Pharmaceutical and Biomedical Analysis of Terpene Constituents in *Salvia miltiorrhiza*

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Abstract: The present paper reviews the most relevant experimental data on miltirone, a diterpene pigment and one of the major active constituents with phenanthrene-quinone structure of the roots of Salvia *miltiorrhiza*. *Radix Salvia miltiorrhizae Bunge* (*Labiatea, Laminaceae)*, a native plant in China, is listed in the Chinese Pharmacopoeia with the name of Dan-shen and used in Chinese folk medicine for the treatment of different pathologies. In fact, two types of major bioactive components in Dan-shen, including water soluble phenolic acids and lipophylic diterpenoid quinines, have the effectiveness in treating coronary heart disease, heart-stroke, cerebrovascular diseases, menstrual disorders, miscarriage, hepatitis and insomnia.

Moreover, recent studies demonstrated the ability of different extracts of *S. miltiorrhiza* to suppress alcohol drinking in selectively bred Sardinian alcohol-preferring (sP) rats. Administration of miltirone to sP rats produced similar results, suggesting that it is the likely active constituent of *S. miltiorrhiza* responsible for the reducing effect of its extracts on alcohol intake.

However, the study of *S. miltiorrhiza* components is very complex, therefore selective and efficient analytical methods are required to simultaneously determine their structures for further study of the pharmacological effects and to control the quality of the preparations in which are contained.

Currently, there are many methods for the determination of phenolic compounds and diterpenes among which, high-performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry (LC-MS/MS) has been shown to be a powerful method for separation and identification of individual molecules in complex samples.

In the proposal review, we will report an overview about the main results concerning the analysis of the main diterpenes constituents of *S. miltiorrhiza* in medicinal preparations and biological fluids, and our recent data on quantitative determination of miltirone in rat plasma.

Keywords: Miltirone, *S. miltiorrhiza*, Diterpenes, Tanshinones, Liquid chromatography mass spectrometry.

1. INTRODUCTION

Salvia miltiorrhiza Bunge (*Labiatea, Laminaceae*) is an annual sage plant, that mainly grows in hilly areas of China and neighboring countries. *Radix Salvia miltiorrhiza Bunge*, also known as Dan-shen, is one of the most commonly used medicinal herb in China and is officially listed in the Chinese Pharmacopeia [1]. Its dried roots are widely adopted in traditional Chinese medicinal preparations for the treatment of several pathologies due to their better performance and fewer side effects as confirmed in the long-time clinical use [2,3]. A great number of finished herbal products containing Dan-shen as its major component have been developed and marketed widely in China for the treatment above all of cardiovascular diseases [4-6]. As reported in this review in recent years many efforts have been made by different groups and with the support of different kind of instruments to create selective and efficient analytical methods for the identification and the structural characterization of the constituents of *S. miltiorrhiza* [7,8]. In particular, it was found that the bioactive constituents of *S. miltiorrhiza* cover two chemical types: water soluble phenolic acids and lipophilic diterpenoid quinones [9,10]. The water soluble phenolic acids in *S. miltiorrhiza* include single phenolic and polyphenolic acids. The single phenolic acids mainly include protocatechuic acid, ptotocatechuic aldehyde, caffeic acid, isoferulic acid and Danshensu (3,4- diydroxyphenyllactic acid). The polyphenolic acids are mainly the conjugate of danshensu, caffeic acid or its dimer. Other polyphenolic acids are rosmarinic acid, lithospermic acid and salvianolic acid A-G. In recent years, the water-soluble components (including phenolic acids and their esters) have attracted increasing attention because of their antioxidant [11], ant-platelet aggregation [12], antitumor [13], antithrombosis, and antiviral activities [14,15].

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The lipophilic diterpenoid quinones, know as tanshinones in *S. miltiorrhiza,* include tanshinone I, tanshinone IIA, cryptotanshinone and miltirone. It has been reported that tanshinones possess pharmacological actions of anti-inflammation, dilating coronary arteries, increasing coronary flow and protecting the myocardium against ischaemia [16,17]. In addition, many works also reported that tanshinones have exhibited significant cytotoxic, antibacterial, anti-dermatophytic, anti-neoplastic and anti-platelet aggregation activities [18,19].

All these above mentioned pharmacological activities have been widely described in literature, so the present paper briefly reviews the most recent findings on the effects of *S. miltiorrhiza* extract on voluntary alcohol intake in animal models of alcoholism. In particular we focused our attention on miltirone, the active ingredient of *S. miltiorrhiza* that is probable responsible for this effect but for which few structural and pharmacokinetics studies are documented.

In addition, we report an overview about analytical methods concerning the assay of *S. miltiorrhiza* components.

2. ANTI – ALCOHOL PROPERTIES OF *S. miltiorrhiza* EXTRACTS AND MILTIRONE

Different lines of experimental evidence have demonstrated that extracts from the dried roots of *S. miltiorrhiza* reduced alcohol drinking in Sardinian alcohol-preferring (sP) rats, one of the few rat lines selectively bred worldwide for high alcohol preference and consumption. Specifically, acute or repeated administration of *S. miltiorrhiza* extracts have been found to: (a) delay the acquisition of alcohol drinking behavior in alcohol-naive rats given alcohol under the home-cage 2-bottle "alcohol *vs* water" choice regimen (specifically, exposure to the 2-bottle choice regimen started immediately after the first administration of the *S. miltiorrhiza* extract) [20]; (b) reduce alcohol intake under the 2-bottle choice regimen in rats which were alcohol-experienced at the time of extract administration (a model of the "active drinking" phase of human alcoholism) [21,22]; (c) suppress the temporary increase in alcohol intake occurring after a period of deprivation from alcohol (a phenomenon named "alcohol deprivation effect", which has been proposed to model the relapse episodes occurring in human alcoholics) [23].

