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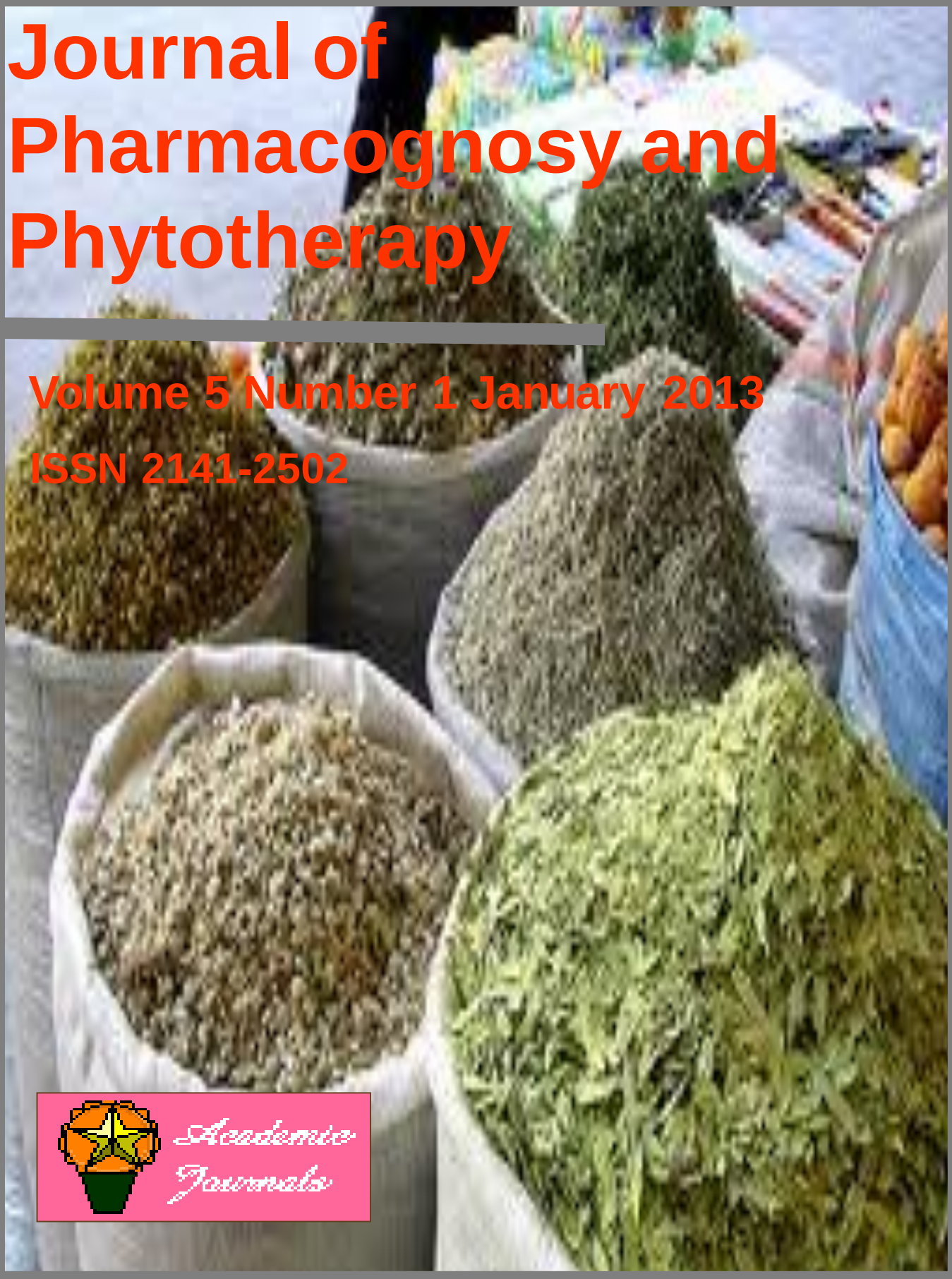

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Charnley AK (1992). Mechanisms of fungal pathogenesis in insects with particular reference to locusts. In: Lomer CJ, Prior C (eds), Pharmaceutical Controls of Locusts and Grasshoppers: Proceedings of an international workshop held at Cotonou, Benin. Oxford: CAB International. pp 181-190.

