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Research Paper

The synthetic antioxidant Butylated Hydroxytoluene, a naturally occurring constituent of the broom C*ytisus triflorus* L'Hérit

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ABSTRACT

BHT chemically named 2,6-Bis(1,1-dimethylethyl)-4-methylphenol is one of the synthetic antioxidant agents commonly used in processed Food, Cosmetics and Pharmaceuticals. A naturally occurring BHT was determined for the first time in extracts of different aerial parts of the shrub *Cytisus triflorus* L'Hérit., by GC/MS and RP-HPLC–UV/Vis with gradient elution. BHT was extracted in this study by three methods. The different extracts were tested for their Radical-Scavenging activity assayed by the DPPH method. Both polar (hydroalcoolic extract) and non-polar (ethyl acetate-hexane and diethyl ether-hexane) extracts exhibited moderate to high free scavenging activity; the strongest one was exhibited respectively by the hydroalcoolic extracts of leaves 81,91% and the diethyl ether-hexane volatile fraction of fruits 87,01%.The analysis of the volatile fractions carried out by GC/MS allowed us to resolve the BHT other constituents in the volatiles fractions and provides positive identification of the BHT with mass spectral data. The RP-HPLC data provides positive confirmation of BHT in the hydroalcoholic extracts from stems and leaves.

Keywords: Natural BHT; *Cytisus triflorus* L'Hérit; GC/MS; RP-HPLC.

INTRODUCTION

Due to its ability to scavenge free radicals, Butylated Hydroxytoluene (BHT; synonyms: 2,6-di-*tert*-butyl-4-methylphenol*;* 2,6-di-*tert*-butyl-*p*-cresol) was used as a synthetic antioxidant in many lubricating oils, fats containing foods and in cosmetic preparation (Leng and Gries, 2010). However, the US Food and Drug Administration (FDA) have restricted the use of BHT because it have also been found to be toxic at higher levels (Schilderman, et al., 1995) and for its suspected carcinogenicity (Witschi and Morse, 1983; Madhavi and Salunkh, 1995). Therefore, several studies aim actually to the search for new natural antioxidant biomolecules contained in vegetables, fruits and medicinal plants, as substitutes for synthetic ones (Veeru, et al., 2009, Reihani and Azhar, 2012). According to Pratt and Hudson (1992), most natural antioxidants can be found in wood, bark, stem, leaf, fruit, root, flower and seed.

The production of a natural BHT was reported for the first time in the vascular plant *Mesembryanthemum cristallinum* L. (*Aizoaceae*) (Bouftira, et al., 2007). Afterwards, a bioavailable BHT has been determined by (Bahu and Wu, 2008) in non vascular plants green algae (*Botryococcus braunii* Kütz.) and three Cyanobacteria [*Cylindrospermopsis raciborskii* (Wollosz) Seenaya et Sabba Raju, *Mycrocystis aeruginosa* (Kütz.) and *Oscillatoria sp*.]. Whereas, the presence of BHT in volatile fractions of *Azadirachta indica* (*Meliaceae*) (Siddiqui, et al., 2004) and *Camellia sinensis* (*Theaceae*) (Pripdeevech and Machan, 2011), was simply reported as 2,6-di-*tert*-butyl-4-methylphenol and BHT respectively. This naturally occurring of BHT is reported for the first time here.

The hairy broom *Cytisus triflorus* (Family-*Fabaceae*), is used in traditional medicine in Algeria for treating abdominal pain, wound healing and as haemostatic. Biological activities as well as the phytochemistry of *Cytisus triflorus* are almost unexplored only some studies on various aspects of aerial part of the species were carried out by our previous work (Ait-kaci, et al., 2000; Ait-kaci, 2001; Mohandkaci, et al., 2008; Ait-kaci, et al., 2011). In the present study, we report for the first time the production of a natural BHT by all aerial parts of *Cytisus triflorus* L'Hérit., using different processes of extraction and identification.

MATERIALS AND METHODS

Plant Material: Aerial parts of *Cytisus triflorus* were collected in May 2010, in eastern Algeria and authenticated by Dr M. Zaoui, Department of Biology, Normal Superior School, Algiers (Algeria).

Extraction procedures:

Method 1: Using an ultrasound bath type Bandelin HF, 35 KHz, powdered dried leaves (5g), shoots (5g), fruits (5g) and of flowers (2g) were respectively sonicated in 135ml, 120ml, 80ml and 60ml of distilled water for 45 minutes below 42°C. The aqueous extracts were filtered and then extracted twice with respectively 35ml, 35ml, 25ml and 20ml diethyl ether/Hexane (2:1). The extract solutions were dried over anhydrous sodium sulfate and then evaporated to dryness using a rotary evaporator at 40°C. The same procedure was followed using ethyl acetate/Hexane (2:1) solvent.