A more recent study investigated whether miltirone is the active ingredient responsible for the reducing effect of *S. miltiorrhiza* extracts on alcohol intake [24]. To this aim, a first experiment compared the effect of four different extracts of *Salvia miltiorrhiza*, varying as to the content of miltirone, on alcohol intake in sP rats. Specifically, 100 mg/kg of four extracts containing approximately 0%, 2%, 3%, and 7% miltirone, respectively, were acutely administered to alcohol-experienced sP rats exposed to the 2-bottle choice regimen. Notably, the reducing effect of the different *S. miltiorrhiza* extracts on alcohol intake positively and significantly correlated with their miltirone content (r=0.9833).

Subsequent experiments tested the effect of pure miltirone on alcohol intake in sP rats [24]. Specifically, miltirone was administered at doses (0, 2.5, 5, and 10 mg/kg, i.g.) comparable to its content in the 100 mg/kg dose of the four extracts tested in the correlation experiment (see above). In the first experiment, the repeated daily administration of miltirone resulted in a significant and dose-dependent delay in acquisition of alcohol drinking behavior in alcohol-naive sP rats. Miltirone effect on alcohol intake was completely selective, as daily water intake was compensatorily higher in miltirone than vehicle-treated rats, while daily food intake was virtually unaltered by treatment with miltirone. In the second experiment, the acute administration of miltirone to alcohol-experienced sP rats (i.e., rats exposed to the 2-bottle choice regimen for several weeks before the experiment with miltirone whose alcohol drinking behavior was well consolidated at the time of the miltirone experiment) resulted in a significant and dose-dependent reduction in alcohol intake. Miltirone-induced reduction in alcohol intake was fully compensated by an increase in water intake; conversely, food intake was not altered by miltirone treatment. The results of these two experiments closely replicate those previously collected with *S. miltiorrhiza* extracts, strengthening the hypothesis that miltirone is likely the active ingredient of *S. miltiorrhiza* responsible for the reducing effect of its extracts on alcohol intake.

In terms of the mechanism of miltirone action, the anxiolytic effect of miltirone [25] might play a role in the drug capacity to reduce alcohol intake in sP rats, as: (a) anxiety is an inherent trait likely predisposing sP rats to high alcohol drinking; (b) alcohol is likely consumed by sP rats to self-medicate anxiety [26]. Accordingly, it can be proposed that the anxiolytic effect of miltirone may have substituted for the anxiolytic effect of alcohol usually sought by sP rats, resulting in a less urgent need of alcohol and, in turn, in the observed reduction of alcohol intake.

Previous work in rats demonstrated that *S. miltiorrhiza* extracts produced significant decrements in blood alcohol levels when alcohol was given intragastrically but not intraperitoneally, suggesting that *S. miltiorrhiza* extracts exerted an inhibitory action on alcohol absorption from the gastrointestinal tract [21,22]. This study was subsequently extended to miltirone, in an attempt to evaluate whether also this effect of *S. miltiorrhiza* extracts was due to miltirone. To this aim, sP rats were initially treated with vehicle or 10 mg/kg miltirone (i.g.) and then with 1 g/kg alcohol, given intraperitoneally or intragastrically in two independent groups of rats. Blood samples were collected from the tip of the rat's tail at different time intervals after alcohol administration. Similarly to the results of the experiments testing the *S. miltiorrhiza* extracts, miltirone administration resulted in a pronounced reduction in blood alcohol levels when alcohol was given intragastrically, and lack of any effect when alcohol was administered intraperitoneally. At present, it is unknown whether, and eventually to which extent, the decreasing effect of *S. miltiorrhiza* extracts and miltirone on alcohol absorption may impact alcohol intake.

3. STRUCTURAL CHARACTERIZATION OF MAIN COMPOUNDS *S. miltiorrhiza*

In chemical studies over several decades, it has been found that there are a variety of diterpenoids, phenolics, flavonoids, triterpenoids and sterols in *Radix S. miltiorrhiza* [27].

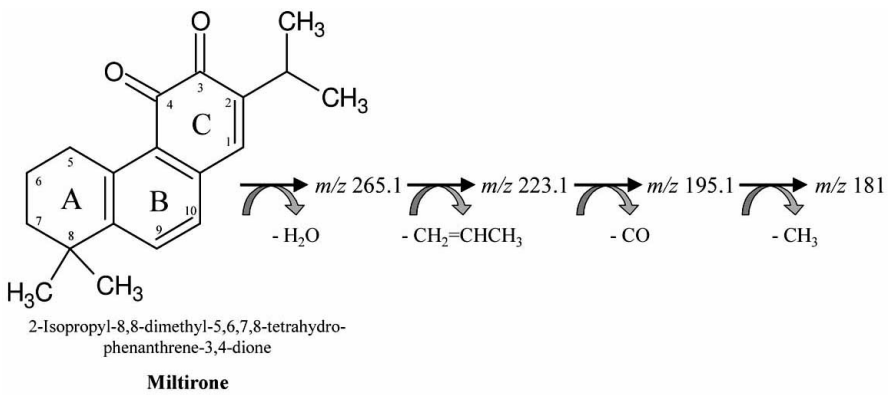

Fig. (1). Chemical structure of Miltirone with the proposed fragmentation pathway.