Jake OO (2002). Pharmaceutical Interactions between *Striga hermonthica* (Del.) Benth. and fluorescent rhizosphere bacteria Of *Zea mays*, L. and *Sorghum bicolor* L. Moench for Striga suicidal germination In *Vigna unguiculata*. PhD dissertation, Tehran University, Iran.

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Research Articles

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Full Length Research Paper

Chemical composition and antinociceptive activity of California sagebrush (*Artemisia californica)*

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***Artemisia californica*, California sagebrush, has been reported to have pain relieving activity and is a traditional medicine of the Chumash Indians of California. Pain relieving activity of a traditional sagebrush preparation was examined in patients suffering from arthritis and other pain. The preparation was examined by gas chromatography-mass spectrometry (GC-MS) and high performance liquid chromatography-mass spectrometry (HPLC-MS) to identify the compounds present. A traditional tincture of sagebrush was produced and used on 42 patients with moderate to severe pain. All patients reported pain relief within 10 to 20 min. Sagebrush was examined by GC-MS and HPLC-MS and was found to contain monoterpenoids, lipids, flavonoids and sesquiterpenes. The major monoterpenoid found is eucalyptol. Of the monoterpenoids, camphor and eucalyptol have reported pain relieving activity. They interact with transient receptor potential cation channel vanilloid 3 (TRPV3), transient receptor potential ankyrin-repeat 1 (TRPA1) and transient receptor potential melastatin 8 (TRPM8) receptors to produce pain relief that lasts for several hours.**

Key words: California sagebrush, *Artemisia californica*, Asteraceae, pain relief, anti-inflammatory, arthritis, transient receptor potential cation channels, transient receptor potential melastatin 8 (TRPM8), transient receptor potential ankyrin-repeat 1 (TRPA1), transient receptor potential cation channel vanilloid 3 (TRPV3).

INTRODUCTION

Artemisia californica Less. also known as California sagebrush (*khapshikh* in Chumash), is a species of the genus *Artemisia*, and belongs to the Asteraceae family. It grows in chaparral in the foothills near the coast from San Francisco to Baja California. *Artemisia* plants are very important medicinal plants throughout the world. The Costanoan Indians of California use the leaves for tooth aches and to poultice wounds (Garcia and Adams, 2012). The Chumash Indians use a decoction of the leaves and stems externally for colds, asthma and arthritis (Garcia and Adams, 2012). In fact, a tincture of *A. californica* has been recommended for use by arthritic patients. Several patients suffering from moderate or severe pain were treated with a sagebrush tincture; case reports were presented.

The chemistry of *A. californica* has not been described previously, except for a report of finding a sesquiterpene named artecalin (Geissman et al., 1969) and a recent review article written to complement the present work

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Table 1. Characteristics of the six different fractions collected from the *A. californica* extract.

Fraction number	Color	Volume (ml)
1	Translucent	10
2	Dark green	15
3	Light green	15
4	Yellow	50
5	Brown-yellow band	5
6	Light yellow	10

(Adams, 2012). The goal of the present work was to find out if antinociceptive compounds are present in *A. californica* that may help explain the use of the tincture for pain control. Tinctures of the plant were examined by gas chromatography-mass spectrometry (GC-MS), high performance liquid chromatography-mass spectrometry (HPLC-MS) and other techniques to characterize the compounds present.

MATERIALS AND METHODS

General experimental procedures

Isolated compounds were characterized by spectroscopic methods. Nuclear magnetic resonance (NMR) spectra (^{1}H and ^{13}C NMR) were recorded at room temperature on a Varian Mercury Plus instrument at 400 MHz. Chemical shifts (δ) are reported in ppm relative to tetramethylsilane (TMS). GC-MS employed a Thermo-Fisher FOCUS-DSQ II gas chromatograph with a mass-selective detector. The column temperature was: 40°C for 10 min, which increased 2°C per min to a final temperature of 250°C for 5 min. HPLC-MS analysis involved a Thermo Finnigan LCQ DECA with a reverse phase column. The solvent system consisted of 10% MeOH in water that increased at 2% per min to 100% MeOH. Column chromatography was performed with Silicagel 60 columns (EBM, Germany) that were developed with the following solvents, AcOEt-Ether (90:10), AcOEt-MeOH (50:50) and pure MeOH and it enabled the fractionation of the *A. californica* extract into six different fractions. The six fractions collected had the characteristics presented in Table 1. Thin layer chromatography (TLC) was performed with Silicagel 60 plates (EBM, Germany). The mobile phase consisted of AcOEt-Ether (90:10). The plant extract gave the following spots on TLC plates, Rf = 0, 0.06, 0.68, 0.83, and 0.95.