***Method* 2**: Sixty grams of powdered shoot and leave samples were individually hydrodistillated for 3 hours using a modified Clevenger apparatus. The oil was isolated from the distillate with 10ml diethyl ether, dried over anhydrous sodium sulfate and the solvent was evaporated by rotary vacuum at 40°C.

***Method* 3**: Ten grams of powdered leafs and powdered shoots were individually sonicated respectively in 160ml and 110ml hydroalcoholic solvent (ethanol-water, 8:2), during 60 minutes below 42°C. The solvents were completely removed by rotary vacuum evaporator at 40°C and further removal of water was carried out by freeze drying.

***DPPH free-scavenging assay*:** The antioxidant potencies of hydroalcoolic extracts and volatile fractions were evaluated by the DPPH method of Thakral et al. (2010).

1 ml at the concentration 200 µg/ml of each sample in ethanol was added to 1 ml of 0.1 mM ethanol solution of DPPH. The reaction mixtures were shaken vigorously and the tubes were allowed to stand at room temperature (27°C) for 30 min. The control was exempt of any extract and ethanol was used as the blank. Inhibition of DPPH was evaluated by changes in absorbance at 517 nm of the samples, using a Shimadzu-1800 UV-vis spectrophotometer. The scavenging ability was expressed as the percentage of inhibition of DPPH and was calculated as follows:

DPPH scavenging effect (%) = $[(A_0-A_1)/A_0]$ x 100

- A_0 is the absorbance of the control at 30 min,
- A1 is the absorbance of the sample at 30 min.
- The assay was carried out in triplicate and the results are the mean values.

GC-EIMS analysis: The gas chromatography coupled with the mass spectrometry was performed with a Hewlett-Packard 6890 gas chromatograph combined with an Agilent 5973 mass spectrometer and equipped with an HP-5 MS (5% phenyl-polymethylsiloxane) capillary column (30m x 0.25mm x 0.25µm). The column temperature was programmed from 40°C (10 min) to 220°C at a ramp of 3°C per minute with holding time 30 min. The injector temperature with splitting ratio or splitless was kept at 250°C. Helium was used as carried gas at a flow rate of 1ml/min.

The mass spectrometer was operated in EI mode with ion source and quadripole temperature 230°C and 150°C, respectively. Mass spectra survey was performed using MS-libraries (Nist, 1998; 2001).

RP-HPLC analysis: The determination of BHT in the hydroalcoholic extracts of leaves and stems was carried out on Agilent HPLC system model 1100, equipped with four pumps and Shimadzu SPD-20 AV- UV/Vis detector. 20µl samples of hydroalcoholic extracts previously dissolved in MeOH was injected. The chromatographic separation was performed on a 250 mm x 4.6 mm, 5µm Hypersil BDS RP-C_{18} column at ambient temperature. The mobile phase consisted of acetonitrile (Solvent A) and water with 2% acetic acid (Solvent B) with the following gradient program described by Noumi, et al., 2011 : 15% A/85% B 0 to 12 min, 40% A/60% B 12 to 14 min, 60% A/ 40% B 14 to 18 min, 80% A/ 20% B 18 to 20 min, 90% A/10% B 20 to 24 min, 100% A 24 to 28 min. The flow rate was kept at 0.5 ml/min and UV detection was performed at 280 nm.

RESULTS AND DISCUSSIONS

Antioxidant activity: The results of the antioxidant properties of various extracts of *C. triflorus* are summarized in table1. The study revealed that all extracts from different aerial parts of the plant have a potential antiradical activity; among them the hydroalcoholic extracts of leaves (81,91%), hydroalcoholic extracts of shoots (78,13%) and the Et2O/Hexane fraction of fruit (87,01%) were the most effectives and comparable to that of synthetic BHT (94,80 %).

Based on the results obtained, it is highly possible that several compounds of different polarity may contribute to the antioxidative activity of *C. triflorus.* The differences in term of radical scavenging ability between the different extracts could be due to the different total phenolic content of the samples and also to their bioactive constituents. The hydroalcoholic extracts may include phenolic and hydrophenolic ompounds with acids, alcohols, sugars or glycosides. Several research groups reported a linear correlation between phenolic content and antioxidant activity (Cai, et al., 2004; Alali, et al., 2007). However, according to Lu and Foo (1995), most natural antioxidative compounds often work synergistically with each other to produce a broad spectrum of antioxidative activities that creates an effective defense system against free radical attack.

Identification of BHT using GC-EIMS: GC-MS analysis was carried out on volatile oil and solvent volatile fractions. A total of sixty volatiles were identified comprising oxygenated and hydrocarbon compounds. Among those bioactive molecules such us linalool, α-Terpineol, eugenol, fatty acids, which have previousely been reported (Lograda, et al., 2009). However, even more surprising, all chromatograms of the analysed samples exhibited a distinct peak, which the corresponding mass spectrum

showed a molecular ion $[M]^+$ at *m/z*= 220. The fragmentation of this ion peak was similar to all the samples and exactly identical to the mass spectrum of thesynthetic BHT published in the NIST spectral library. The base peak observed at m/z = 205 is mainly due to loss of a methyl group corresponding to $[M - CH^3]^+$ and a second fragment at m/z = 177 corresponding with $[M – C_2H_3O]^+$ (Bahu and Wu, 2008). The molecular BHT structure corresponds to a phenol in which the aromatic ring contains alkyl groups.