Up to now, bioactivity researches on this herb have showed that the two major interesting compounds are the phenolic acids and the diterpenes. Moreover, to evaluate the fingerprint of these constituents it was necessary to develop analytical methods that can simultaneously analyze both water-soluble and non-polar bioactive compounds.

In recent years, liquid chromatography hyphenated with tandem mass spectrometry (LC-MS/MS) has been successfully applied to evaluate the structures of the constituents of different herbal extracts and in literature it is possible to find many works on the multi-component fingerprint of *S. miltiorrhiza* [4,28-31].

As an example Yang *et al.* [4] studied the fragmentation behavior of tanshinones and applied HPLC/ESI–MS^n to their structural characterization. The authors of this work isolated 11 reference compounds with diterpenoid structure from Dan-shen extract and introduced them into the electrospray ionization source (ESI) by continuous infusion.

For each of these reference compounds the $[M+H]^+$ ions was selected as precursor ion to produce MS/MS spectra, in which the most abundant product ion was then selected for further MS^n analysis. On the basis of these mass spectra obtained it was possible dividing diterpenes constituents of *S. miltiorrhiza* into 6 groups according to their chemical structures and fragmentation patterns. In fact, tanshinones have either a furano-orthonaphthaquinone or a furano-para-naphtaquinone skeleton and the different position of π-conjugation extensions result in different fragmentation pathways. The rules deduced by this study were successfully implemented in the identification and structural characterization of 27 tanshinones, including miltirone. The fragmentation pathway of miltirone described by Yang *et al.* [4], was confirmed in our laboratory (Fig. **1**). In particular, the MS/MS spectrum (collision energy 40% and isolation width 1), obtained from the miltirone molecular ion ($[M+H]^+$ 283.1 *m/z*), gives a prominent ion product at 265.1 *m/z*, due to the loss of H_2O, and a less intense product ion at 223.1 *m/z*, due to the loss of propylene from 265.1 *m/z*. Then 223.1 *m/z* lost another CO group to m/z 195.1, that subsequently lost CH_3 to give *m/z* 181. A loss of propylene (283.1 to 241.1 *m/z*) was also observed in the MS/MS spectrum (Fig. **2**).

The MS^3 spectrum of the 265.1 *m/z* ion produces fragment 223.1 *m/z*, due to a loss of propylene group. In the MS^3 spectrum of 265.1 *m/z* it is possible to observe the presence of a less intense ion at 237.1 *m/z* due to the loss of CO group (Fig. **3**).

On the basis of our previous experience [32], about the possibility to obtain quantitative data using flow injection coupled to tandem mass spectrometry (FI/ESI-MS/MS), we have also developed an analytical method to assay miltirone (IDN 5477, supplied by Indena S.p.a, Milan, Italy). In particular, a volume of 1μL for each standard concentration of miltirone (10-100 ng/mL) was injected into the ion trap mass spectrometer (LCQ_{DECA}, Thermo Electron Corporation, San-Josè, CA, USA) and its molecular ion was isolated (width 1) and fragmented (collision energy 40%). In this way, it was possible to monitor the product ion 265.1 *m/z*, increasing the LOD sensitivity and to observe a linear relationship between the peak area (average of quadruplicate injection) and the injected amounts of miltirone, with a regression coefficient (r^2) around 0.95 (25, 50, 75, 100, 200 ng/mL). This preliminary study evidences that it is possible to analyze each sample (replication n=5) in short time (3-4 min) with a limit of detection (LOD) around 5 ng/mL, corresponding to 20 fmol injected (signal-to-noise S/N=5). In this way a rapid evaluation of miltirone is possible, of course, using LC-MS/MS method, both reproducibility and regression coefficient are better (see section 5).

4. ANALYTICAL METHODS FOR QUALITY CONTROL OF *S. miltiorrhiza* EXTRACTS

Most herbal medicines are composed of complex costituents and proper methods are required for quality control of them to ensure their stability, efficiency and safety. As described above, phenolic acids and diterpenes are the main bioactive compounds present in a *S. miltiorrhiza* extract.

The most published methods for analyzing these compounds are mainly on liquid chromatography as separation method and on UV or MS detectors as identification ones (see Table **1**). High-performance liquid chromatography coupled to diode array detection (HPLC-DAD) is used to achieve the fingerprinting analysis of the components from plant extracts [4]. For example in the study proposed by Ma *et al.* [33] is developed a method that, using HPLC combined to UV detection, permits the quality evaluation of Dan-shen through simultaneous determination of four phenolic acids and three diterpenes in only 60 min of analysis time. Li *et al.* [34] developed a rapid, sensitive and reproducible ultraper-