Plants

A. californica is a perennial shrub that grows to 1.5 to 2.5 m high and branches from the base. The branches are about 1 m long, flexible and canescent. The leaves are thread like, light green, about 5 cm long and may be 2 to 4 pinnately lobed. Flower heads are less than 5 mm wide, yellow or white, contain 6 to 10 pistillate flowers and 15 to 30 disk flowers. The branches of *A. californica* used in this study were collected near Pasadena, California. White sage (*Salvia apiana*) leaves were also collected. All plant materials were collected in early May, 2010. Voucher specimens of *A. californica* are available at the Rancho Santa Ana Botanic Garden, Claremont.

Extraction and tincture preparation

The alcoholic sagebrush tincture was prepared following the methods of traditional Chumash healers (Garcia and Adams, 2012). Eighty-six grams of *A. californica* branches, one leaf of *S. apiana*, one avocado seed (*Persea americana*) and 500 ml of 70% isopropanol were introduced into an amber-colored glass bottle. A total of 10 L of tincture were produced. This tincture was used to treat patients.

A sagebrush extract, for chemical analysis, was prepared with 290 ml of 99.8% isopropanol as well as 29.0 g of *A. californica* leaves and stems. This *A. californica* extract was used to identify, characterize and isolate the compounds present.

Patient treatment

Patients were recruited from a senior citizen center and from the community. Each patient reported pain of moderate to extreme intensity using an Osteoarthritis Research Society (OARSI) pain scale (Hawker et al., 2008) modified for sites in addition to the hips and knees. Patient data is presented in the results subsequently. Each patient was allowed to apply sagebrush tincture topically to the painful site with a cotton ball. Patients were then questioned at 10 min intervals and were asked to rate their pain as nonexistent, mild, moderate, severe or extreme. Results were recorded at the time of each interview.

RESULTS

Phytochemical characterizations by GC-MS

In total, 19 compounds were found by GC-MS and were characterized with a GC-MS database. Compounds found by GC-MS data were identified by comparison of mass total ion count (TIC) fragmentation spectra with authentic samples reported in the NIST/EPA/NIH Mass Spectral Library (Version 2.0 d) build 26 April, 2005 using the NIST Mass Spectral Search Program (Stein et al., 2005). Out of the 19 identified, 15 turned out to be known monoterpenes-camphene, menthadiene, β-pinene, eucalyptol, isopropenylmethylcyclohexanol, trimethylheptadienol, isopropylmethylbicyclohexanol (also called 4-methyl-1-propan-2-yl-bicyclo[3.1.0]hexan-5-ol), 3-thujanone (also called α-thujone), β-thujone, chrysanthenone, camphor, borneol, carene, menthenol and menthadienol (Figure 1). The remaining 4 compounds were characterized as known lipids and diterpenoids, retinol acetate, dimethylmethylene cyclohexenyl diene methylbutylester acetic acid, methylhexadecanol and tetratetracontane. The retention times (RTs), characteristic fragments and base peaks of each of these 19 chemical compounds are presented in Table 2. Four of them, all monoterpenoids, appeared to be the main compounds present in the *A. californica* tincture since they corresponded to the peaks with the highest relative abundances (> 8% of the total), eucalyptol (24%), camphor (18%), carene (14%), and menthadienol (9%).

