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Protective effect of *Hypericum calabricum* Sprengel on oxidative damage and its inhibition of nitric oxide in lipopolysaccharide-stimulated RAW 264.7 macrophages

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ABSTRACT

The present study shows for the first time the phenolic composition and the in vitro properties (antioxidant and inhibition of nitric oxide production) of *Hypericum calabricum* Sprengel collected in Italy. The content of hypericins (hypericin and pseudohypericin), hyperforin, flavonoids (rutin, hyperoside, isoquercetrin, quercitrin, quercetin and biapigenin) and chlorogenic acid of *H. calabricum*, have been determined. The ethyl acetate fraction from the aerial parts of *H. calabricum* exhibited activity against the radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) with IC_{50} value of 1.6 μg/ml. The test for inhibition of nitric oxide (NO) production was performed using the murine monocytic macrophage cell line RAW 264.7. The ethyl acetate fraction had significant activity with an IC_{50} value of 102 μg/ml and this might indicate that it would have an anti-inflammatory effect in vivo.

Key terms: antiradical activity, hypericaceae, *Hypericum calabricum* Sprengel; phenolic compounds.

INTRODUCTION

Plant species of the genus *Hypericum* are well known for their use in traditional medicine due to their therapeutic efficacy. One of the most important and commercially recognized species of the genus is *H. perforatum* L. (St. John's wort), which has been used in herbal medicine, externally for the treatment of skin wounds, eczema and burns, and internally for disorders of the central nervous system, the alimentary tract and other ailments (Bombardelli and Morazzoni, 1995; Barnes et al., 2001). The main constituents of the *Hypericum* species are: naphthodianthrones, primarily represented by hypericin and pseudohypericin; flavonoids, e.g., hyperoside, rutin or quercitrin; and phloroglucinol derivatives, e.g., hyperforin and adhyperforin (Nahrstedt and Butterweck, 1997; Smelcerovic et al., 2006).

The use of traditional medicine is widespread and plants still represent a large source of natural antioxidants that might serve as leads for the development of novel drugs. Therefore, the *Hypericum* genus has attracted much attention in the investigation of metabolites from this genus.

The aim of present study was to determine the chemical composition, antioxidant potential and inhibition of nitric oxide (NO) production of *Hypericum calabricum* Sprengel (Hypericaceae). Therefore, the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activities and production of NO in murine monocytic macrophage cell line RAW 264.7 of ethyl acetate fraction were determined.

METHODS

Chemicals

Ethanol and dimethyl sulfoxide were obtained from VWR International s.r.l. (Milan, Italy). Ascorbic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), Griess reagent [1% sulfanamide and 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride in 2.5% H_3PO_4], 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Dulbecco's modified Eagle's medium (DMEM), L-glutamine, fetal bovine serum, antibiotic/antimycotic solution (penicillin/streptomycin), lipopolysaccharide (LPS), indomethacin, standard compounds were obtained from Sigma-Aldrich S.p.A. (Milan). All other reagents, of analytical grade, were products of Carlo Erba (Milan).

Plant materials

H. calabricum is a herbaceous perennial plant, the distribution area of which is limited to Calabria, Italy (Fig. 1) (Conti et al., 2005; Brullo et al., 2007). The aerial parts of *H. calabricum* used in this study were collected in June 2002 in Calabria (Southern Italy) and authenticated by Dr. Carmen Gangale, Natural History Museum of Calabria and Botanic Garden, University of Calabria (Italy). A voucher specimen was deposited in the Botany Department Herbarium at the University of Calabria (CLU-9307).

High-performance liquid chromatography analysis

The dried aerial parts of *H. calabricum* (720 g) were extracted with methanol, and successively fractioned with n-hexane, dichloromethane and ethyl acetate (1.1 L each). The ethyl acetate fraction was subjected to HPLC analysis (JASCO - PU-980 pumps; JASCO-MD-910 Multiwavelength Detector with UV-Diode Array) water-H_3PO_2 mixture (10:90, 20:80, 40:60, 60:40, 80:20 and 100:0). The preparative reversed-phase HPLC analysis (C18 column 250 x 4.6 mm, 5 mm; mobile phase: 0-15 min, isocratic 100% water; 15-45 min gradient 30% ACN in water; 45-55 min, gradient 80% ACN in water; isocratic 100% ACN; flow-rate: 1 ml/min) was used.