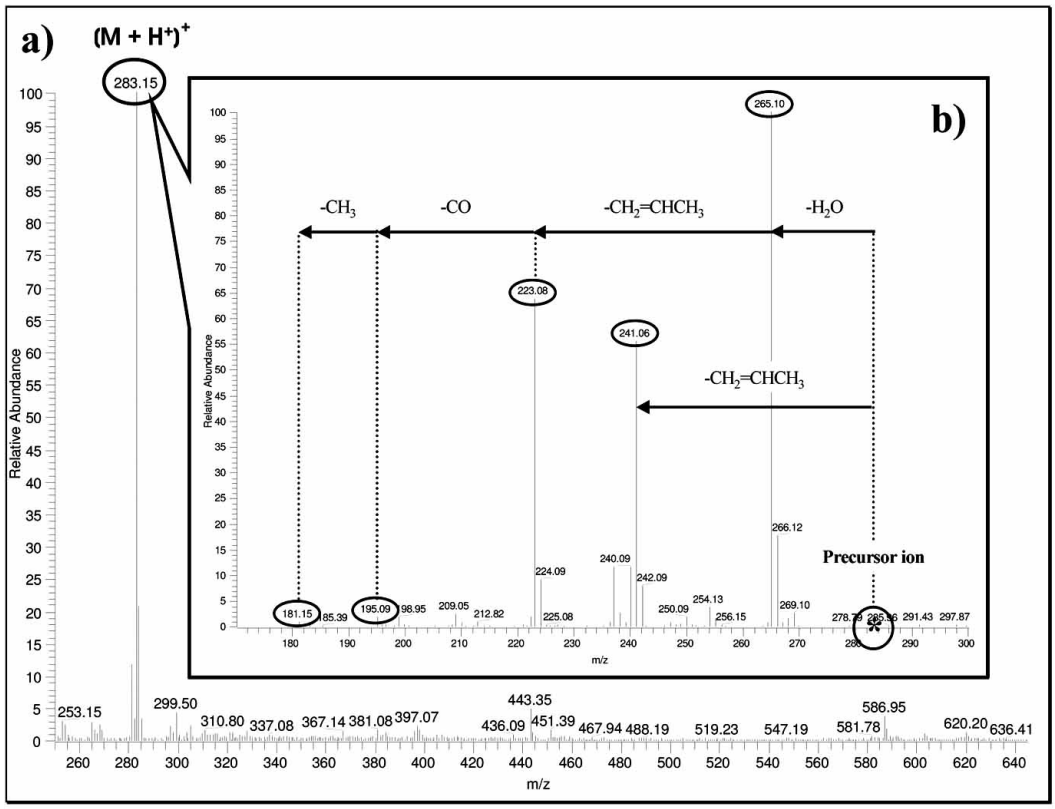

Fig. (2). a) ESI-MS and **b)** ESI-MS/MS spectra of $[M+H]^+$ at 283.1 *m/z*.

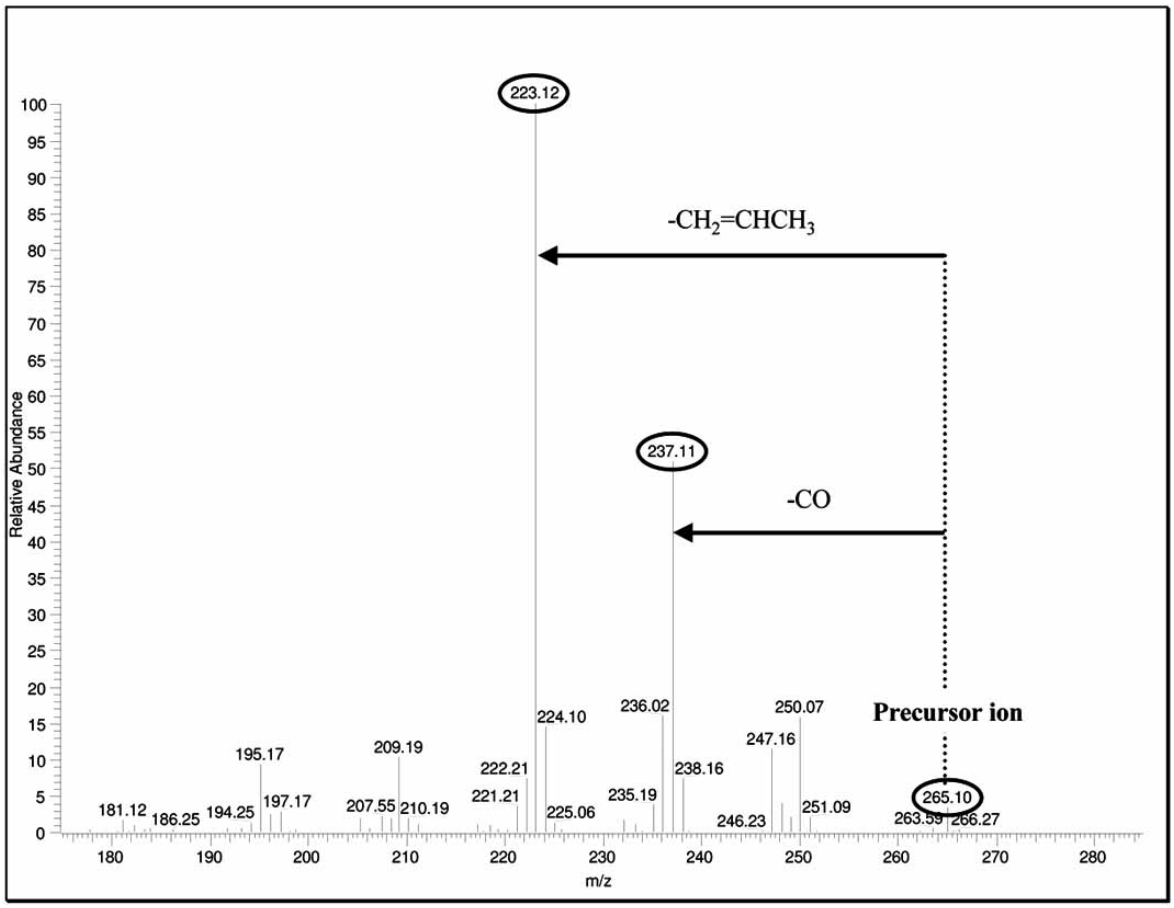

Fig. (3). MS^3 spectrum of *m/z* 265.1 (283.1 → 265.1).