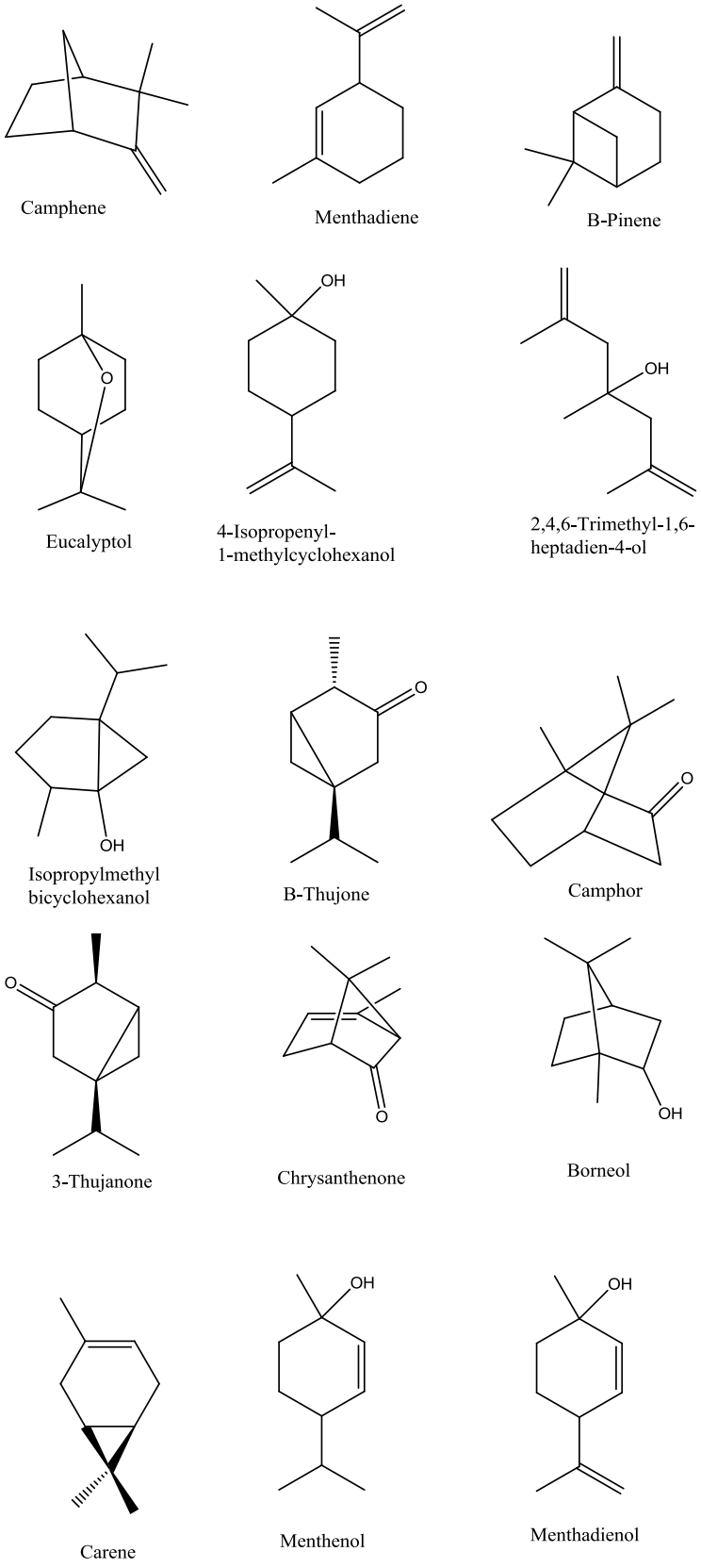

Figure 1. Monoterpenoids found in *A. californica*.

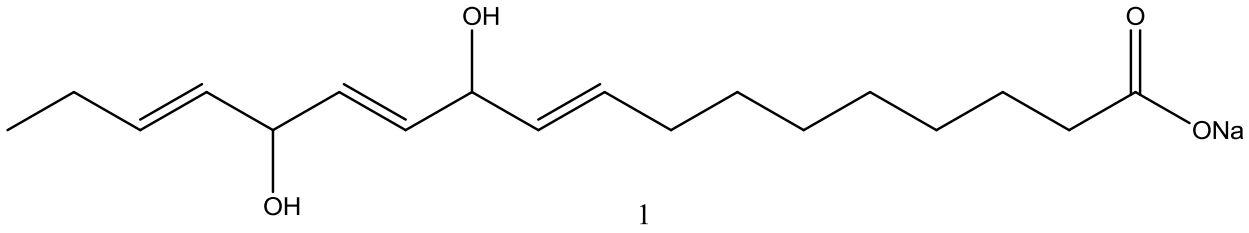

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Figure 2. Dihydroxylinolenic acid found in *A. californica*.

