Journal of Applied Pharmaceutical Science

Available online at www.japsonline.com

Artemisia herba alba & *Artemisia monosperma:* The Discovery of the first potential Egyptian plant sources for the Pharmaceutical Commercial Production of Artemisinin and Some of Its Related Analogues

El Motaz Bellah El Maggar

El Motaz Bellah El Maggar
*Department of Pharmacognosy,
Faculty of Pharmacy, Damanhour
University, Damanhour City, Egypt.*

ABSTRACT

Artemisinin has received considerable attention in the last few decades as the current drug of choice for treatment of malaria and a number of other diseases. Because of the development of resistance against other malarial drugs, the demand for artemisinin has rapidly increased during the past decade. However, the supply of artemisinin is troublesome as neither total nor semi-synthesis is economically feasible and the only plant species known to produce artemisinin, *Artemisia annua* L., contains only low amounts of this compound ranging from (0.01-0.6%) of dry weight. The wish to improve the overall supply of artemisinin at a reduced market price has encouraged interest to hit upon novel plant sources for artemisinin production as alternative to *A. annua*. In our current study a fingerprint profile method was developed for the detection and quantification of artemisinin and its related analogues in the methanolic extract of *Artemisia monosperma* and *Artemisia herba alba* using a high-performance liquid chromatography (HPLC) with diode-array detector (DAD) and ion trap mass spectrometry. Artemisinin, dihydroartemisinin (α and β isomers), artemisitene, dihydroartemisinic aldehyde, dihydroartemisinic acid, dihydroartemisinic alcohol were detected and quantified in the methanolic extract of *Artemisia monosperma* using LC-MS peak at concentrations of 3.6, 1.9, 0.27, 0.06, 0.08 and 1.95 % of dry plant weight respectively in addition to the detection of arteannuin B and artemisinic acid at a trace levels. While artemisinin, artemisitene, dihydroartemisinic acid, and artemisinic acid were detected and quantified in the methanolic extract of *Artemisia* herba alba at concentrations of 4.9, 0.35, 0.08 and 0.04 % of dry plant weight respectively . The high unexpected concentration of artemisinin and some of its related analogues detected in this study reported *Artemisia herba alba* and *Artemisia monosperma* for the first time as a novel potential plant sources for artemisinin and some of its related analogues that may be helpful for its commercial pharmaceutical production and could lead to the improvement of the overall supply of artemisinin at a reduced market price offering an acceptable price for most patients.especially that these *Artemisia* species are abundant in distribution in Egyptian desert.

Keywords: Artemisinin, Artemisinin Intermediate precursors involved in its biosynthesis pathway; *Artemisia monosperma*; *Artemisia herba alba*, High performance liquid chromatography–Mass spectrometry; Quantification.

INTRODUCTION

Malaria is one of the planet's deadliest diseases and one of the leading causes of sickness in poor developing countries. The World Health Organization reports that there are 300–500 million clinical cases of malaria each year resulting in 1.5–2.7 million deaths, particularly of children below the age of 5 years in African Countries (far more than AIDS) (Avula *et al.,* 2009; Hartl, 2004*;* ElSohly, 2002).

For Correspondence
**Pharm. Dr. El Moataz Bellah El
Naggar**
E-mail :elnaggarpharm@yahoo.com

The first effective antimalarial drug was quinine, which was isolated from the barks of several South American species of *Cinchona*. For a long time this was the ideal drug for prophylaxis since it is not toxic, works fast and efficiently and has also a long half-life time. However, *Plasmodium* parasites have built resistance against this drug. Emergences of multi-drug resistance of the *Plasmodium* strains to the cheapest and most widely used antimalarials such as chloroquine, mefloquine and sulfadoxine-pyrimethamine is one of the biggest challenges in the fight against malaria (White, 1992). The interest in plants as potential sources of new antimalarial drugs has been stimulated by the isolation and clinical use of the endoperoxide sesquiterpene artemisinin, the active principle of the Chinese medicinal herb *Artemisia annua* (Phillipson and Wright, 1991).

Artemisinin, an endoperoxide-containing sesquiterpene lactone of the cadinane type, a first naturally occurring 1,2,4-trioxane: Fig.(1).with an endoperoxide moiety, a functionality rare in natural products is the antimalarial principle isolated by Chinese scientists in 1972 from *Artemisia annua* L., (sweet wormwood), an herb of the Asteraceae family (Compositae), is an effective antimalarial drug against both chloroquinine-resistant and chloroquinine- sensitive strains of *Plasmodium falciparum*, as well as against cerebral malaria (Liu *et al.,* 1979; Luo and Shen, 1987; Klayman, 1985). It has also been found to be a potential agent for treating skin diseases (Thornfeldt, 1991) and as a natural herbicide (Chen *et al.,* 1987; Duke *et al.,* 1987). This novel compound has a unique structure among antimalarial agents, lacking the nitrogen-containing heterocyclic ring which is found in most antimalarial compounds. The endoperoxide sesquiterpene lactone moiety of this class of compounds is found to be indispensable for the erythorocytic schizontocidal activity and reacts with the intraparasitic heme and form free radicals. These free radicals appear to damage intracellular targets and perform their antimalarial activity. However, artemisinin and its derivatives or analogues are currently regarded as the most promising and potent antimalarial drug weapons against malaria as part of the ideal strategy for malaria in Africa by WHO, which meets the dual challenge posed by drug-resistant parasites and rapid progression of malarial illness (Cheng *et al.,* 2002; Perez-Souto *et al.,* 1992; Robert *et al.,* 2002; Yikang, 2002). The supply of this promising antimalarial compound is troublesome as neither total nor semi-synthesis is economically feasible and the only plant species known to produce artemisinin is *Artemisia annua* L., which contains only very low amounts of this compound making the production of this promising antimalarial compound, remains expensive and is hardly available on a global scale (De Vries and Dien, 1996).

The significant biological activity, novel chemical structure, and low yield from natural sources have prompted efforts directed at the isolation of related compounds with similar activities (Klayman, 1985). Several research programs have been set up trying to identify artemisinin in other *Artemisia* species (Klayman *et al.,* 1984; Samuelsson, 1999). Many *Artemisia* species have been examined to find the sesquiterpene lactone, artemisinin,

or any of its related analogues. Which so far has been isolated only from *A. annua* growing in China in very minute amounts ranging from (0.01-0.6%) of dry weight (Klayman *et al.,* 1984; Perez-Souto *et al.,* 1992). Making the production of this promising antimalarial compound remains expensive and is hardly available on a global scale .Although its total synthesis has been achieved, the synthesis gives low yields and is uneconomical due to its complex structural properties. Therefore, the isolation of artemisinin from the plant still represents the best alternative (De Vries and Dien, 1996; Klayman, 1985; Samuelsson, 1999). However, according to the outcomes obtained from previous researches on ten *Artemisia* species (*A. santonicum* L., *A. taurica* Willd., *A. spicigera* K. Koch, *A. herba-alba* Asso, *A. haussknechtii* Boiss., *A. campestris* L., *A. araratica* Krasch., *A. armeniaca* Lam., *A. austriaca* Jacq. and *A. abrotanum* L.) collected from different localities throughout Turkey. However, these studies showed that artemisinin or any of its related analogues were not detected in all investigated *Artemisia* species of Turkish origin (Nurgün *et al.,* 2007).

The ability to detect artemisinin and its known analogues in plant extracts is as critical as the isolation of new compounds. This task is especially difficult when these compounds are unstable, sensitive to acid and base treatment (Lin *et al.,* 1985), thermally labile and lack UV or fluorescent chromophores (Fig. 1.) and are present at trace levels in a complex matrix making the standard UV detection methodology ineffective in the identification of these compounds. Rapid methods of detection at trace levels that are applicable to mixtures are still needed. Tandem mass spectrometry (ms/ms) (Busch *et al.,* 1988) is a powerful tool for rapid identification of target chemical constituents in complex matrices, including plant extracts (Ranasinghe *et al.,* 1993) and biological fluids (Richter *et al.,* 1983). The high sensitivity of MS as an LC detector facilitates to discover new minor constituents, which are difficult to obtain by conventional means. The tandem mass spectrometric fragmentation behavior of compounds has been investigated extensively, which allows the characterization of unknown compounds even without the reference standards (De Rijke *et al.,* 2003; Fabre *et al.,* 2001; Hughes *et al.,* 2001; Ma *et al.,* 2000; Stevens *et al.,* 1997).

In the present study, we successfully developed a very simple extraction method followed by HPLC–DAD–ESI-MSn to study the content of artemisinin and its related analogues in the methanol extracts of *Artemisia herba alba* and *Artemisia monosperma* for the first time. Identifications were performed by comparing mass spectra of samples with earlier publications. To our knowledge, we report the first MS/MS detection of artemisinin and its related analogues in the selected Egyptian *Artemisia* species.

MATERIALS AND METHODS
Plant material

Artemisia herba alba and *Artemisia monosperma* aerial parts were collected from western desert of Egypt, in spring of 2010 and authenticated by the author according to its

morphological and taxonomically aspects. Voucher specimens were deposited in the Herbarium of Faculty of Pharmacy, Damanhour University, Damanhour City, Egypt.

Chemicals

All solvents and reagents from various suppliers were of the highest purity needed for each application. Methanol and water were of HPLC grade (J.T. Baker, Phillipsburg, NJ, USA). Absolute methanol and formic acid were purchased from Merck (Darmstadt, Germany).

Preparation of Extracts

An aliquot of 15 gram of grinded air dried aerial parts of *Artemisia herba alba* and *Artemisia monosperma* aerial parts were separately exhaustively extracted with 750 ml of methanol in a Soxhlet apparatus at 60 °C then filtered through 0.45 µm membrane prior to use. The collected methanolic extracts were evaporated via rotavapour at 40-50 °C under reduced pressure yielding 3.1 and 2.98 gm respectively. The residue of each extract was dissolved in HPLC grade methanol in a concentration of 10mg/ml. Aliquots of 10µl of each were injected into the LC-DAD/MS analysis system

HPLC conditions

Analyses were performed using a Hewlett-Packard 1100 (Waldbronn, Germany) composed of a quaternary pump with an on line degasser, a thermostatted column compartment, a photodiode array detector (DAD), an auto sampler, and 1100 ChemStation software. The HPLC separation was performed on Eclipse XDB C18 column (50 mm×2.1 mm, 1.8 µm, Agilent Company, USA). Mobile phase consisted of two solvents, (A) methanol and (B) 0.2 % formic acid. Separation of compounds was carried out with gradient elution profile: 0 min, A: B 10:90; 36 min, A: B 100:0; 40 min, A: B 100:0. Chromatography was performed at 30 °C with a flow-rate of 0.2 ml/min. UV traces were measured at 290, 254 and 350 nm and UV spectra (DAD) were recorded between 190 and 900 nm.