Table 1. Summary of Main Methods Used for the Analysis of Phenolic Acids and Diterpenes in *Salvia miltiorrhiza* Extracts

Reference	Extraction Method	Separation System	Detector	Identified Compounds	LOD
[53]	Methanol : dichloromethane (4:1, v/v) for 60 min by sonication	HPLC- Alltech, Allitima C18column (2.1 mm x 150 mm, 5μm)	ESI positive	4 diterpenes	N.A.
[54]	Methanol : chloroform (7:3, v/v) for 30 min by sonication	HPLC- an Agilent Zorbax Extend reversed-phase C18column (250 x 4.6 mm, 5μm)	UV	4 diterpenes	0.02 - 0.05 μg/mL
[4]	Methanol : chloroform (7:3, v/v) for 60 min a 100°C	HPLC- an Agilent Zorbax Extend reversed-phase C18column (250 x 4.6 mm, 5μm)	DAD ESI positive	27 diterpenes	N.A.
[30]	65% Ethanol	HPLC- Inrtsil ODS3 column (250 x 4.6 mm, 5μm)	PDA ESI negative	28 phenolic acids	N.A.
[33]	Methanol for 60 min a 75°C	HPLC- Agilent Zorbax SB-C18column (250 x 4.6 mm, 5μm)	UV	4 phenolic acids 3 diterpenes	< 0.9 ng/mL 0.2 - 0.6 ng/mL
[31]	70% Methanol for 30 min by sonication	HPLC- Agilent Zorbax XDB-C18column (3 x 50 mm, 1.8 μm)	DAD ESI-TOF positive/negative ESI-IT positive/negative	22 phenolic acids 18 diterpenes	N.A.
[42]	70% Methanol for 1h in an ultrasonic bath	HPLC- Agilent Zorbax Extend reversed-phase C18column (250 x 4.6 mm, 5μm)	UV ESI/TOF positive	9 phenolic acids 6 diterpenes	0.02 - 0.09 μg/mL 0.02 - 0.06 μg/mL
[34]	Ethanol for 10 min a 100°C	UPLC- UPLC BEH C18 column (50 mm x 2.1 mm i.d., 1.7 μm)	PDA	10 diterpenes	0.02 - 0.21 μg/mL

N.A.= Not Available

formance liquid chromatography (UPLC) method for the identification of 10 diterpenoid compounds in S. *miltiorrhiza* for the first time, among which miltirone. In addition to chromatography (including thin-layer chromatography, TLC, gas chromatography, GC, high-performance chromatography, HPLC), high-performance capillary electrophoresis (HPCE) has recently been developed as an effective method for the quality control of traditional Chinese medicine (TCM). In the last years an application of capillary electrophoresis, non-aqueous capillary electrophoresis (NACE) [35], has been developed rapidly and has proven to be a promising approach for the separation of a large range of compounds. As an example, Gu *et al.* [35] compared NACE with high-speed counter chromatography (HSCCC) [36-38], in the development of fingerprint of *S. miltiorrhiza Bunge.* The authors of this work observed that both HSCCC and NACE, with their own advantages and disadvantages were necessary to investigate the complicated costituents traditional Chinese plants. Unfortunately, the assignment of peaks, corresponding to the active constituents and toxic ingredients is usually difficult due to the unavailability of reference standard. In fact, isolation and purification of some constituents from *S. miltiorrhiza* are not easy due to their instability under normal conditions or to their presence in trace amounts [4,28]. Therefore, the development of simple and rapid methods for the analysis of diterpenes and phenolic acids is of great significance for the quality control of Danshen. High-performance liquid chromatography/mass spectrometry (HPLC/MS) equipped with electrospray ionisation (ESI) ion source is one of the most powerful technique for the rapid identification of constituents of plant extracts [39,40]. Furthermore, the mass spectrometer is a sensitive and selective detector and allows detection of minor or even trace amounts of constituents from a microscale sample.

As reported by Hu *et al.* and Li *et al.* [28,41], the MS response of phenolic acids is more sensitive using negative ion mode while the detection of diterpenoid quinones is more sensitive in positive ion mode. Zeng *et al.* [30] report the identifications of phenolic compounds in *S. miltiorrhiza* extracts using an HPLC separation, based on an Ivertsil ODS3 analytical column (250 x 4.6 mm i.d., 5 μm; GL Science, Japan) coupled to UV photodiode-array detector and triple quadrupole MS. The use of the negative ion mode combined to the MS/MS analysis lead to the complete characterization

of 28 phenolic acids. In this case, the most abundant constituents resulted rosmarinic acid and lithospermic acid derivatives.

As another example Zhu *et al.* [31] used a 30 min HPLC separation, based on a reversed-phase C_{18} column, coupled to a mass spectrometer equipped with a TOF analyzer or to an ion trap analyzer for characterizing simultaneously 22 phenolic acids and 18 diterpenes constituents from *Radix S. miltiorrhiza*. Specifically, the authors used three different detectors to obtain three specific goals: UV detector to optimize chromatographic conditions, TOF-MS to obtain accurate molecular weights and ion trap MS to perform fragmentation of molecular ions and to confirm structural identifications. In this way, coupling electrospray ionization mass spectrometry (ESI-MS) with HPLC, it was possible to detect many target components in a complex mixture with an high sensitivity and a fast screening compatibility. The HPLC method coupled to UV and ESI-TOF/MS developed by Cao *et al.* [42] confirms the possibility to simultaneusly evaluate phenolic acids and diterpenes. In particular, this work describes the qualitative and quantitative determination of 9 major phenolic acids and 6 main diterpenoids in 21 samples of *S. miltiorrhiza*.