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The quantification of the constituents was done by the external standard method, using a solution containing 15 μg/ml of each reference compound in methanol. The reference compounds were chlorogenic acid, rutin, hyperoside, isoquercitrin, quercetin, biapigenin, hypericin and hyperforin salt. Flavonols and flavones were quantified at 350 nm, hyperforins at 260 nm and hypericins at 590 nm. Other flavonols and cinnamic acid type compounds were quantified at 350 nm, such as quercetin and chlorogenic acid equivalents, respectively. All the samples were analyzed in triplicate.

Free radical-scavenger activity

Free radical-scavenger activity was determined by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, as described previously (Silva and Dias, 2002). The antiradical activity of each fraction was evaluated using a dilutions series, in order to obtain a large spectrum of sample concentrations. Additionally, relative free radical scavenging activity was assessed using fractions with equivalent dry-weight biomass concentrations. The reaction solution consisted of 0.2 ml of sample and 0.8 ml of a DPPH stock solution (1.0×10^{-4} M, methanol 100%). The absorbance was monitored continuously at 517 nm with a Perkin-Elmer UV/VIS Spectrometer Lambda, assuring that the reaction was complete (plateau state). Methanol was used as a blank and ascorbic acid was used as a positive control. All determinations were performed in triplicate. The percentage of reduced DPPH at steady state (DPPH R) was calculated and these values were plotted against the log10 of the concentrations of individual fractions. A decrease by 50% of the initial DPPH concentration was defined as the IC_{50}. The amount of reduced DPPH was estimated after 30 min at the dilution factor closest to the estimated value IC_{50}. This activity is given as a percentage of DPPH radical scavenging, which is calculated with the equation:

% DPPH radical-scavenging = [1-(sample absorbance with DPPH-sample absorbance without DPPH/control absorbance)] x 100.

The DPPH solution without sample solution was used as control. All the parameters were calculated graphically using the software GraphPad 4.0 (Prism, USA). All tests were performed in triplicate.

Fig. 1: Distribution of *Hypericum calabricum* Sprengel in Italy.

Cell culture

The murine monocytic macrophage cell line RAW 264.7 (European Collection of Cell Cultures, London, UK) was grown in plastic a culture flask in DMEM with L-glutamine supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic solution (penicillin/streptomycin) under 5% CO_2 at 37°C. After 4-5 days cells were removed from the culture flask by scraping and centrifuged for 10 minutes. The medium was then removed and the cells were resuspended with fresh DMEM. Cell counts and viability were assessed using a standard trypan blue cell counting technique. The cell concentration was adjusted to 1×10^6 cells/ml in the same medium. One hundred microliters of the above concentration was cultured in a 96-well plate for 1 day to become nearly confluent. Concentrations of the samples ranging from 10 to 100 μg/ml were prepared from the stock solutions by serial dilution in DMEM to give a volume of 100 μl in each well of a 96-well microtiter plate. Then cells were cultured with vehicle or *H. calabricum* ethyl acetate fraction in the presence of 1 μg/ml LPS for 24 hours.

Assay for cytotoxic activity

Cytotoxicity was determined using the MTT assay reported by Tubaro and co-workers (1996) with some modifications. The assay for each sample analyzed was performed in triplicate, and the culture plates were kept at 37°C with 5% (vol/vol) CO_2 for 1 day. After 24 hours of incubation, 100 μl of medium was removed from each well. Subsequently, 100 ml of 0.5% (wt/vol) MTT, dissolved in phosphate-buffered saline, was added to each well and allowed to incubate for a further 4 hours. After 4 hours of incubation, 100 μl of dimethyl sulfoxide was added to each well to dissolve the formazan crystals. Absorbance values at 550 nm were measured with a microplate reader (model DV 990 B/V, GDV, Rome). Cytotoxicity was expressed as 50% inhibitory concentration (IC_{50}), which is the concentration to reduce the absorbance of treated cells by 50% with reference to the control (untreated cells).