Mass spectrometric conditions

The HPLC–MS system consisted of electrospray ionization (ESI) interfaced Bruker Daltonik Esquire-LC ion trap mass spectrometer (Bremen, Germany) and an Agilent HP1100 HPLC system equipped with an autosampler and a UV-Vis absorbance detector. The ionization parameters were as follows: positive ion mode; capillary voltage 4000 V, end plate voltage −500 V; nebulizing gas of nitrogen at 35.0 p.s.i.; drying gas of 10 l/min nitrogen at 350 °C. Mass analyzer scanned from 15 to 1000 u. The MS–MS spectra were recorded in auto-MS–MS mode. The fragmentation amplitude was set to 1.0 V. MS2 data were acquired in positive mode.

Standard-Free Relative Quantification by Peak area of LC-MS

In classical chromatography, reference standards are not only used for correct amount determinations, but they are also essential for compound identification. Thus, an analytical solution free from reference standards must also have structural information to identify the analytes of interest. Here, high-resolving MS is a valuable tool for providing elemental composition by exact mass determination, and for structure elucidation by fragmentation (Dionex Corporation, 2011).

The structural information provided by a high-resolution mass spectrometer for compounds , offers the highly attractive opportunity of an easy quantitation without a large number of reference compounds based on the relative peak areas that delivers both qualitative and quantitative results within one run and without the need for extensive external calibration becomes feasible. However, obtaining calibration curves for each single component is time consuming, expensive, or simply not possible because of the lack of most standards (Lisa *et al.,* 2007; Lisa and Holcapek, 2008).

In LC-MS, an ion with a particular m/z is detected and recorded with a particular peak area and intensity, at a particular time. It has been observed that signal intensity and peak area from electrospray ionization (ESI) correlates with ion concentration (Voyksner, 1999). The label free quantification of compounds via peak area in LC-MS was first studied by LC/MS/MS (Chelius and Bondarenko, 2002). When the chromatographic peak areas of the identified compound were extracted and calculated, the peak areas were found to increase with increased concentration of injected compounds. The results of quantitative profiling were further improved by normalizing the calculated peak areas (Chelius and Bondarenko, 2002; Bondarenko *et al.,* 2002).

RESULTS AND DISCUSSION

Selection of optimal chromatographic conditions

Optimal chromatographic conditions were obtained after running different mobile phases with different reversed-phase C18 columns. Indeed, the crude methanolic extract of the studied Compositae plant was found to contain a wide range of compounds ranging from water-soluble to lipid-soluble components. It is well established that for the separation of different compounds, choice of column, mobile phase and an appropriate gradient elution sequence are critical. The use of methanol-water (0.2 % formic acid) as mobile phase with a C18 column was found to be the most effective procedure for analysis showing a satisfactory separation for compounds of interest (Justesen *et al.,* 1998; Justesen., 2000).

In our study the best results were observed with Eclipse XDB C18 column (50 mm×2.1 mm, 1.8 µm, Agilent Company, USA) using methanol and water containing 0.2 % formic acid as mobile phase. Variation of the column temperature between 25 and 40 _C did not cause significant change in the resolution, however changes in retention time were observed. The column was used at 30 _C at a flow rate of 0.2 mL min-1 for LC–DAD–ESI method. This method allowed for the separation of all compounds in 40 min (Fig.2). Separation of compounds was carried out with gradient elution profile. All the peaks of interest were further confirmed by LC–MS.

HPLC-DAD and HPLC-MS analysis of the extracts

The developed analytical system made it possible to separate artemisinin as well as its related analogues clearly. However the ability to detect artemisinin and its known analogues in plant extracts using UV detection methodology at 215, 254, 290 and 334 nm was ineffective because these compounds lack UV or fluorescent chromophores showing poor extinction coefficients in the ultraviolet (UV) regions in comparison with the registered base peak chromatogram (BPC) profile that allows the identification of artemisinin and its known analogues. Artemisinin and its related compounds are known to produce significant fragments even under soft ionization conditions. ESI was chosen for the study of artemisinin because it has proven effective in generating molecular ions of a variety of compounds without causing much fragmentation (D'Acquarica *et al.,* 2010). Because it is a rapid heating technique, thermal degradation is also minimized.

Chemical structures of the identified compounds are shown in **(Fig. 1).**

A typical HPLC/DAD and MS chromatograms of the methanolic extract of *Artemisia monosperma* is shown in **(Fig. 2),** and their Extracted ion chromatogram, mass spectra, and MSn fragmentations pattern were shown in Table 1.

A typical HPLC/DAD and MS chromatograms of the methanolic extract of *Artemisia herba alba* is shown in **(Fig. 3),** and their Extracted ion chromatogram, mass spectra, and MSn fragmentations pattern were shown in **Table 2.**
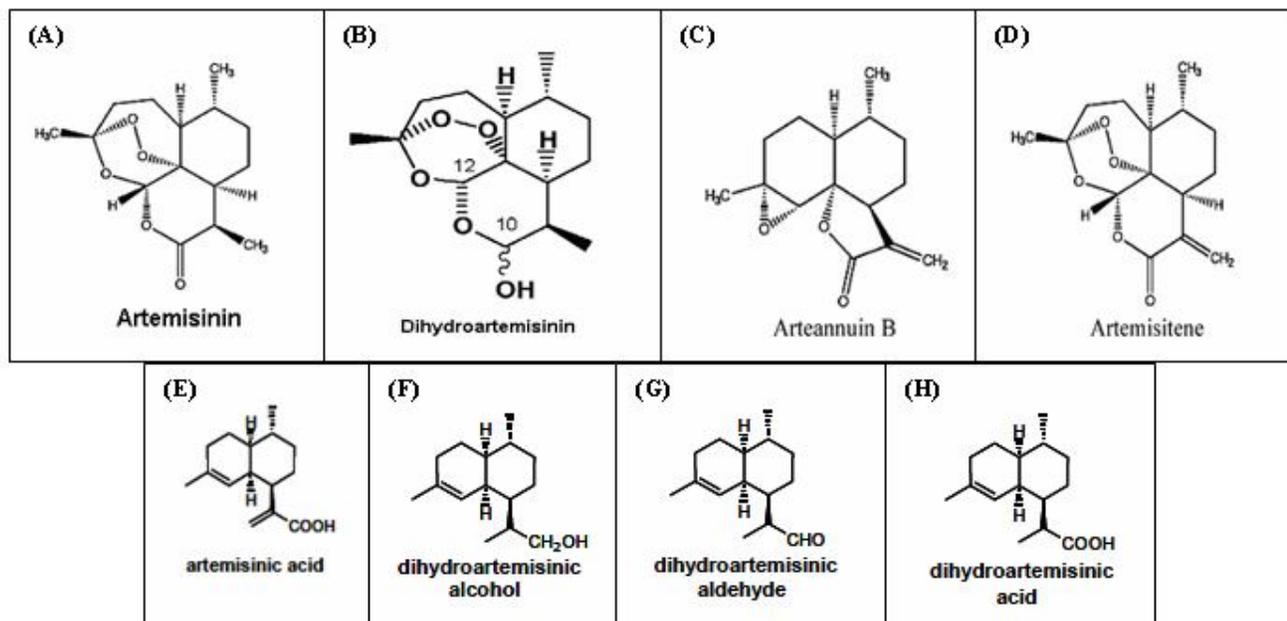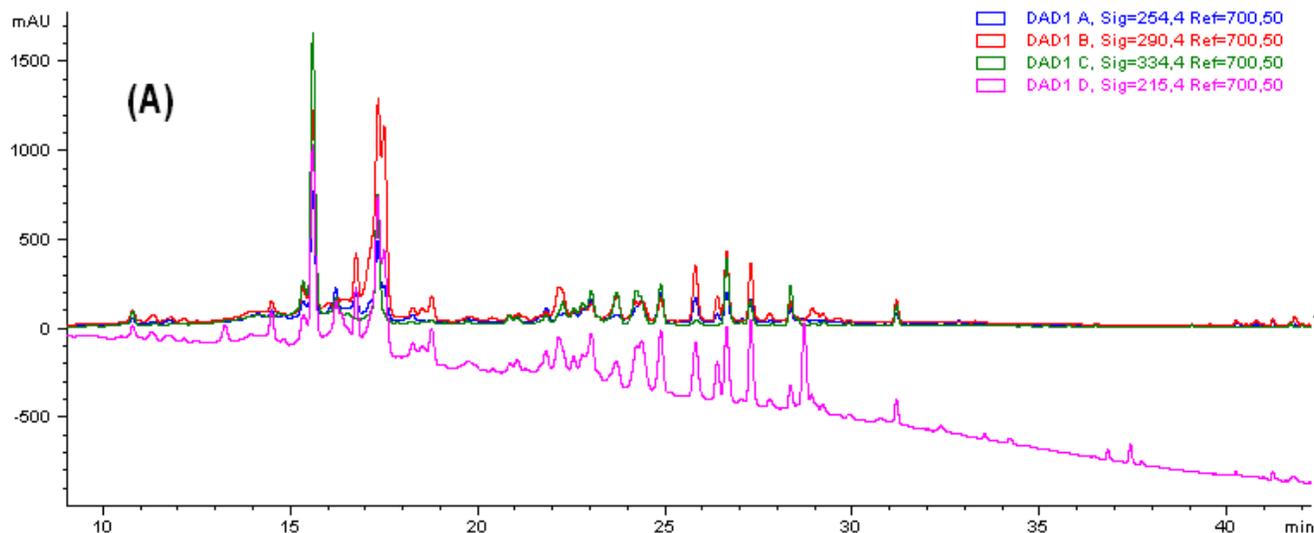