In our laboratory, we have used a modified HPLC method to perform stability studies of three tanshinones. Three purified tanshinones (Tanshinone IIA, Miltirone, Tanshinone I) were separately treated for 24 hours at reflux in 95% ethanol solution. Each sample was analyzed by HPLC-PDA/ESI-MS employing a Spectra System P4000 pump system controller equipped with a Spectra System UV 6000 LP DAD detector and a LCQ mass spectrometer (Thermo Electron Corporation, SanJosè, CA, USA). The chromatographic column was an Agilent Zorbax XDB C_8 (250 x 4.6 mm, 5 μm). The detection wavelength range was set from 215 to 600 nm, the flow rate was 1.0 ml/min and the column temperature maintained at 35°C. The mobile phases consisted of water (eluent A), methanol (eluent B) and tetrahydrofuran (eluent C). Gradient elution was as follow: initially 25% B and 10 % C at 0 min, linearly changing to 82% B and 3% C in 50 min, then to 90% of B and 10% of C in two min, maintaining 90% of B and 10% of C in the last four minutes of the run. After each analysis, the initial solvent conditions were maintained for 12 min to re-equilibrate the system for baseline stability.

Tanshinone IIA heated at reflux in 95% ethanol yields two main degradation peaks at Rrt 0.64 (Rrt: relative retention time normalized to standard, tanshinone IIA) and 0.91 (respect to tanshinone IIA). The peak (at Rrt 0.64) possessing the ions $[M+Na^+]$ at *m/z* 333 and $[2M+Na^+]$ at *m/z* 643 was identified as hidroxytanshinone which was previously described as an oxidation product of tanshinone IIA [43, 44]. The other degradation product (Rrt 0.91) having $[M+Na^+]$ and $[2M+Na^+]$ ions at *m/z* 361 and 699 respectively, and a fragment ion at *m/z* 293 $[M+H-C_2H_5OH]^+$ was tentatively identified as ethoxytanshinone.

About stability studies, miltirone (95% ethanol) resulted stable at 50°C for 24h; while at 60°C for 24h recovery was around 80%. Stability decreased rapidly when miltirone was kept in solid state at 80°C (recovery around 35%).

Miltirone heated at reflux in 95% ethanol showed the presence of three new peaks at Rrt 0.67, 0.91 and 1.01 (respect to miltirone). The MS spectrum of peak at Rrt 0.67 exhibited the $[M+Na^+]$ and $[2M+Na^+]$ ions at *m/z* 321 and 619 respectively, and a fragment ion at *m/z* 281 $[M+H-H_2O]$. This peak was tentatively identified as hydroxymiltirone (5-hidroxy-2-isopropyl-8, 8-dimethyl-5,6,7,8-tetrahydrophenanthrene-3,4-dione). The peak at Rrt 0.91 had the $[M+Na^+]$ and $[2M+Na^+]$ ions at *m/z* 349 and 675 respectively and a fragment ion at *m/z* 281 $[M+H-C_2H_5OH]^+$. This degradation product was tentatively identified as ethoxymiltirone. To further prove this hypothesis, Miltirone was heated at reflux for 24 h in 95% methanol. Two degradation products were observed at Rrt 0.67 and 0.81 (respect to miltirone). The peak at Rrt 0.67 was already identified as hydroxymiltirone. The HPLC-MS spectrum of the second peak exhibited the $[M+Na^+]$ and $[2M+Na^+]$ ions at *m/z* 335 and 647 respectively and a fragment ion at *m/z* 281 $[M+H-CH_3OH]^+$ due to the loss of methanol, confirming the influence of the solvent on the structure of the main degradation product. The compound eluting at Rrt 1.01 exhibited a different UV spectrum of both miltirone and the other degradation products with maxima at 277, 362 and 410 nm. Its MS spectrum had the $[M+H^+]$ ion at *m/z* 299. The UV and the MS spectra allowed to identify this degradation product as Arucadiol [45] already described in *Salvia argentea*.

When tanshinone I was heated at reflux in 95% ethanol no degradation products were observed.

From these data it is possible to conclude that in hydroalcoholic solutions, tanshinones possessing a 8,8-dimethyl-5,6,7,8-tetrahydro-phenanthrene-3,4-dione moiety, such as tanshinone IIA and miltirone, give rise to degradation products stemming from hydroxylation and alkoxylation in position 5. This is in agreement with the literature [44] which reports that the photoxidation of tanshinone IIA yields hydroxytanshinone and 6,7,8,9-tetrahydro-1,6,6-trimethylfuro [3,2-c]naphth[2,1-e]oxepine-10,12-dione. In addition, when the oxidation occurs in ethanol or methanol the corresponding ethers are the major degradation products.