BHT was found in appreciable amounts in all samples (Table 2). The Et2O/Hexane fractions afforded the highest abundances (6,06% - 43,58%) whereas the lesser amounts were detected in the EtOAc/Hexane fractions (0.32% - 0,27%). In the other hand, it is interesting to note that BHT content stem was found in general to be higher than in leaves whatever the extraction method.

Several investigations have been conducted on the influence of the solvent on the yield extraction of synthetic phenolic antioxidants from vegetable oils and food (Phillips, 1973; Lin, et al., 2003). Among polar solvents and less polar solvents, diethyl ether exhibited the best extraction effect of BHT from chewing gum.

According to the results of the DPPH free-scavenging assay, there was apparently a positive correlation between antioxidative activity and the content of the BHT for the same volatile fraction.

Identification of BHT using RP-HPLC: In order to confirm the natural presence of the BHT in the plant *Cytisus triflorus*, a joint extraction method was applied and the hydroalcoholic extracts obtained from vegetative organs were analysed by HPLC-RP at 280nm. HPLC profiles of stem and leave HA extracts showed a distinct peak at 29,861mn and 29,909mn respectively. The HPLC chromatogram of the standard BHT exhibited a peak with a retention time 29,986mn highly similar to those mentioned above.

The abundance of BHT in the stem HA extract was 1,65% and 0.63% in the leave HA extract. The relative abundance of BHT in the stems is in agreement with the data obtained by GC/MS analysis. However and contrary to the volatile fractions, the antioxidative activity of HA extracts was not related to the BHT content. This result suggested that the BHT worked synergistically with other effective antioxidant compounds present in the HA extracts.

To the best of our scientific knowledge, this is the first time that the synthetic phenolic antioxidant BHT is detected in a natural form in the shrub *Cytisus triflorus*. The natural occurring of this molecule has already been reported from plants and fresh water algae using different solvents and procedures. BHT determined by GC-MS in volatile oils from both vascular plants is less abundant compared to that found in *C. triflorus*. It represented 0.009%-0.68% in the volatile oil samples of *Camellia sinensis* (Pripdeevech and Machan, 2011) and 0.2%-0.4% in different leaf essential oils of *Juniperus oxycedrus* var. *oxycedrus* (Adams et al., 2005). In Cyanobacteria, the content of BHT quantified by GC-MS correlated positively with the irradiation intensity of the growth conditions; thus the bioproduction of BHT was considered to protect the cells from photooxidation (Babu and Wu, 2008). In the other hand, the concentration of this phenol was dependent on the plant growth stage of the halophyte species *Mesembryanthemum crystallinum* (Bouftira, et al., 2007).

Finally, other synthetic molecules can also be found in the future in natural form. That is the case for instance for Tramadol, a wholly synthetic drug produced by the pharmaceutical industry that is used world-wide as an analgesic, which has

recently been isolated in strong concentrations in root bark of an African medicinal plant *Nauclea latifolia* (Boumendjel, et al. 2013).

CONCLUSION

Our study has clearly revealed that all aerial parts of *C. triflorus* (leaf, stem, flower and fruit) have different potential antioxidative activities which varied according to the plant extract. Our results converge and confirm that the potential antioxidative activity of the plant *C. triflorus* was partly due to the naturally occurring of buthylated hydroxytoluene in all aerial parts of the shrub. Extraction yield was dependent on the solvent and method of extraction. Further studies should be conducted on other species of the genus *Cytisus* in order to determine which species contain BHT.

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Table-1: Percentage inhibition by DPPH method of *Cytisus triflorus* extracts.

Extract	DPPH percentage inhibition
EtOH-H_2O L	81,91
EtOH-H_2O S	78,13
Et2O-Hexane L	33,73
Et2O-Hexane S	40,78
Et2O-Hexane Fl	Ne
Et2O-Hexane Fr	87,01
EtOAc-Hexane L	69,29
EtOAc-Hexane S	57,8O
VO L	Ne
VO S	Ne
BHT	94,80

- L: leave; S: stem; F: fruit; Fl: flower; VO: volatile oil, Ne: no evaluated.

Table-2: BHT content (%) in different *Cytisus triflorus* volatile fractions analysed by GC/MS.

	Et2O-Hexane				EtOAc-Hexane		VO	
	Leave	Stem	Flower	Fruit	Leave	Stem	Leave	Stem
BHT (%)	22,62	43,58	6,06	24,39	0,32	0,27	4,34	10,40