Fig. 1: Chemical structures of artemisinin (A), dihydroartemisinin (B), arteannuin B (C), artemisitene (D), and four possible intermediate precursors involved in artemisinin biosynthesis pathway : artemisinic acid (E), dihydroartemisinic alcohol (F), dihydroartemisinic aldehyde (G) and dihydroartemisinic acid (H).
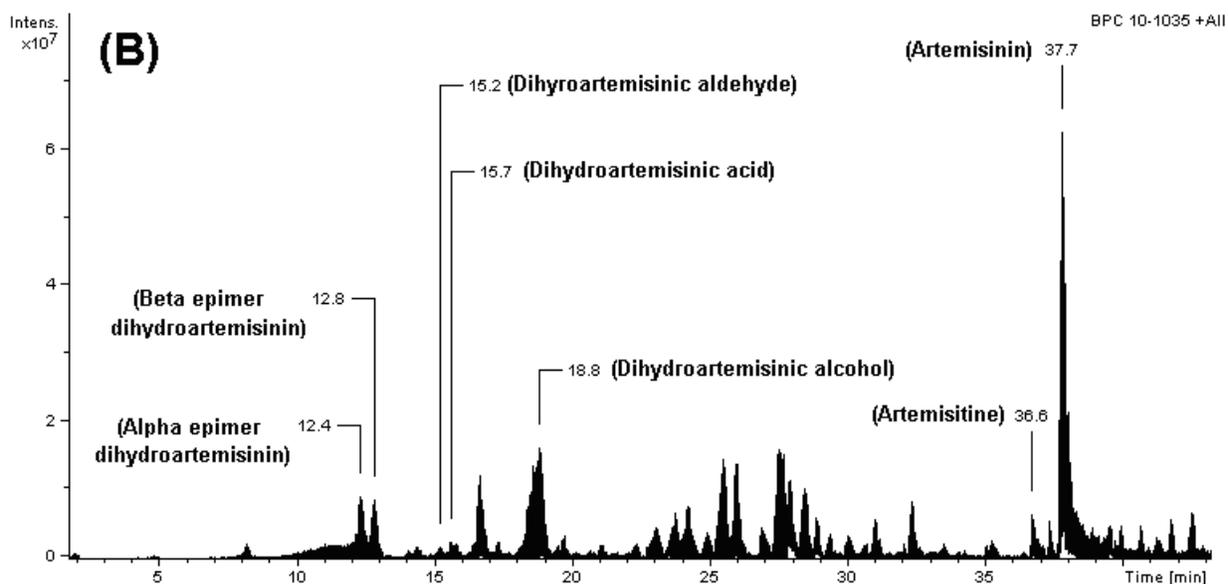
Fig. 2. (A): HPLC-DAD chromatogram of the crude methanolic extract of *Artemisia monosperma* monitored at different wave lengths (215, 254, 290, and 334 nm) showing a poor extinction coefficients in the ultraviolet (UV) regions of artemisinin as well as most of its first generation analogs. (B): LC-positive mode ESI-MSn base peak chromatogram (BPC) profile allows the identification and confirmation of all peaks of interest.
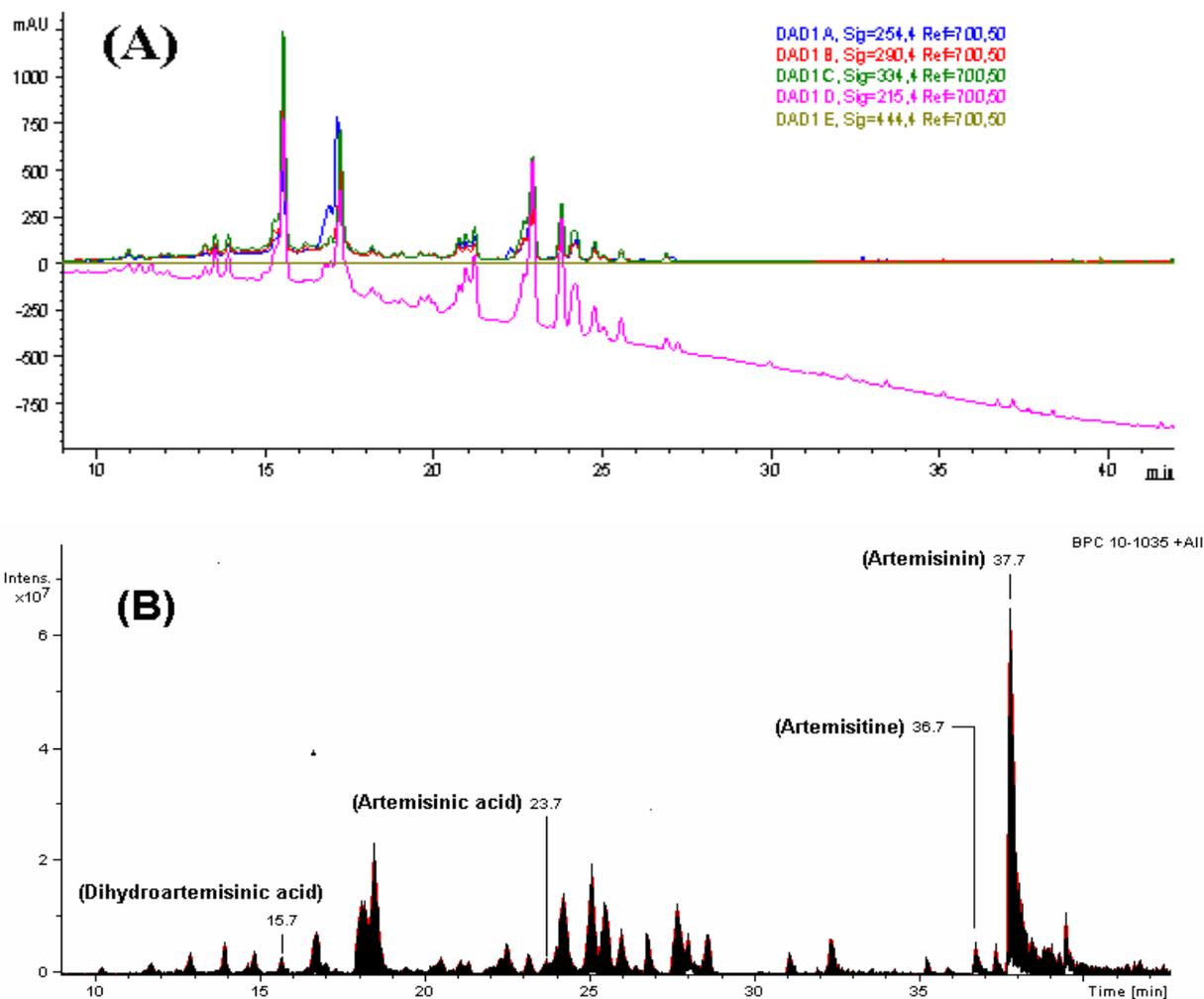

Fig. 3. (A): HPLC-DAD chromatogram of the crude methanolic extract of *Artemisia herba alba* monitored at different wave lengths (215, 254, 290, and 334 nm) showing a poor extinction coefficients in the ultraviolet (UV) regions of artemisinin as well as most of its first generation analogs. (B): LC-positive mode ESI-MSn base peak chromatogram (BPC) profile allows the identification and confirmation of all peaks of interest.

When plant sample of *Artemisia monosperma* (10μl) were analyzed by HPLC–MS, dihydroartemisinin epimers (α-DHA eluted at 12.4 min and β-DHA eluted at 12.8 min), dihydroartemisinic aldehyde eluted at 15.2 min, arteannuin B eluted at 15.5 min (not shown; trace amount), dihydroartemisinic acid eluted at 15.7 min, dihydroartemisinic alcohol eluted at 18.8 min, artemisitene eluted at 36.6 min and artemisinin eluted at 37.7 min, (Fig. 2). Their MS/MS product spectra and their molecular weights, obtained by mass selecting corresponding parent ions generated from the plant extract are shown in TABL.1.

When (10μl) of plant sample of *Artemisia herba alba monosperma* (10 mg/ml) were analyzed by HPLC–MS, arteannuin B eluted at 15.5 min (not shown; trace amount), dihydroartemisinic acid eluted at 15.7 min, artemisinic acid eluted at 23.7 min, artemisitene eluted at 36.6 min and artemisinin eluted at 37.7 min, (Fig. 3). Their MS/MS product spectra and their molecular weights, obtained by mass selecting corresponding parent ions generated from the plant extract are shown in TABL.2.

Identification of compounds

Both electrospray (ESI) and atmospheric pressure chemical ionisation (APCI) have been used previously for identification and quantification of artemisinin and its derivatives in both plant extracts and biological fluids (Gu *et al.,* 2008; Hanpithakpong *et al.,* 2008; Naik *et al.,* 2005; Rajanikanth *et al.,* 2003; Souppart *et al.,* 2002; Xing *et al.,* 2006; Xing *et al.,* 2007). Previous investigations revealed that (ESI) was superior to APCI mainly because of improved linearity (Hanpithakpong *et al.,* 2008). MS/MS analysis performed using electrospray ionization technique in positive mode was used for the detection of artemisinin (*m/z* 283→219 + 229 + 247 + 265), arteannuin B (*m/z* 249→185 + 189 + 203 + 231), artemisitene (*m/z* 281→217 + 227 + 245 + 263), dihydroartemisinin (*m/z* 284→267) and artemisinic acid (*m/z* 234→217 + 201 + 199 + 189). The fingerprint profile and mass spectra of the analyzed compounds were consistent with published data (Avula *et al.,* 2009; Ranasinghe *et al.,* 1993; Van Nieuwerburgh *et al.,* 2006).

In addition to other Possible Intermediate Precursors Involved In Artemisinin Biosynthesis Pathway, including dihydroartemisinic acid (*m/z* 235→219 + 201 + 175 + 161 +162 + 147 +133 +121 +107), dihydroartemisinic aldehyde (*m/z* 220→218 + 205 + 187 +175 + 164 + 163 + 162 + 147 + 135 + 121 + 91), dihydroartemisinic alcohol (*m/z* 222→204 + 191 + 161 + 149 + 133 + 119 + 107 + 91). Their Peak identification was performed by comparing their molecular weights, fingerprint profiles and MS/MS product spectra obtained by mass selecting corresponding parent ions generated from the plant extract with published data (Li *et al.*, 2006; Brown, 2010; Avula *et al.,* 2009; Teoh *et al.,* 2006).

Identification of Dihydroartemisinin (present only in *Artemisia monosperma*)