5. PHARMACOKINETIC ANALYSIS

Concerning pharmacokinetic studies some works are reported in literature [46-50]. Specifically, Park *et al.* [46] developed a sensitive and reliable LC-MS/MS method for assaying the plasma levels of four tanshinones in rats using oral administration of an enriched extract of *S. miltiorrhiza* and a liquid-liquid extraction as sample clean-up method. LC-MS/MS methodology was also applied by Li *et al.* [47] to analyze plasma level of tanshinone IIA and cryptotanshinone after oral administration of total tanshinones with a dose of 150 mg/Kg. These methods don't determine the quantitative presence of miltirone in biological samples or tissue, even if this diterpene is described to be one of the main active compound of *S. miltiorrhiza* [51]. For this reason, we have developed an analytical method, based on the use of liquid chromatography coupled to tandem mass spectrometry, to evaluate plasma levels of miltirone after acute oral administration of an enriched miltirone extract with a dose of 20 mg/Kg (IDN 5477, supplied by Indena S.p.A, Milan, Italy). In particular, the plasma samples of treated rats (Colombo

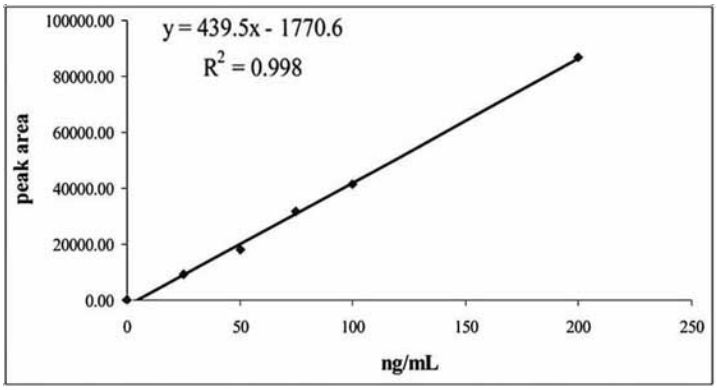

Fig. (4). Typical calibration curve obtained by injecting miltirone standard solutions.

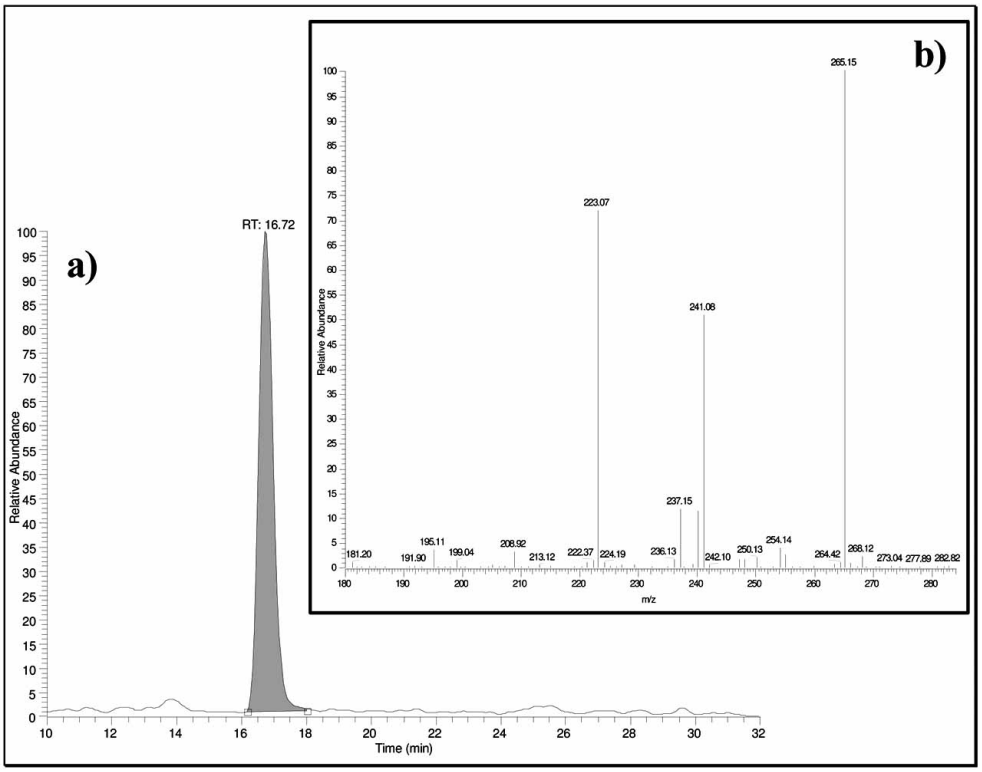

Fig. (5). a) Extracted ion chromatogram of 265.1 *m/z* in rat plasma sample collected at 60 min. after acute oral administration of miltirone enriched extract and **b)** its related MS/MS spectrum at 16.7 min.

et al. 2006) [24] were collected for a period of 12 hours (0, 30, 60, 120, 180, 360, 720 min) in heparinized syringes and centrifuged at 2000 *x g* for 10 min at 4°C. Each collected fraction (300µL) was extracted with the same volume of ethylacetate and, after centrifugation at 2000 *x g* for 2 min, the supernatant was evaporated to dryness under vacuum and the residue was dissolved in 150µL of 0.1% formic acid (Mauri *et al*. 2006) [52]. In this case, it was not possible to apply the FI/ESI-MS/MS approach (see section 3) because plasma samples are more complex than *S. miltiorrhiza* extracts and a chromatographic step is necessary to separate miltirone from plasma impurities. In particular, 10µL of plasma extracted with ethylacetate were injected, using a 1090 Hewlett Packard pump, in a Vydac MS C_{18} column (1 i.d. x 150mm, 5µm, 300 A°, Alltech Ass. Inc., Deerfield, USA) and eluted by an acetonitrile gradient (eluent A, 0.1% formic acid in water; eluent B, 0.1% formic acid in acetonitrile; 0-2 min 15% B, 2-7 min from 15 to 55% B, 7-25 min from 55 to 95% B). The flow rate was 70 µL/min. Detection of miltirone was performed by means of a LCQ_{Deca} ion trap mass spectrometer (Thermo Electron Corporation, San Josè, CA, USA), equipped with an ESI ion source. ESI parameters were optimized by flow injection of miltirone standard solution. LC-MS/MS analyses were carried out in the positive ion mode, with isolation (width 1) of miltirone molecular ion (*m/z* 283.1), its fragmentation (energy 40%) and monitoring of fragment ions 265.1, 223 and 241 *m/z*. The content of miltirone in plasma samples was obtained by external standardization from calibration curves prepared by injecting miltirone standard solutions (15 - 500 ng/mL) (Fig. **4**). In this range all plots were linear (r^2 = 0.996). In plasma samples, miltirone resulted stable at 4°C for 24h. Fig. (**5**) reports a typical chromatogram of rat plasma sample (col-