Inhibition of NO production in LPS-stimulated RAW 264.7 cells

The presence of nitrite, a stable oxidized product of NO, was determined in cell culture media by Griess reagent [1% sulfanamide and 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride in 2.5% H_3PO_4] (Green et al., 1982). One hundred microliters of cell culture supernatant was removed and combined with 100 ml of Griess reagent in a 96-well plate followed by spectrophotometric measurement at 550 nm using

the DV 990 B/V microplate reader. Nitrite concentration in the supernatants was determined by comparison with a sodium nitrite standard curve.

Statistics

Data were expressed as mean ± SD values. Statistical analysis was performed by using Student's *t* test or by one-way analysis of variance followed by the Dunnett's test for multiple comparisons of unpaired data. Differences were considered significant at $P \leq .05$. The IC_{50} was calculated from the Prism (GraphPad, San Diego, CA, USA) dose-response curve (statistical program) obtained by plotting the percentage of inhibition versus the concentrations.

RESULTS

Phenolic composition

Several papers have reported the analysis of *H. perforatum* extracts. However, most of them focus only on some individual compounds, such as the hypericins or the phloroglucinols; LC-MS studies have also been done (Fuzzati et al., 2001; Tolonen et al., 2002). In this work, we fully analyzed the ethyl acetate fraction of *H. calabricum* by HPLC-DAD and the major compounds were identified and characterized. Phenolic compounds were identified by their UV spectra. Ethyl acetate fraction was composed of a complex mixture of compounds, most of them already known to be present in *Hypericum* extracts (Dias et al., 1999; Erdelmeier et al., 2000; Jürgenliemk and Nahrstedt, 2002). Figure 2 shows the data obtained by HPLC-DAD of the most representative phenolics present in *H. calabricum*.

A major group of compounds was identified as flavonols, due to their characteristic UV spectra. Compound 1 has similar UV-spectra, characteristic of chlorogenic-type acids. The utilization of chlorogenic acid commercial standards confirmed the identification of compound 1. Compounds 2 and 3 have UV-spectra characteristic of flavonols glycosylated at C3 (257, 265sh, 355 nm). Compound 2 was putatively identified as rutin while compound 3 as hyperoside. The utilization of rutin, hyperoside commercial standards confirmed the identification of compounds 2 and 3. Compounds 4 and 5 have an UV-spectrum (255, 265sh, 301sh, 349) consistent with those of isoquercetrin and quercetrin. The identification was confirmed by spiking with a commercial standard of quercetin 3-rhamnoside. Compound 6 has a UV-spectrum (255, 265sh, 370 nm) consistent with those of quercetin. The identification was confirmed by spiking with a commercial standard of quercetin. Compound 7 has a UV-spectrum (268, 333 nm), similar to that of amentoflavone. These data and its localization in the chromatogram are compatible with biapigenin. Compounds 8 and 9 were identified as hypericins according to their characteristic UV-Vis spectra (Kurth and Spreemann, 1998). Confirmation of hypericin identity was supported by data consistent with that already published (Tolonen et al., 2002). Hyperforin was identified as described elsewhere (Dias and Ferreira, 2003).

The preparative reversed-phase HPLC analysis of ethyl acetate fraction resulted in the quantification (mg/g) of phenolic compounds: flavonoids: rutin 0.72, hyperoside 10.94, isoquercetrin 3.35, quercitrin 2.17, quercetin 1.04, biapigenin (not detected); naphthodianthrones: pseudohypericin (not detected), hypericin 0.03, hyperforin 3.45; chlorogenic acid 1.27.