The mass spectrum of the peaks eluted at 12.4 min and 12.8 min corresponds to the dihydroartemisinin epimers (α-DHA and β-DHA) respectively are shown in Fig. 4. According to previous studies (Cabri *et al.,* 2008; D'Acquarica *et al.,* 2010) on the equilibrium between α-DHA and β-DHA showed that interconversion of the two epimers occurred in a chromatographic time scale which is known as the on- column epimerization process of DHA during the analysis. The conversion of the lactone carbonyl group at C-10 of artemisinin into the hydroxyl (hemiacetal) group in DHA yields a newsterically labile centre in the molecule, which, in turn, provides two lactol hemiacetal epimers, namely, α and β (Fig. 4.).
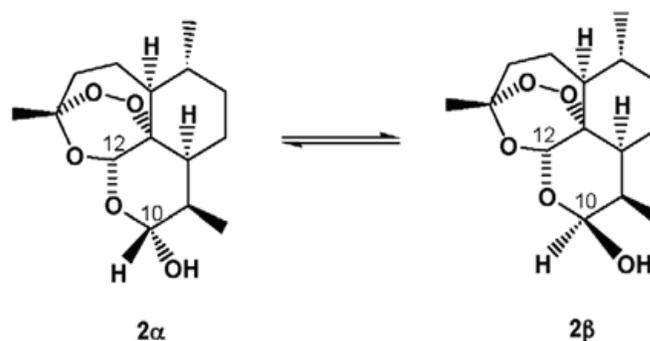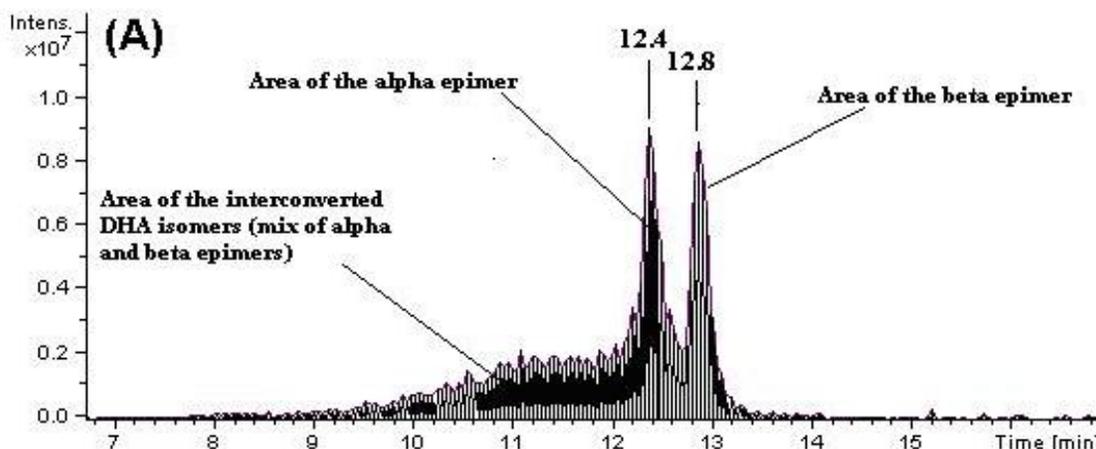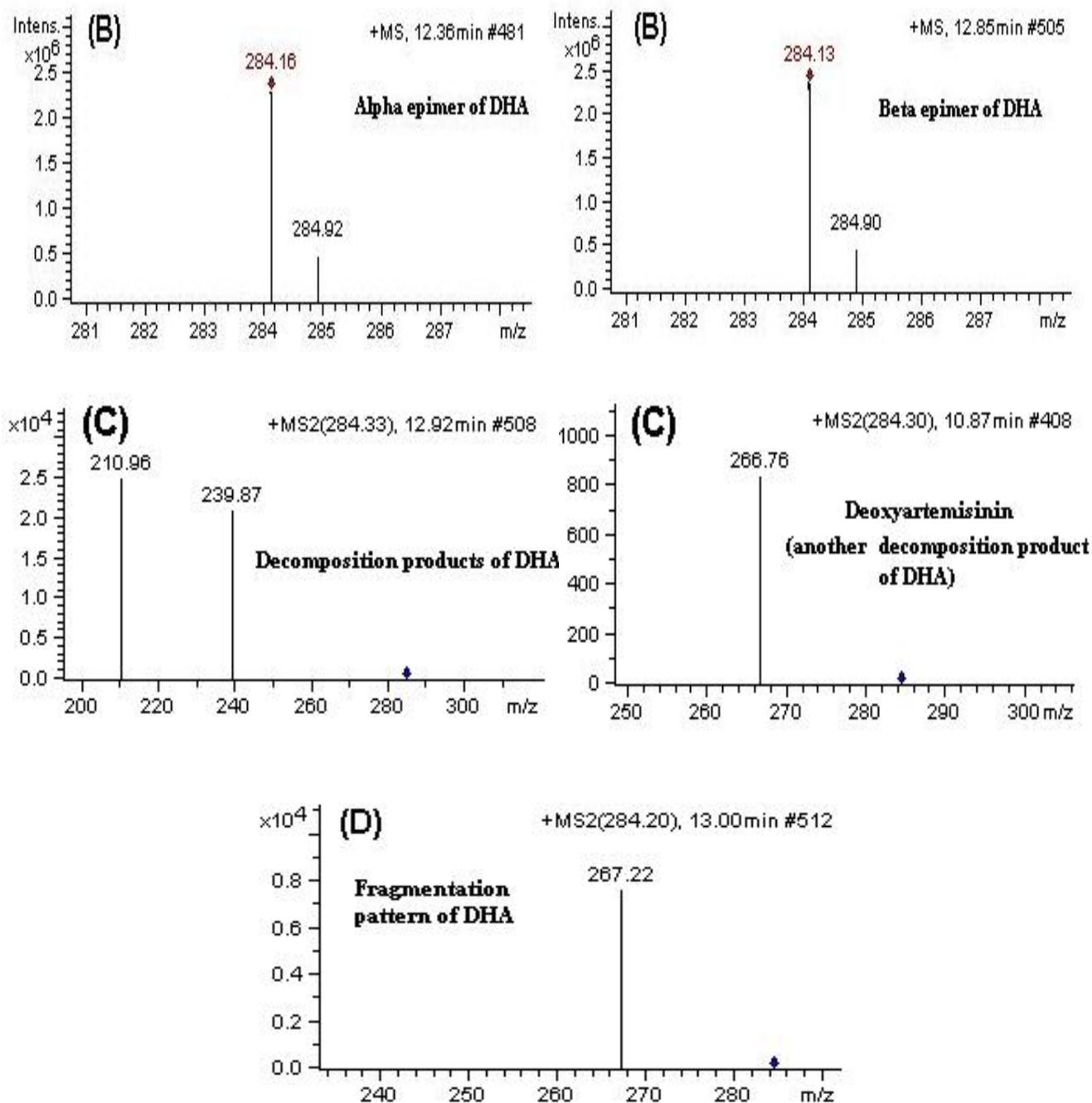

Fig. 4: Chemical structures of the interconverting epimers of dihydroartemisinin:

The α-epimer bears the hydroxyl group in the equatorial position (absolute stereo-chemistry at C-10: R), whereas the β-epimer possesses an axial hydroxyl group (Cabri *et al.,* 2008).

Fig.5. (A): Extracted ion chromatogram showing the interconverting epimers (α and β epimers) of dihydroartemisinin that occurs in a chromatographic time scale (on-column epimerization). (B) mass spectra, (C) decomposition product and (D) fragmentation pattern of DHA.

Samples of dihydroartemisinin were found to contain a decomposition product with a molecular weight of 238, as evidenced by MS analysis. This decomposition product was isolated and identified as 2-(3-oxobutyl)-3-methyl-6-(2-propanal)-cyclohexanon (Fig.5.C.), known to be formed by thermolysis of dihydroartemisinin at 190 degrees C.

However, during work-up of this compound a hitherto unknown decomposition product of dihydroartemisinin with a molecular weight of 210 was obtained and identified by 1H, 13C and two-dimensional NMR spectroscopy as 2-(3-oxobutyl)-3-methyl-6-ethyl-cyclohexanon. Most probably this product was formed by oxidation of the aldehyde functionality of the former decomposition product and subsequent decarboxylation. (Dhooghe et al., 2007). The fingerprint profile and mass spectra of the analyzed compound was consistent with published data (Mohamed et al., 1999; Rajanikanth et al., 2003).
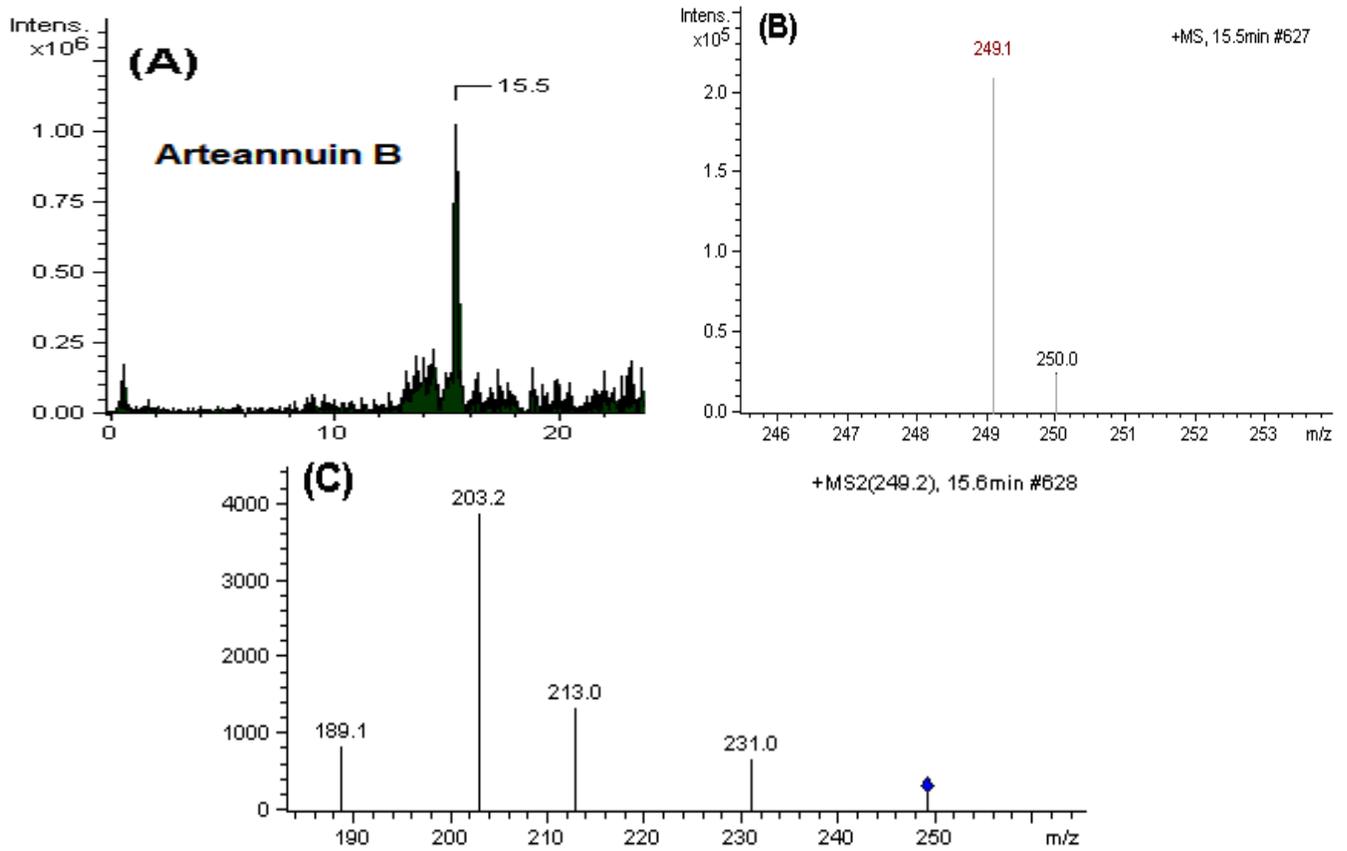
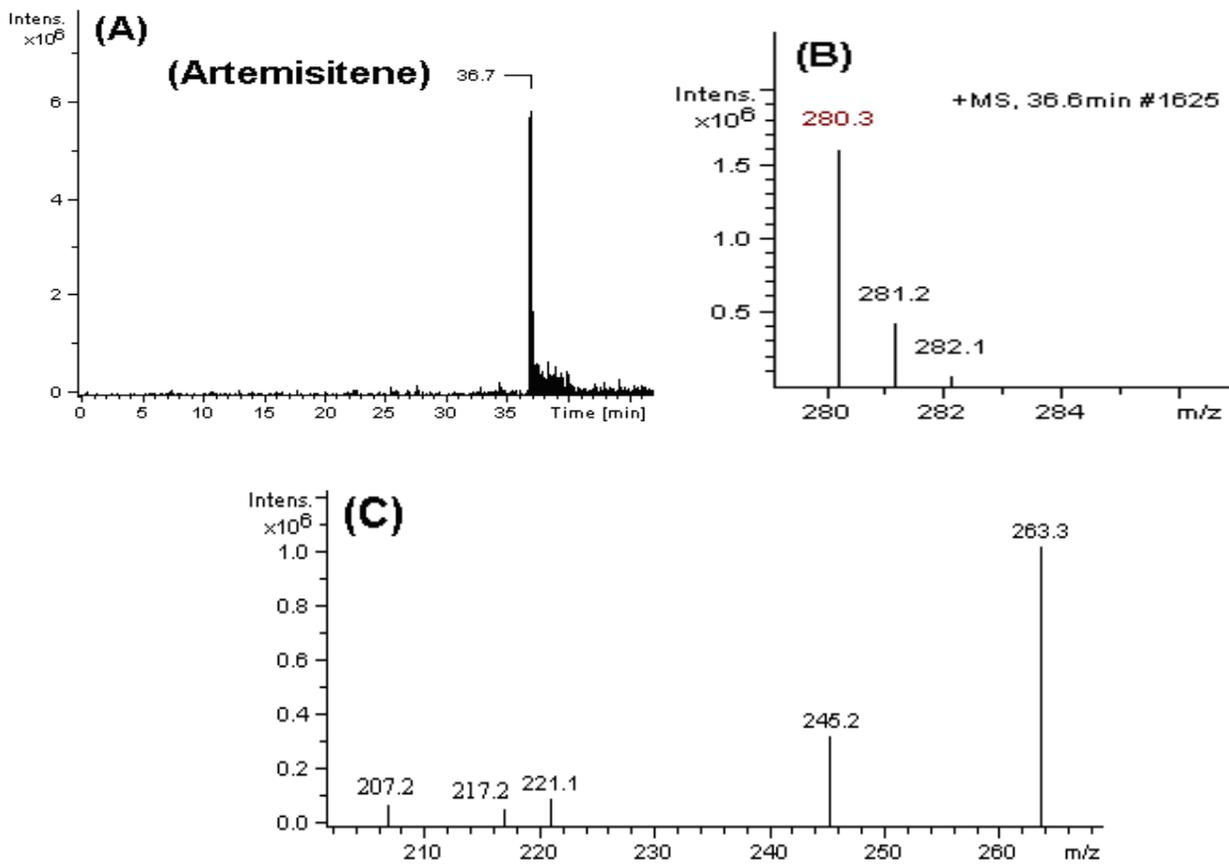Fig. 6. (A): Extracted ion chromatogram, (B) mass spectra, and (C) fragmentation pattern of arteannuin B.