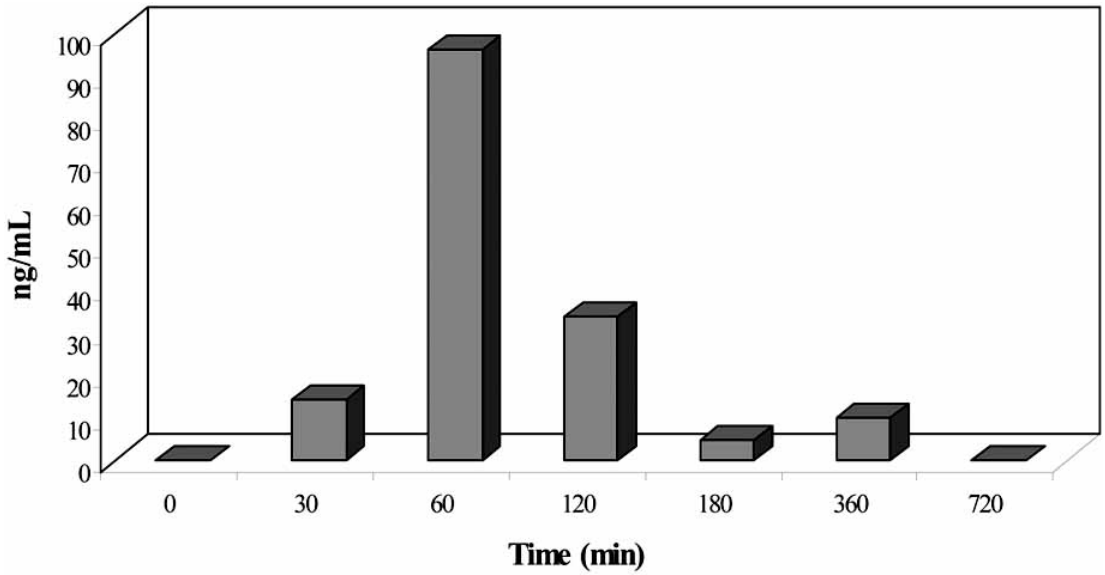

Fig. (6). Mean plasma concentration time curve in rats after acute oral administration of enriched miltirone extract of 20 mg/Kg.

lected at 60 min) after acute oral administration of miltirone enriched extract. Miltirone was identified both from the retention time and positive on line mass spectra (full and fragmentation spectra). The maximum plasma concentration of miltirone was observed to be around 90 ng/mL around 60 min (Fig. **6**) and AUC resulted around 16 mg*min/mL.

6. CONCLUSION

S. miltiorrhiza extract has used for the treatment of different diseases, but in the recent years its interest is increased in relation to the effects on Sardinia alcohol-preferring rats. In fact, different experiments evidenced the reduction of alcohol drinking behaviour due to the administration of *S. miltiorrhiza* extract. Specifically, miltirone seems to be responsible to decrease alcohol intake in sP rats due to its anxiolitic action.

In order to develop future investigations, concerning bioactive actions of *S. miltiorrhiza* compounds, it is of primary importance to get analytical methods for characterizing them in complex matrices, such as plasma. In the recent years different analytical methods have been proposed for characterizing both water soluble (such as phenolic acids) and lipophilic (such as terpenes) compounds present in *Salvia miltiorrhiza* extracts. Specifically, the methods reported in literature are mainly based on liquid chromatography coupled to tandem mass spectrometry by means of an electrospray ion source. However, these methods do not include miltirone assay. For this reason we have developed two analytical approaches to be able to identify and quantify miltirone in different matrices. In particular, flow injection electrospray mass spectrometry (FI-ESI-MS) method can be used for a rapid and simple analysis of miltirone in *S. miltiorrhiza* extract. It is suitable for automation and can be useful for routine quality control of pharmaceutical preparations. On the contrary, for pharmacokinetic studies concerning miltirone it is necessary to use LC-MS/MS approach to allow quantification of this bioactive compound in plasma samples, due to the presence of many impurities.

In conclusion, there are useful analytical methods for characterizing and assaying the different components of *S. miltiorrhiza* in pharmaceutical preparations and plasma samples. These methods can be used to develop future investigations concerning the major biological activities of the components of *S. miltiorrhiza*.

ABBREVIATIONS

EIC	=	Extracted ion chromatogram
ESI	=	Electrospray ionisation
FI	=	Flow injection
HPLC	=	High performance liquid chromatography
LOD	=	Limit of detection
MS	=	Mass spectrometry
MS/MS	=	Tandem mass spectrometry
PDA	=	Photodiode-array detector
TOF	=	Time of flight
UPLC	=	Ultra performance liquid chromatography

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