Phytochemical characterizations by low resolution HPLC-MS

The first fraction from silica gel column chromatography was found to contain a fatty acid that appeared to be dihydroxylinolenic acid (1) (Figure 2). Purity was demonstrated by HPLC-MS as subsequently shown. The ^{1}H NMR signals of the compound were observed at δ 1.5 to 1.2 as a multiplet characteristic of alkyl protons, 2.1 for the proton on C17, 2.2 for the proton on C2, 3.6 for the proton on C14, 3.9 for the proton on C11, 5.0 to 5.6 as a multiplet characteristic of protons for C9, OH, C10, C12, C13, C15 and C16. Positive ion HPLC-MS showed an ion of *m/z* value of 610.3 (base peak) that corresponds to a dimer of dihydroxylinolenic acid (1). Other column fractions were impure and were not analyzed by NMR.

HPLC-MS enabled the identification of several sesquiterpenes and flavonoids, all of which are known chemical compounds often found in the *Artemisia* genus. Some compounds were retrieved in both the positive and negative ion modes whereas some others were either found in the negative or the positive ion mode. For instance, quercetin hexose (Yin et al., 2008) was found in both modes (RT = 18.01 min) and they demonstrated the characteristic fragment of quercetin itself (MW = 302). In the positive ion mode, there were also the two following fragments, 655.35 which corresponds to the addition of a molecule of ketohexose ($C_7H_9O_6$, MW = 190) (Tan et al., 2008) to quercetin hexose and, 433.67 that is the loss of CH_4O from quercetin hexose. However, in the negative ion mode there was only one additional fragment, 615.25, characteristic of the addition of a pentose (MW = 150) onto quercetin hexose.

A few other compounds were detected in both positive and negative ion modes tamarixetin glycoside (Avula et al., 2009), jaceosidin (Kazuno et al., 2005; Yin et al., 2008), 6-methoxytricin (ACD/Labs Mass Spectrometry Database, National Institute of Standards mass spectrometry database), and chrysosplenetin (Wollenweber et al., 1991). Tamarixetin glycoside showed several different fragments in the two modes. In the positive ion mode (RT = 21.35 min), a 259.05 ion that could be attributed to the loss of $C_4H_8O_2$ (MW = 88) was found (ACD/Labs Mass Spectrometry Database). Isomers of tamarixetin glycoside were however found twice in the negative ion mode at two successive RTs of 20.97 and 21.35 min. Many higher molecular weight fragments than in the positive mode were found in the isomer at RT = 20.97 min in the negative ion mode such as 1332.62, which could be called tamarixetin quadra hexose, dipentose (MW = 1336); 647.62 that could be tamarixetin hexose, pentose diglycoside followed by 579.42 and 445.67 ions that are probably tamarixetin diglycoside fragments.

Jaceosidin (RT = 23.38 min) had a fragment at 286.37, which is the loss of $C_3H_5O_2$ in negative ion mode. However, a fragment at 168.21 for the neutral loss of a flavone glycoside fragment (MW = 162, Kazuno et al., 2005) was found in positive ion mode. 6-Methoxytricin was identified in the two modes. In positive ion mode, 331.46 was found, which is the result of the loss of two methyls ($2\times CH_3 = 30$) and, 229.23 that corresponds to the loss of $C_5H_9O_2$ (ACD/Labs Mass Spectrometry Database, MW = 101).

Chrysosplenetin in the negative ion mode, led to a 343.65 ion, which is a characteristic of the loss of a molecule of CH_2O. In positive ion mode, a molecule of H_2O was lost to produce a 299.55 ion. Several chemicals were detected only in negative ion mode. Isoorientin (RT = 15.10 min) and tanapartholide A (RT = 15.70 min) (Wen et al, 2010) that both led to respective ions at 293.29 and 243.13 by loss of H_2O_2. Secogorgonolide (Ortet et al., 2008) at a RT of 15.99 min, gave an ion at 192.98, which is equivalent to loss of C_5H_9O (ACD/Labs Mass Spectrometry Database). Finally, methoxyflavone hexose (MW = 462) was found at a RT of 22.47 min. Several ions were found including 1120.24 methoxy-flavone trihexose, dipentose; 1064.53 methoxyflavone hexose, quadra pentose; 1049.18 methoxyflavone glycoside fragment; 763.87 methoxy-flavone hexose, dipentose; 749.36 which may be a multiple pentose fragment; and 299.36, which is the loss of a flavone glycoside fragment (MW = 162) (Kazuno et al., 2005).

