array detector (DAD), Waters 2996 (PDA), and a triple quadrupole spectrometer (TQ) (Micromass® Quattro microTM, Waters) with an ESI source operating in negative mode. The chromatographic separation was performed on a LiChroCART RP-18 column (250 x 4 mm, particularly from size 5 µm, Merck) at 35°C. For data acquisition and treatment of data MassLynx® software, version 4.1 was used.

Chemical composition of carbohydrates: The individual sugars were determined by gas chromatography (GC), after acid hydrolysis, and conversion to the corresponding alditol acetates [20b, 21a-21b].

Statistical analysis: The data were analysed with ANOVA using Statistica, V.7 software. The experiments were repeated 3 times and the significance among means was carried out using the Scheffé's multiple range test at $\alpha \leq 0.05$.

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