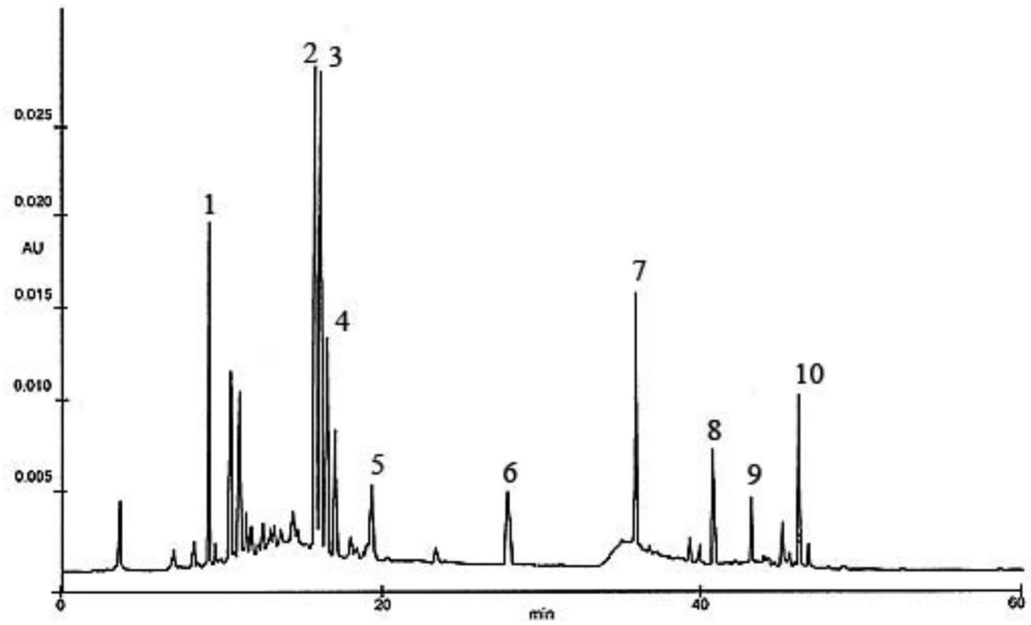

Fig. 2: HPLC chromatogram of *H. calabricum* ethyl acetate fraction. Compounds are identified in the figure by numbers: 1 - chlorogenic acid; 2 - rutin, 3 - hyperoside; 4 - isoquercetrin, 5 - quercitrin, 6 - quercetin, 7 - biapigenin; 8 - pseudohypericin, 9 - hypericin, 10 - hyperforin.

Radical scavenging activity

The model of scavenging stable DPPH free radicals can be used to evaluate antioxidant activity in a relatively short time. The absorbance decreases as a result of a color change from purple to yellow as the radical is scavenged by antioxidants through donation of hydrogen to form the stable DPPH-H molecule (Gadov et al., 1997), although a recent article suggests that, on the basis of kinetic analysis of the reaction between phenols and DPPH, the reaction in fact behaves like a single electron transfer reaction (Foti et al., 2004). It was found that the rate-determining step for this reaction consists of a fast electron transfer process from the phenoxide anions to DPPH. The hydrogen atom abstraction from the neutral ArOH by DPPH becomes a marginal reaction path because it occurs very slowly in strong hydrogen-bond-accepting solvents, such as methanol and ethanol. The scavenging effects of extract on DPPH were examined at different concentrations (range between 0.1 and 100 μg/ml). Ethyl acetate fraction from the aerial parts of *H. calabricum* was able to reduce the stable free radical DPPH to the parent yellow-colored DPPH with an IC_{50} value of 1.6 μg/ml (Table 1). This result is very significant in comparison to the positive control, ascorbic acid, which showed an IC_{50} value of 2 μg/ml (Fig. 3).

TABLE 1
IC_{50} values of antioxidant and inhibition of NO production of H. calabricum: IC_{50} values.

Assay	IC_{50} μg/ml[a]
Inhibition of NO production	102 ± 1.2
Indomethacin[b]	53 ± 0.8
DPPH	1.6 ± 0.001
Ascorbic acid[b]	2.0 ± 0.001

[a] ± S.D. (n = 3).
[b] Positive control.