Fig. 7. (A): Extracted ion chromatogram, (B) mass spectra, and (C) fragmentation pattern of artemisitene.

Identification of Arteannuin B (present in *Artemisia monosperma*)

The mass spectrum of arteannuin B eluted at 15.5 min is shown in Fig. 6. The largest ion (249) is consistent with [M+H] of arteannuin B showing the following fragments (*m/z*→**249 231+203+189+185**). The structure of that compound is shown in Fig. 1. This compound is likely not present at a significant level. The fingerprint profile and mass spectra of the analyzed compound was consistent with published data (Avula *et al.,* 2009; Ranasinghe *et al.,* 1993; Van Nieuwerburgh *et al.,* 2006).

Identification of Artemisitene (present in both *Artemisia monosperma* and *Artemisia herba alba*)

The mass spectrum of the peck eluted at 36.6 min corresponds to artemisitene is shown in Fig. 7. The largest ion (281) is consistent with [M+H] of artemisitene showing the following fragments:

(*m/z*→ **281   263+245+227+217**). The structure of that compound is shown in Fig. 1. This compound has UV absorbance showing a poor extinction coefficient comparable to artemisinin. The fingerprint profile and mass spectra of the analyzed compound was consistent with published data (Avula *et al.,* 2009; Ranasinghe *et al.,* 1993; Van Nieuwerburgh *et al.,* 2006).

Identification of Artemisinin (present in both *Artemisia monosperma* and *Artemisia herba alba*)

However, artemisinin is not observed in HPLC-DAD chromatograms Fig 2 (A) showing poor extinction coefficients in the ultraviolet (UV) regions, it is well registered in the base peak chromatogram (BPC) profile shown in Fig 2 (B) that allows the identification of artemisinin as well as some of its known analogues. Its mass spectrum and fragmentation pattern is shown in Fig. 8.

The peak eluted at 37.7 min using LC-positive mode ESI-MSn base peak chromatogram at m/z 283 corresponds to protonated artemisinin itself showing the following fragments:

(*m/z* →**283 265+247+229+223+219**). The fragments ions at mlz 265, 247, and 209 correspond to loss of H_2O [M-18+H+], loss of H_2O and loss of H_2O and two CO, respectively. The most characteristic fragment occurs with the loss of a neutral molecule(s) of mass 60 producing the ion, *mlz* 223. The 223.14 ion gives a $C_{13}H_{19}O_3$ molecular formula which would arise from a loss of $C_2H_4O_2$ from artemisinin under both (ESI) and (APCI) conditions. The fingerprint profile and mass spectra of the analyzed compound was consistent with published data (Avula *et al.,* 2009; Ranasinghe *et al.,* 1993; Stevens *et al.,* 1997 Van Nieuwerburgh *et al.,* 2006).

Identification of Artemisinic acid (present in *Artemisia herba alba*)

The mass spectrum of the peck eluted at 23.7 min corresponds to artemisinic acid is shown in Fig. 9. The largest ion (236) is consistent with [M+H] of artemisinic acid showing the following fragments:

(*m/z*235 →   **217+199+189+177+157+145+133+123+105+91**). The structure of that compound is shown in Fig. 1. The fingerprint profile and mass spectra of the analyzed compound was consistent with published data (Brown, 2010; Bertea *et al.,* 2005).
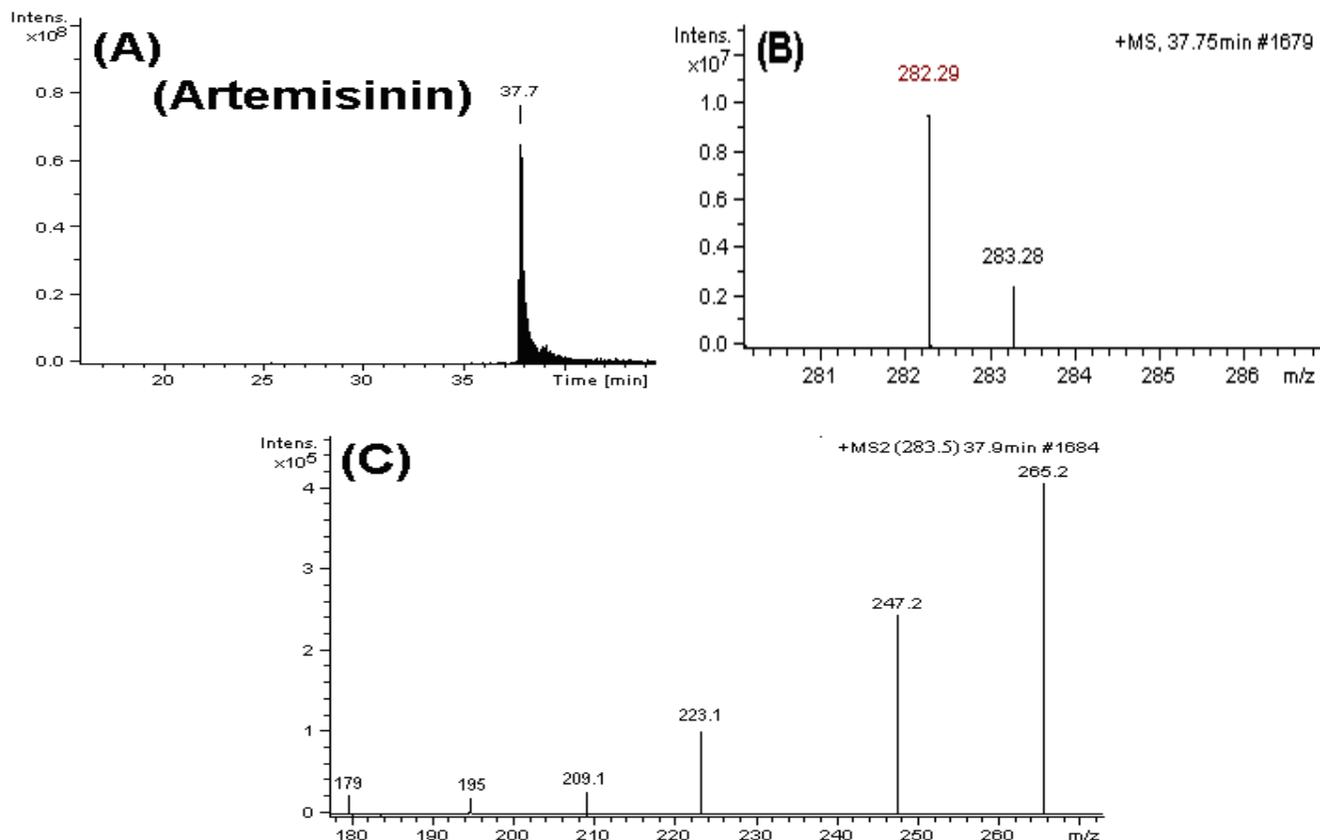