Finally, a compound that could be luteolin gentiobioside or quercetin glucoside rhamnoside was eluted at RT = 6.73 min in the positive ion mode (Sakushima et al., 1988; Kazuno et al., 2005). Usaramine was found at RT = 14.93 min (ACD/Labs Mass Spectrometry Database). Leucodin was only retrieved in the positive mode and had

Table 2. Retention times, characteristic ions and base peaks of the nineteen *A. californica* monoterpenes and lipids found by GC/MS.

Retention Time (min)	Fragment 1 (Relative Abundance)	Fragment 2	Fragment 3	Fragment 4	Fragment 5	Base Peak	Compound
7.01	93.05 (100)	28.03 (63)	-	-	-	93.05	Camphene
8.21	93.05 (100)	28.04 (83)	-	-	-	93.05	Menthadiene
9.5	93.03 (92)	28.03 (100)	-	-	-	28.03	β- Pinene
12.82	81.00 (52)	71.00 (52)	43.04 (100)	-	-	43.04	Unknown
12.96	43.02 (100)	-	-	-	-	43.02	Eucalyptol
15.41	82.99 (100)	-	-	-	-	82.99	Isopropenylmethylcyclohexanol
16.87	85.02 (100)	-	-	-	-	85.02	Trimethylheptadienol
17.46	93.06 (100)	70.99 (72)	43.07 (82)	28.03 (56)	-	93.06	Isopropylmethylbicyclohexanol
17.71	110.10 (82)	95.05 (76)	81.06 (100)	69.06 (74)	67.04 (92)	81.06	Thujanone
18.48	110.10 (92)	95.02 (100)	81.05 (98)	69.08 (78)	67.05 (90)	95.02	Thujone
19.08	107.05 (100)	91.00 (78)	79.05 (52)	-	-	107.05	Chrysanthenone
20.24	95.02 (100)	81.06 (56)	41.06 (52)	-	-	95.02	Camphor
21.74	95.06 (100)			-	-	95.06	Borneol
22.97	93.05 (92)	91.02 (74)	79.05 (100)	77.02 (58)	42.99 (94)	79.05	Carene
23.84	136.12 (64)	121.07 (56)	93.06 (100)	59.01 (98)	43.05 (60)	93.06	Menthenol
30.27	119.04 (69)	43.04 (100)		-	-	43.04	Menthadienol
77.04	-	-		-	-	-	Retinol, acetate
86.91	173.09 (72)	43.05 (100)	28.03 (56)	-	-	43.05	Dimethylmethylene cyclohexenyl diene methylbutylester acetic acid
96.53	97.10 (78)	83.08 (55)	69.07 (68)	57.03 (76)	55.05 (54)	43.09	Methylhexadecanol
103.34	71.07 (90)	57.05 (100)	43.09 (71)	-	-	57.05	Tetratetracontane

two characteristic fragments, a 243.30 ion that can be named dehydroleucodine (MW = 244) and 215.51 that is the result of loss of O_2 (Glasl et al., 2002; Ando et al., 1994). Near the RT of leucodin, pestalodiopsolide A was found to loose H_2O_2 to give a fragment at 261.15 (Magnani et al., 2003; Huang et al, 2009). A few minutes later, echinolactone B eluted and was characterized by its fragment at 243.39 that results from the loss of H_2O (Suzuki et al., 2005). Marmin (ACD/Labs Mass Spectrometry Database), chromonar (ACD/Labs Mass Spectrometry Database), and xanthohumol disaccharide were identified through the positive ion mode and had only molecular ions without other fragments. Apigeninidin glucoside (Swinny et al., 2000) was found and had a fragment that came from the loss of either C_5H_{11} or $C_3H_3O_2$ (MW = 71). Rupestine (MW = 245) (Su et al., 2008) had a characteristic ion at 227.51 that can be attributed to the loss of a molecule of H_2O.

The characteristic ions, base peaks and RTs of all the compounds retrieved in *A. californica* are presented in Tables 3 and 4. Ultraviolet (UV) spectral characteristic peaks for each compound are reported in Table 5.

Out of all the known flavonoids, sesquiterpenes and alkaloids found in *A. californica* tincture, four of the flavonoids, appeared to be the primary non-monoterpenoid compounds present in the extract. These compounds correspond to the peaks with the highest relative abundances on the total ion current, jaceosidin, 6-methoxytricin, chrysosplenetin, and quercetin hexose.