Inhibition of NO production

An activity of *H. calabricum* ethyl acetate fraction relative to reduction of inflammation was studied in vitro by analyzing their inhibitory effects on the chemical mediator NO released from macrophages. Once activated by inflammatory stimulation, macrophages produce a large number of cytotoxic molecules. The treatment of RAW 264.7 macrophages with LPS (1 μg/ml) for 24 hours induces NO production, which can be quantified by utilizing the chromogenic Griess reaction measuring the accumulation of nitrite, a stable metabolite of NO. NO is considered to play a key role in inflammatory response, based on its occurrence at inflammatory sites and its ability to induce many of the hallmarks in the inflammatory response. The beneficial effect of *H. calabricum* extract on the inhibition of production of inflammatory mediators in macrophages can be mediated through oxidative degradation of products of phagocytes, such as O^{2-} and HOCl. As shown in Figure 4, incubation of RAW 264.7 cells with extract of *H. calabricum* induced a significant inhibitory effect on the LPS-induced nitrite production. The extract of *H. calabricum* showed significant inhibition of LPS-induced NO production in RAW 264.7 cells in a dose-dependent manner, with an IC_{50} value of 102 μg/ml. Cytotoxic effect of the sample in the presence of LPS (1 μg/ml) was also evaluated. *H. calabricum* extract did not show any cytotoxicity up to 500 μg/ml concentration.

DISCUSSION

Oxidative damage may initiate and promote the progression of a number of chronic diseases, including inflammation. The present work showed for the first time the in vitro activity of *H. calabricum* extract and its chemical composition. Further in vivo investigations are needed for a possible usefulness of this extract in the treatment of inflammation. In this study we have demonstrated that the extract of *H. calabricum* exhibited significant antioxidant activity and an inhibitory effect on production of NO (an inflammatory mediator) in macrophages. The observed in vitro activities suggest that the investigated plant extract might also exert in vivo protective effects against oxidative and free radical injuries occurring in different pathological conditions.

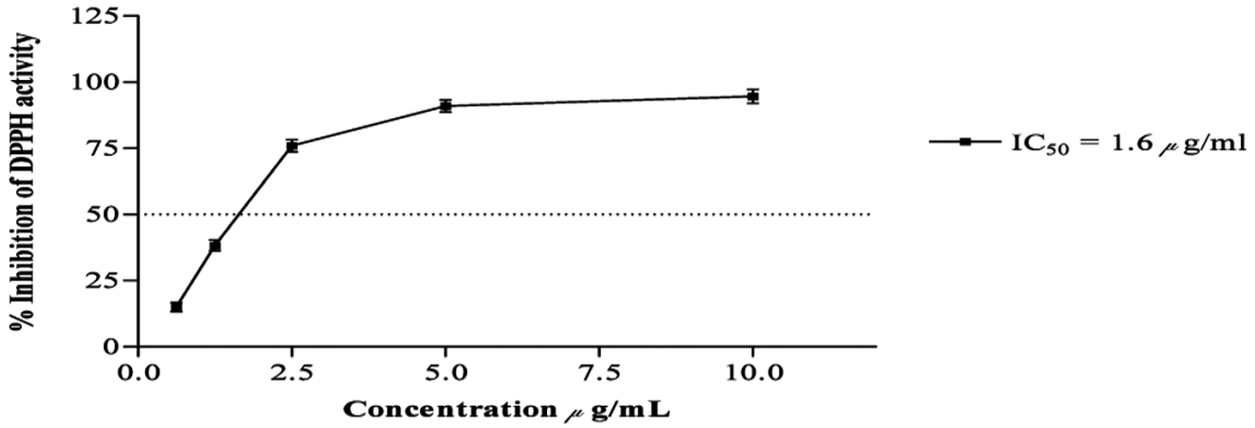

Fig. 3: Dose-dependent activity of *H. calabricum* ethyl acetate fraction using DPPH radical. Data are mean ± S.D. (n = 3).