Fig. 8. (A): Extracted ion chromatogram, (B) mass spectra, and (C) fragmentation pattern of artemisinin .
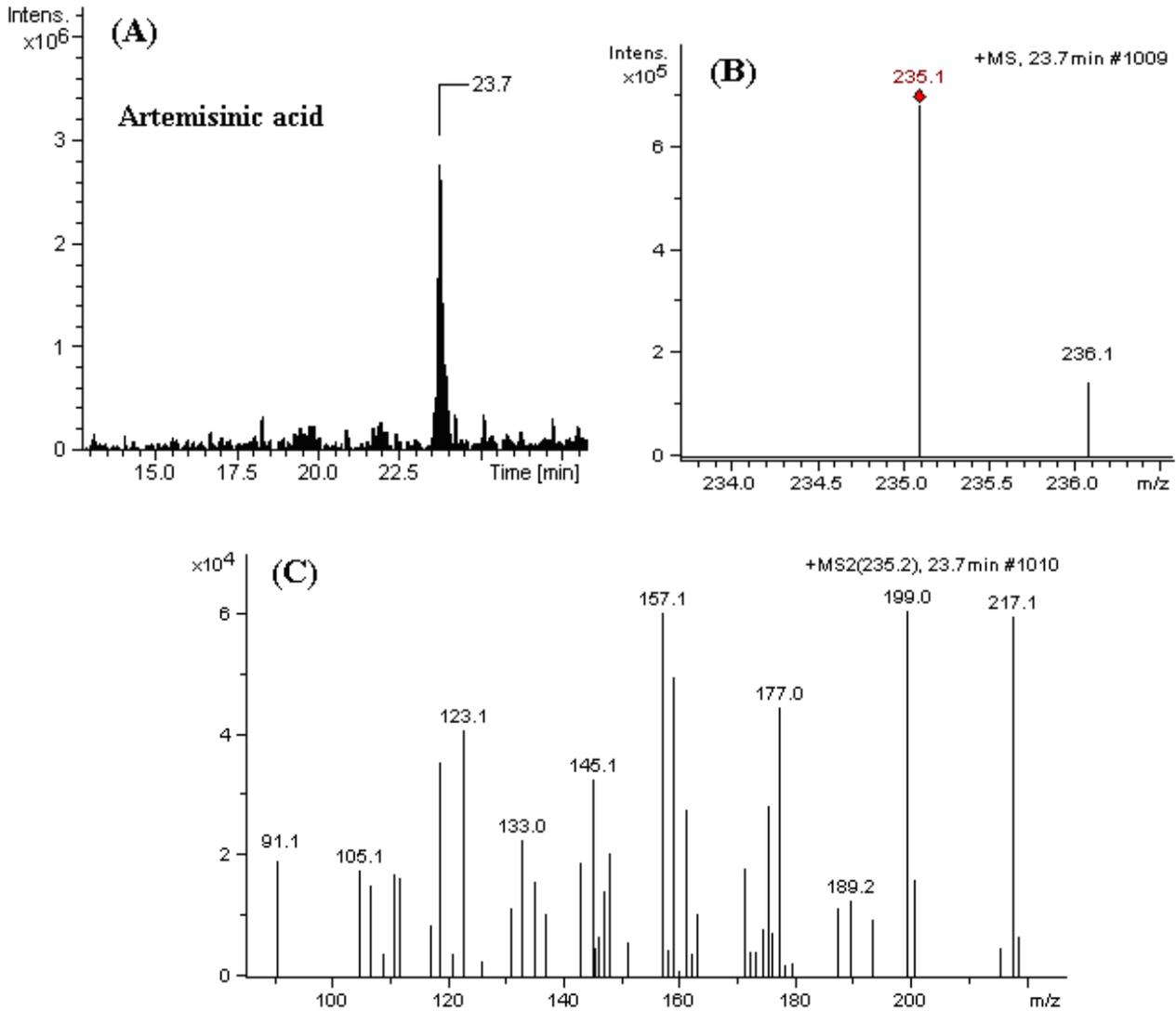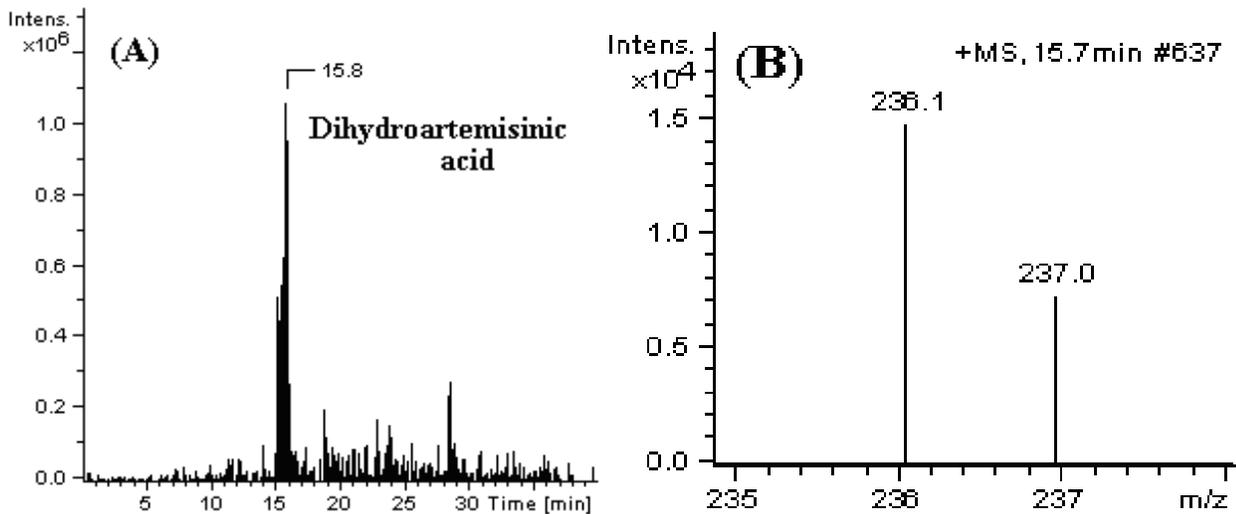
Fig. 9. (A): Extracted ion chromatogram, (B) mass spectra, and (C) fragmentation pattern of artemisinic acid.
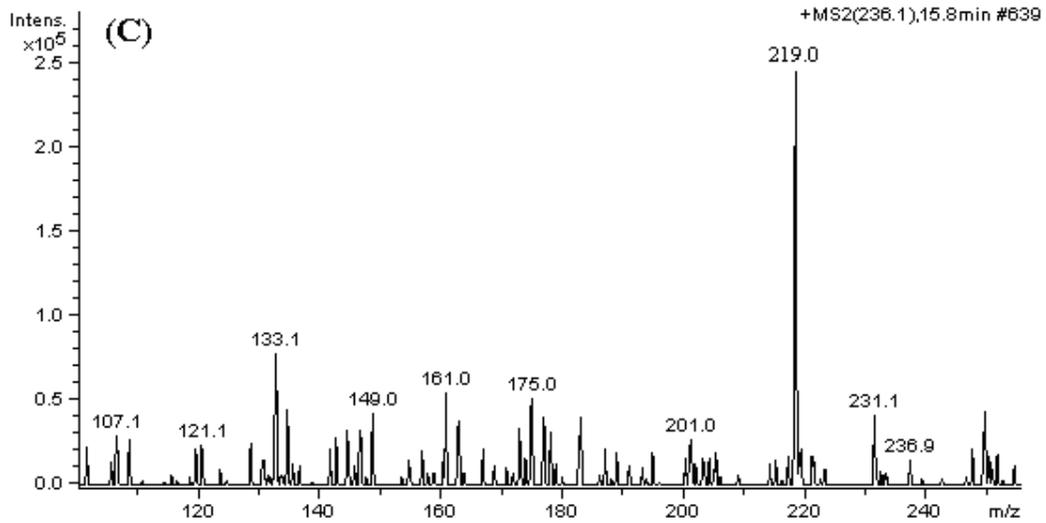
Fig. 10. (A): Extracted ion chromatogram, (B) mass spectra, and (C) fragmentation pattern of dihydroartemisinic acid.
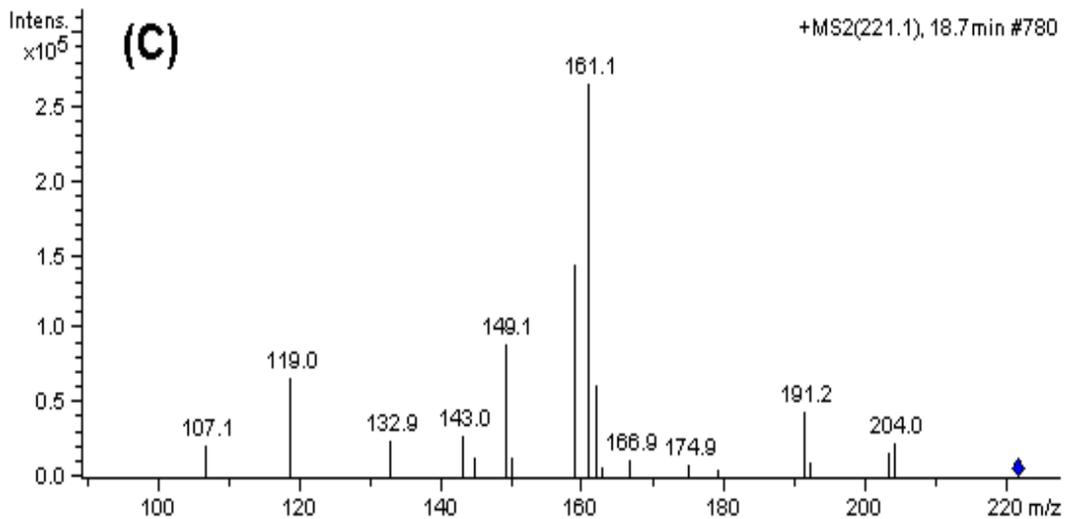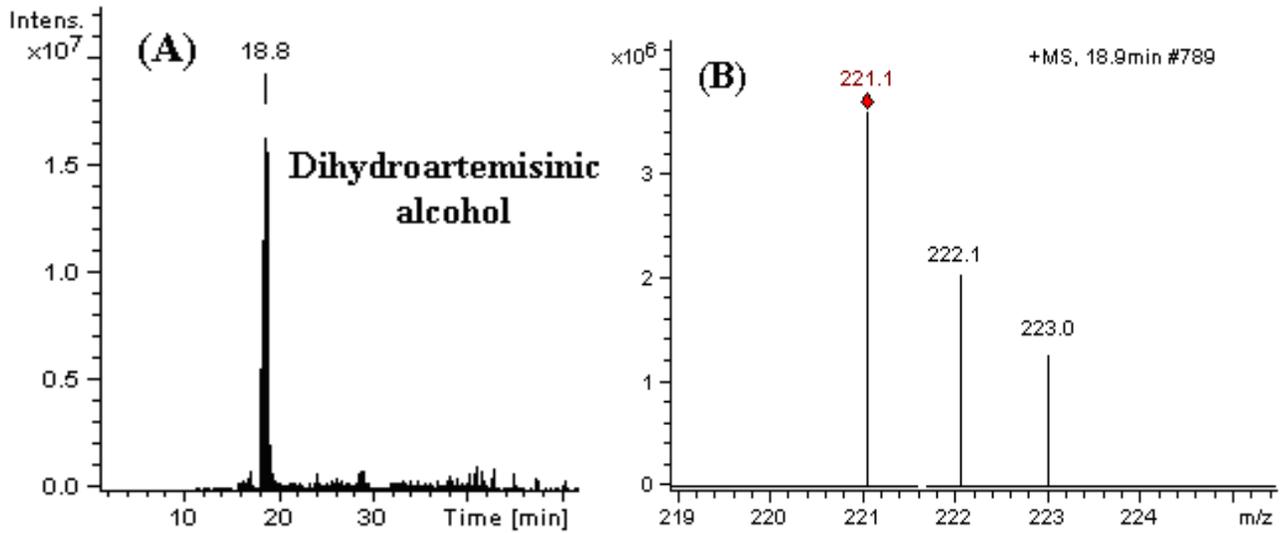

Fig. 11. (A): Extracted ion chromatogram, (B) mass spectra, and (C) fragmentation pattern of dihydroartemisinic acid.

Identification of Dihydroartemisinic acid (present in both *Artemisia herba alba* and *Artemisia monosperma*)

The mass spectrum of the peck eluted at 15.8 min corresponds to dihydroartemisinic acid is shown in Fig. 10. The largest ion (237) is consistent with [M+H] of dihydroartemisinic acid showing the following fragments:

(m/z→236 219+201+175+161+162+147+133+121+107). The structure of that compound is shown in Fig. 1. The fingerprint profile and mass spectra of the analyzed compound was consistent with published data (Avula *et al.,* 2009; Brown, 2010; Teoh *et al.,* 2006; Wallaart *et al.,* 1999).