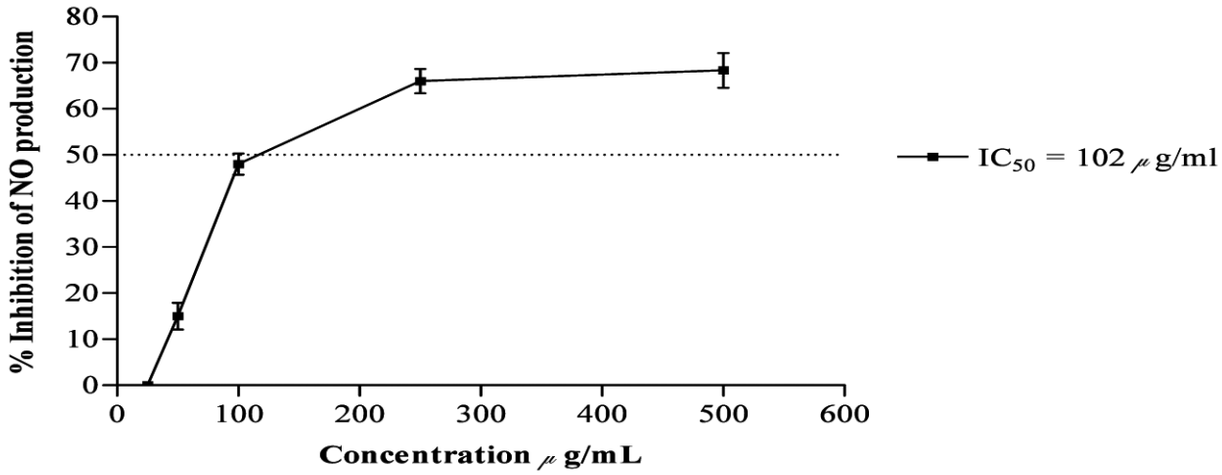

Fig. 4: Dose-dependent activity of *H. calabricum* ethyl acetate fraction on NO production in the murine monocytic macrophage cell line RAW 264.7. Data are mean ± S.D. ($n = 3$).

According to a study of Silva et al. (2005), phenolic compounds other than hypericins should be relevant for DPPH activity. Taking into account the activity of the *H. perforatum* fractions tested, the hydroxycinnamic acids and flavonoids were highly relevant for both radical-scavenging and inhibition of lipid peroxidation activities. Our previous study (Conforti et al., 2002) evaluated the antioxidant potential of *H. triquetrifolium* and we showed that this activity could be related to the content of flavonoids. The antioxidant activity of an *H. androsaemum* infusion was also related to its phenolic content (Valentao et al., 2002). Some of the identified phenols in the *H. androsaemum* infusion extract were flavonoids, specifically quercetin and glycosylated derivatives, also present in ethyl acetate fraction of *H. calabricum*. These types of compounds are well known antioxidants. They have structural aspects, such as the presence of a catechol moiety in the ring, a 2,3-double bond in conjunction with a 4-oxo group in the C-ring, and the presence of hydroxyl groups at positions 3 and 5, which are all determinants of high antioxidant activity (Halliwell et al., 1995; Rice-Evans et al., 1997).

Oxidative stress has been implicated in exacerbated inflammation, a process of cellular aggression mainly mediated by reactive oxygen/nitrogen species (ROS/RNS). Our results suggest that ethyl acetate fraction of *H. calabricum* could have a beneficial role in inflammatory disorders by trapping an important mediator of inflammatory processes: nitric oxide (NO). Previous studies have reported that compounds also present in *H. calabricum* extracts, such as chlorogenic acid and flavonoids, are efficient scavengers of NO (Firuzi et al., 2004; Kono et al., 1997).

Therefore, we propose here the potential benefits of *H. calabricum* extract on the basis of the phytochemical characteristics and the observed bioactive properties. The antioxidative and anti-inflammatory properties of naturally occurring compounds appear to contribute to their chemopreventive or chemoprotective activity.

The anti-inflammatory activity of *H. calabricum* extract was evaluated to obtain an insight into the beneficial effects of this plant species in conditions related to inflammation, reduced risk for cardiovascular diseases, and cancer prevention by acting as anti-inflammatory agents. Further studies of the plant extracts and/or the identified compounds from *H. calabricum* on the pharmacokinetics or mode of action on mechanisms of chemopreventive properties are warranted. As well, the extraction technique should be investigated more widely, particularly in view of the application of supercritical fluids. Another point that should be strongly evaluated is the use of emulsions instead of solution in real applications, with the aim of preventing degradation of extract activity due to oxygen exposure. In conclusion, this work reveals that *H. calabricum* can be an interesting source of anti-inflammatory and antioxidant principles, with a potential use in different fields (the food, cosmetics, and pharmaceutical).

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