Identification of Dihydroartemisinic alcohol (present in *Artemisia monosperma*)

The mass spectrum of the peck eluted at 18.8 min corresponds to dihydroartemisinic alcohol is shown in Fig.11. The largest ion (223) is consistent with [M+H] of dihydroartemisinic alcohol showing the following fragments:

(m/z →223 204+191+161+149+133+119+107). The structure of that compound is shown in Fig. 1. The fingerprint profile and mass spectra of the analyzed compound was consistent with published data (Brown, 2010; Bertea *et al.,* 2005).

Identification of Dihydroartemisinic aldehyde (present in *Artemisia monosperma*)

The mass spectrum of the peck eluted at 15.3 min corresponds to dihydroartemisinic aldehyde is shown in Fig.12. The largest ion (221) is consistent with [M+H] of dihydroartemisinic aldehyde showing the following fragments: (m/z 221→ 218+205+187+175+164+163+162+135+119+91). The structure of that compound is shown in Fig. 1. The fingerprint profile and mass spectrum of the analyzed compound was consistent with published data (Brown, 2010; Bertea *et al.,* 2005).

Standard-Free Relative Quantification by Peak area of LC-MS.

Signal or peak integration of ions in the MS scans has been used in the quantification technique for decades by small molecule analytical chemists because theoretically the peak intensity of any ion should be proportional to its abundance. Quantification was based on the LC-MS peak area integration of artemisinin and its related analogues. The area inscribed by the peak is proportional to the amount of substance separated in the chromatographic system. The relative quantities of the components were determined from the relative areas under the peaks and expressed as a % of dry plant weight.
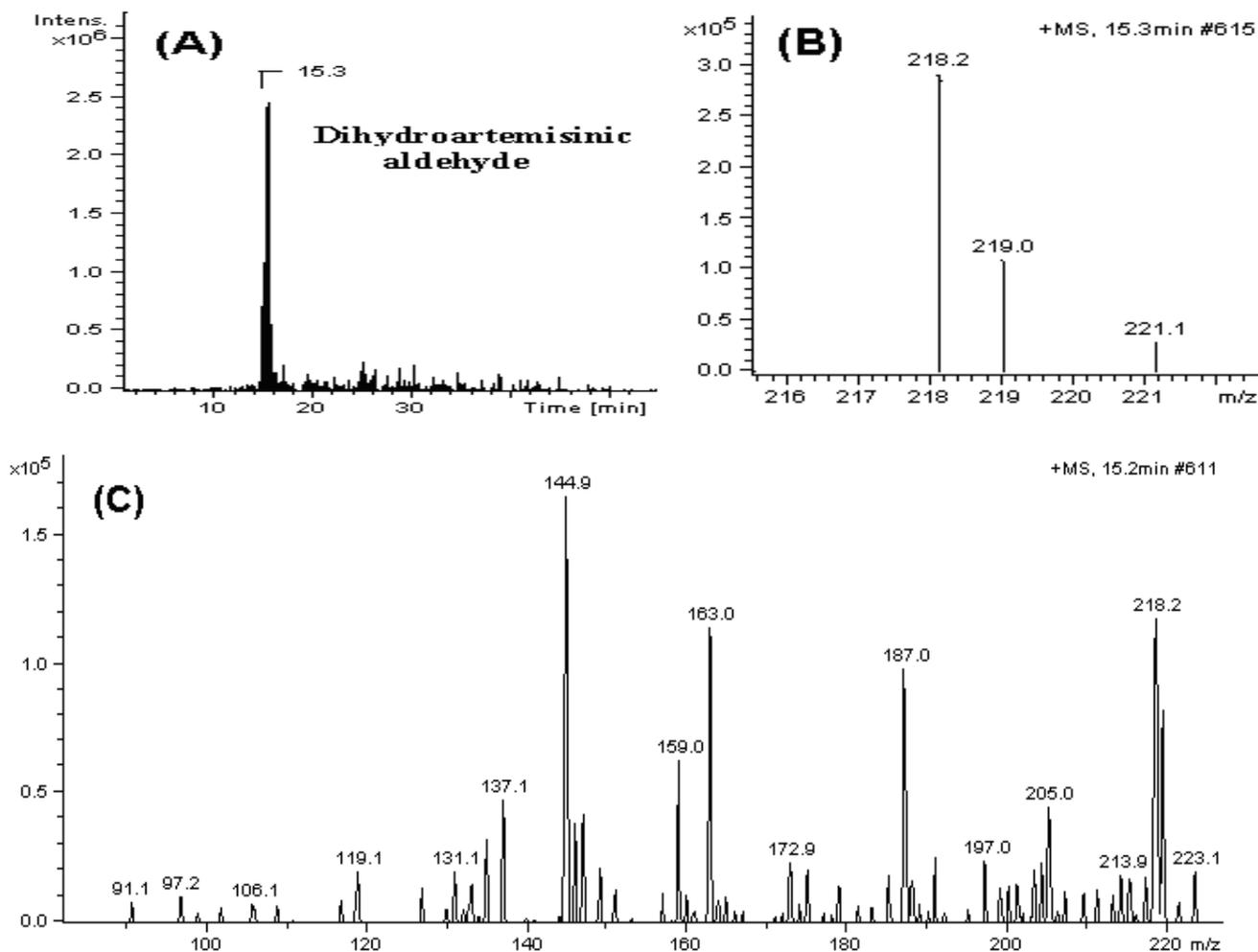

Fig. 12. (A): Extracted ion chromatogram, (B) mass spectra, and (C) fragmentation pattern of dihydroartemisinic acid.

Table. 1: The ms/ms Product Spectra of the Known Artemisinin Analogues of *Artemisia* monosperma crude extract Obtained by Mass Selecting Corresponding Parent Ions Generated from the Plant Extract.

R_t Time (min)	Mol wt m/z	Compound	m/z	Ref.	% Peak Area fraction	% of dry plant weight
12.4 12.8	283	Dihydroartemisinin epimer (α and β DHA)	267	(Mohamed *et al.,* 1999; Rajanikanth *et al.,* 2003)	9.5	1.90
15.2	220	Dihydroartemisinic aldehyde	218, 205, 187, 175,164,163,162,147,135,121,91,79 and 55	(Brown, 2010; Bertea *et al.,* 2005)	0.3	0.06
15.7	235	Dihydroartemisinic acid	219,201,175,161,162,147,133,121 and 107	(Avula *et al.,* 2009; Brown, 2010; Teoh *et al.,* 2006; Wallaart *et al.,* 1999)	0.4	0.08
18.8	222	Dihydroartemisinic alcohol	204,191,161,149,133,119,107, and 91	(Brown, 2010; Bertea *et al.,* 2005)	9.8	1.95
36.6	280	Artemisitene	281, 217, 227, 245 and 263	(Avula *et al.,* 2009)	1.3	0.27
37.7	282	Artemisinin	265, 247, 223 and 209	(Avula *et al.,* 2009)	18	3.6

Table. 2: The ms/ms Product Spectra of the Known Artemisinin Analogues of *Artemisia* herba alba crude extract Obtained by Mass Selecting Corresponding Parent Ions Generated from the Plant Extract.

R_t Time (min)	Mol wt m/z	Compound	m/z	Ref.	% Peak Area fraction	% of dry plant weight
15.7	235	Dihydroartemisinic acid	219,201,175,161,162,147,133,121 and 107	(Avula *et al.,* 2009; Brown, 2010; Teoh *et al.,* 2006; Wallaart *et al.,* 1999)	0.4	0.08
23.7	234	Artemisinic acid	217,199,189,177,157,145,133,123,105 and 91	(Brown, 2010; Bertea *et al.,* 2005)	0.2	0.04
36.6	280	Artemisitene	281, 217, 227, 245 and 263	(Avula *et al.,* 2009)	1.7	0.35
37.7	282	Artemisinin	265, 247, 223 and 209	(Avula *et al.,* 2009)	23.7	4.9

CONCLUSION

This study reported *Artemisia herba alba* and *Artemisia monosperma* for the first time as a novel potential plant sources for artemisinin and some of its related analogues. The quantification of artemisinin and its related analogues in plant samples yielded unexpected results. The high artemisinin concentration detected in both *Artemisia herba alba* (4.9 % of dry weight) and *Artemisia monosperma* (3.6 % of dry weight) compared with the low production yield of artemisinin from *A. annua* (0.01-0.05 % of dry weight), may be helpful for its commercial pharmaceutical production especially that these *Artemisia* species are abundant in distribution in Egyptian desert. Apart from artemisinin, the detection and quantification of other artemisinin precursors including: dihydroartemisinin, artemisitene, arteannuin B, Dihydroartemisinic alcohol, Dihydroartemisinic aldehyde, Artemisinic acid and Dihydroartemisinic acid as shown in table 1 and 2 in a relatively higher concentration than that present in *A. annua*, could lead to the improvement of the overall supply of artemisinin at a reduced market price offering an acceptable price for most patients.

ACKNOWLEDGEMENTS

REFERENCES

Avula B, Wang YH, Smillie TJ, Mabusela W, Vincent L, Weitz F, Khan A K.. Comparison of LC–UV, LC–ELSD and LC–MS Methods for the Determination of Sesquiterpenoids in Various Species of *Artemisia*. Chromatographia. 2009; 70: 797-803.

Bertea CM, Freije JR, Van der WH, Verstappen FW, Perk L, Marquez V, De Kraker J.W, Posthumus, MA, Jansen BJ, De Groot A, Franssen M.C, Bouwmeester H.J. Identification of intermediates and enzymes involved in the early steps of artemisinin biosynthesis in *Artemisia* annua. Planta Med. 2005; 71: 40–47.

Bondarenko PV, Chelius D, Shaler TA. Identification and relative quantitation of protein mixtures by enzymatic digestion followed by capillary reversed-phase liquid chromatography-tandem mass spectrometry. Analytical Chemistry. 2002; 74: 4741–4749.

Bouwmeester, H., Bertea, C., De kraker, J.W., Verstappen, F., Franssen, M. (2006). Research to improve artemisinin production for use in the preparation of anti-malarial drugs. In R.J Bogers, L.E. Craker, D. Langer (Eds.) Medicinal and Aromatic Plants (pp. 275-290). Netherlands: Springer, Inc.

Brown G.D. The Biosynthesis of Artemisinin (Qinghaosu) and the Phytochemistry of *Artemisia annua* L. (Qinghao). Molecules 2010; 15: 7603-7698.

Busch KL, Glish GL, McLuckey SA. Mass Spectrometry: Techniques and Applications of Tandem Mass Spectrometry, Publisher VCH, New York (1988).

Cabri W, Ciogli A, D'Acquarica I, Di Mattia M, Galletti, B, Gasparrini, F, Giorgi, F, Lalli S, Pierini M, Simone P. On-column epimerization of dihydroartemisinin: An effective analytical approach to overcome the shortcomings of the International Pharmacopoeia monograph. Journal of Chromatography B. 2008; 875: 180–191.

Chen PK, Leather GR, Klayman DL. Allelopathic effect of artemisinin and its related compounds from *Artemisia annua*. Plant Physiol. 1987; 83; 406.

Cheng F, Shen J, Luo X, Zhu W, Gu J, Ji R., Jiang H, Chen K. Molecular docking and 3-D-QSAR studies on the possible antimalarial mechanism of artemisinin analogues. Bioorg. Med. Chem. 2002; 10: 2883–2891.

Chelius D, Bondarenko PV. Quantitative profiling of proteins in complex mixtures using liquid chromatography and mass spectrometry. Journal of Proteome Research. 2002; 1: 317–323.

D'Acquarica I, Gasparrini F, Kotoni D, Pierini M, Villani C, Cabri W, Di Mattia M, Giorgi F. Stereodynamic Investigation of Labile Stereogenic Centres in Dihydroartemisinin. Molecules. 2010; 15 : 1309-1323.

De Rijke E, Zappey H, Ariese F, Gooijer C, Brinkman UAT. Liquid chromatography with atmospheric pressure chemical ionization and

electrospray ionization mass spectrometry of flavonoids with triple-quadrupole and ion-trap instruments. J. Chromatogr. A. 2003 ; 984: 45-58.

De Vries P.J, Dien TK. Clinical pharmacology and therapeutic potential of artemisinin and its derivates in the treatment of malaria. Drugs 1996 ; 52 : 818–836.

Dhooghe L, Van Miert S, Jansen H, Vlietinck A, Pieters L. A new decomposition product of dihydroartemisinin. Pharmazie. 2007; 62 : 900-1.

Duke SO, Vaughn KC, Croom Jr EM, Elsohly HN. Artemisinin, a constituent of annual wormwood (*Artemisia annua*), selective phytotoxin. Weed Sci. 1987; 35 : 499-505.

ElSohly, H.N. (2002). Plants-Industrial Profiles. In: C.W. Wright (Ed.). *Artemisia*-Medicinal and Aromatic plants (pp. 24–28). London, UK: Taylor &. Francis, Inc.

Fabre N, Rustan I, De Hoffmann E, Quetin-Leclercq J. Determination of flavone, flavonol, and flavanone aglycones by negative ion liquid chromatography electrospray ion trap mass spectrometry. J. Am. Soc. Mass Spectrom. 2001; 12 : 707-715.

Gu Y, Li Q, Melendez V, Weina PJ. Comparison of HPLC with electrochemical detection and LC-MS/MS for the separation and validation of artesunate and dihydroartemisinin in animal and human plasma. J Chromatogr B Analyt Technol Biomed Life Sci. 2008 ; 867 : 213–218.

Hanpithakpong W, Kamanikom B, Dondorp AM, Singhasivanon P, White NJ, Day NP, Lindegardh N. A liquid chromatographic-tandem mass spectrometric method for determination of artesunate and its metabolite dihydroartemisinin in human plasma. J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 2008 ; 876 : 61–68.

Hartl DL. The origin of malaria: mixed messages from genetic diversity. Nat. Rev. Microbiol. 2004; 2: 15–22.

Heidorn M, Martin M, Steiner F, Plante M, Mcleod F. Towards Standard-Free Quantitative and Qualitative Analysis in Liquid Chromatography. Dionex Corporation, Thermo Fisher Scientific. (2011) Germany: Germering, Inc.

Hughes RJ, Croley TR, Metcalfe CD, March RE. A tandem mass spectrometric study of selected characteristic flavonoids. Int. J. Mass Spectrom. 2001; 210: 371–385.

Justesen UJ. Negative atmospheric-pressure-chemical-ionization low-energy collision activation mass spectrometry for the characterization of flavonoids in extracts of fresh herbs. Chromatogr. A . 2000:902: 369.

Justesen U, Knuthsen P, Leth T. Quantitative analysis of flavonols, flavones, and flavanones in fruits, vegetables, and beverages by HPLC with photodiodearray- and mass spectrometric detection. J. Chromatogr. A. 1998; 799: 101-110.

Klayman DL. Qinghaosu (artemisinin): an antimalarial drug from China. Science. 1985; 228: 1049-1055.

Liu JM, Ni MY, Fan JF, Tu YY, Wu ZH, Wu Y.L, Zhou, W.S . The structure and reaction of arteannuin. Acta Chim. Sin. 1979; 37: 129–141.

Klayman DL, Lin AJ, Acton N, Scovill JP, Hoch JM, Milhous WK, Theoharides AD, Dobek AS. Qinghaosu (artemisinin): An antimalarial drug from China. J. Nat. Prod. 1984; 47: 715–717.

Klayman DL. Qinghaosu (artemisinin): An antimalarial drug from China. Science 1985; 228: 1049-1055.

Li, Y., Huang, H., & Wu, Y.L., (2006). Qinghaosu (artemisinin) a fantastic antimalarial drug from a traditional Chinese medicine. In X.T. Liang & W.S. Fang (Eds.) Medicinal chemistry of bioactive natural products (pp. 183–256). Hoboken: John Wiley & Sons, Inc.

Lin AJ, Klayman DL, Hoch JM, Silverton, JV, George CF. Thermal rearrangements and decomposition products of artemisinin (qinghaosu) 1. J Org Chem. 1985; 50: 4504–4508.

Lisa M, Holcapek M. Triacylglycerols profiling in plant oils important in food industry, dietetics and cosmetics using high-performance liquid chromatography–atmospheric pressure chemical ionization mass spectrometry. J. Chromatography A. 2008 ;115–130: 1198–1199.

Lisa M, Lynen F, Holcapek M, Sandra P. Quantitation of triacylglycerols from plant oils using charged aerosol detection with gradient compensation. J. Chromatography A. 2007;1176: 135–142.

Luo XD, Shen CC. Review of chemistry, pharmacology and clinical applications. Med. Res. Rev. 1987;7: 29-52.

Ma YL, Vedernikova I, Van den Heuvel H, Claeys M. Internal glucose residue loss in protonated O-diglycosyl flavonoids upon low-energy collision-induced dissociation. J. Am. Soc. Mass Spectrom. 2000; 11: 136–144.

Mohamed SS, Khalid SA, Ward SA, Wan TSM, Tang HPO, Zheng M, Haynes RK, Edwards G. Simultaneous determination of artemether and its major metabolite dihydroartemisinin in plasma by gas chromatography–mass spectrometry-selected ion monitoring. Journal of Chromatography B. 1999; 731: 251–260.

Naik H, Murry DJ, Kirsch LE, Fleckenstein L. Development and validation of a high-performance liquid chromatography-mass spectroscopy assay for determination of Artesunate and Dihydroartemisinin in human plasma, J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci. 2005; 816: 233-242.

Nurgün E, lkay O, Murat K, Nezaket A, Barıs B. Determination of Artemisinin in Selected *Artemisia* L. Species of Turkey by Reversed Phase HPLC. Rec. Nat. Prod. 2007;1:2-3, 36-43.

Perez-Souto N, Lynch RJ, Measures G, Hann JT. Use of high-performance liquid chromatographic peak deconvolution and peak labelling to identify antiparasitic components in plant extracts. J. Chromatogr. 1992; 593: 209-215.

Phillipson DJ, Wright CW. Antiprotozoal agents from plant sources. Planta Med. 1991;57 (Suppl 1): S53–S59.

Rajanikanth M, Madhusudanan KP, Gupta RC. An HPLC-MS method for simultaneous estimation of α,β-arteether and its metabolite dihydroartemisinin, in rat plasma for application to pharmacokinetic study. Biomed. Chromatogr. 2003;17: 440–446.

Ranasinghe A, Sweatlock JD, Graham cooks R. A rapid screening method for artemisinin and its congeners using MS/MS: search for new analogues in Artemesia annua. J. Nat. Prod.1993; 56: 552-563.

Richter, W.J., Blum, W., Schlunegger, U.P., Senn, M.(1983). Tandem Mass Spectrometry. In: F.W. McLafferty (Ed). Tandem mass spectrometry of pharmaceuticals (pp. 417). New York: John Wiley and Sons, Inc.

Robert A, Cazelles J, Odile DC, Meunier B. Acc. From mechanistic studies on artemisinin derivatives to new modular antimalarial drugs.Chem. Res. 2002; 35: 167-174.

Samuelsson G. Drugs of Natural Origin. 4nd ed.Swedish Pharmaceutical Press, Kristiandstad Publishers, Sweden(1999).

Souppart C, Gauducheau N, Sandrenan N, Richard F. Development and validation of a high-performance liquid chromatography-mass spectrometry assay for the determination of artemether and its metabolite dihydroartemisinin in human plasma. J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 2002; 774: 195-203.

Stevens JF, Ivancic M, Hsu VL, Deinzer ML. Prenylflavonoids from *Humulus Lupulus*. Phytochemistry 1997; 44: 1575-1585.

Teoh KH, Polichuk DR, Reed DW, Covello PS. Molecular cloning of an aldehyde dehydrogenase implicated in artemisinin biosynthesis in *Artemisia* annua. Botany. 2009;87: 635–642.

Teoh KH, Polichuk DR, Reed DW, Nowak G, Covello PS. *Artemisia annua* L. (Asteraceae) trichome-specific cDNAs reveal CYP71AV1, a cytochrome P450 with a key role in the biosynthesis of the antimalarial sesquiterpene lactone artemisinin. FEBS Lett. 2006; 580: 1411–1416.

Thornfeldt CR. Treatment of skin disease with artemisinin and its derivatives Can. Patent Appl. Chem. Abstr. 1991; 116: 34598.

Van Nieuwerburgh FCW, Vande Casteele SRF, Maes L, Goossens A, Inz´e D, Bocxlaer JV, Deforce DLD. Quantitation of artemisinin and its biosynthetic precursors in *Artemisia annua* L. by high performance liquid chromatography–electrospray quadrupole time-of-flight tandem mass spectrometry. Journal of Chromatography A. 2006;1118: 180–187.

Voyksner RD, Lee H. Investigating the use of an octupole ion guide for ion storage and high-pass mass filtering to improve the quantitative performance of electrospray ion trap mass spectrometry. Rapid Communications in Mass Spectrometry. 1999;13: 1427–1437.

Wallaart T.E, Van Uden W, Lubberink HG, Woerdenbag HJ, Pras N, Quax WJ. Isolation and identification of dihydroartemisinic acid

from *Artemisia annua* and its possible role in the biosynthesis of artemisinin. J. Nat. Prod. 1999;62: 430–433.

White NJ. Antimalarial drug resistance: the pace quickens. J Antimicrob Chemother. 1992; Nov: 30, 571–585.

Xing J, Yan HX, Wang RL, Zhang LF, Zhang SQ. Liquid chromatography–tandem mass spectrometry assay for the quantitation of β-dihydroartemisinin in rat plasma. J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 2007; 852: 202–207.

Xing J, Yan HX, Zhang SQ, Ren G, Gao Y. A highperformance liquid chromatography /tandem mass spectrometry method for the determination of artemisinin in rat plasma. Rapid Commun Mass Spectrom 2006; 20: 1463–1468.

Yikang W. How Might Qinghaosu (Artemisinin) and Related Compounds Kill the Intraerythrocytic Malaria Parasite? A Chemist's View. Acc. Chem. Res. 2002;35: 255–259.