CASE REPORTS

Knee pain was reported by several patients. A 57 year old Oriental woman with a broken left patella reported extreme pain. Her pain was not diminished by 2 tablets of hydrocodone (7.5 mg)/acetaminophen (750 mg). She reported her pain as moderate within 20 min of one topical application of *A. californica* tincture. She also noted that the swelling of her knee diminished somewhat. She applied another topical application 3 h later, when the pain returned. By 10 min, she reported that her pain was gone. The next day, she began to use naproxen as needed. Other knee pain patients included, a 72 year old Caucasian woman with arthritis in the right knee that caused moderate pain and a slight limp. Within 10 min of topical application of the tincture, she reported that her pain was mild. A 60 year old Caucasian woman had

Table 3. Retention times, characteristic ions and base peaks of the *A. californica* compounds found by low resolution HPLC-MS (+ ion mode) in the different fractions.

Retention time (min)	Fragment 1 (relative abundance)	Fragment 2	Fragment 3	Fragment 4	Fragment 5	Fragment 6	Fragment 7	Fragment 8	Base peak
6.73	610.09 (100) Luteolin gentiobioside, Quercetin glucoside rhamnoside (MW=610)	536.15 (60)	520.07 (45)	503.29 (20)	403.21 (15)	355.11 (25)	299.17 (55)	194.18 (45)	610.09
14.93	351.32 Usaramine	346.2	261.33	243.37	215.4	-	-	-	261.33
15.75	565.24 (30)	296.27 (85)	247.32 (100) Leucodin (MW= 246)	243.30 (40) Dehydroleucodine (MW= 244)	215.51 (12) M-O2 (MW= 32)	183.47 (6)	-	-	247.32
15.96	296.15 (100) Pestalodio-psolide A (MW= 295)	261.15 (66) M-H2O2	243.25 (30)	215.37 (13)	199.22 (5)	-	-	-	296.15
18.01	655.35 (100) M+190 Ketohexose (C7H9O6)	608.19 (25)	523.19 (30)	499.35 (45)	465.35 (89) Quercetin hexose (MW= 465)	433.67 (39) M-CH4O	325.27 (20)	303.58 (25) Quercetin (MW= 302)	528.69
18.65	261.42 (100) Echinolactone B (MW=260)	243.39 (8) M-H2O	169.10 (4)	-	-	-	-	-	261.42
20.58	423.49 (90)	418.40 (100) Apigeninidin glucoside (MW= 417)	347.47 (25) M-C5H11 M-C3H3O2 (MW= 71)	277.25 (40)	261.32 (66)	243.45 (38)	237.08 (23)	-	418.4
21.35	400.30 (70)	347.44 (100)	317.44 (20) Tamarixetin (glycoside) (MW= 316)	259.05 (10) M-C4H8O2 (MW= 88)	177.33 (10)	-	-	-	347.44
22.65	1314.16	597.97	332.3 Marmin	302.24	243.39	167.05	-	-	332.3
22.82	362 (20)	331.51 (20)	303.27 (25)	245.40 (100) Rupestine (MW= 245)	227.51 (2) M-H2O	-	-	-	245.4
23.38	361.44	331.48 (100) Jaceosidin (MW= 330)	316.49 (20)	301.68 (7)	229.24	168.21 (1) M-162	-	-	331.48
23.71	361.40 6-methoxy-tricin (MW= 360)	331.46 (15) M-30 (2× CH3)	303.42	229.23 (1) 331-C5H9O2 (MW= 101)	-	-	-	-	361.4
23.73	361.74 Chromonar	303.52	257.34	169.19	-	-	-	-	361.74

Table 3. Contd.

25.69	375.45 (100) Chrysosplenetin (MW= 374)	317.48 (10)	299.55 (2) M-H2O	274.34 (2)	201.08 (1)	194.38 (1)	-	-	375.45
40.77	1369.86 (9)	998.29 (30)	759.33 (25)	685.35 (100) Xanthohumol disaccharide (MW= 685)	355.26 (20)	299.17 (22)	-	-	685.35

arthritis of the left knee that made sitting or straightening her leg extremely painful. Within 10 min of topical application of the tincture, she reported that her pain was mild. A 63 year old Indian man had a swollen, painful knee from rheumatoid arthritis. He applied the liniment and reported that his pain went from 7 to 3 within 20 min. He continued to use the liniment for 3 weeks with continued success and no adverse reactions. A 27 year old Caucasian woman presented with moderate tendon pain due to having the left leg longer than the right. She applied the tincture and within 10 min reported nonexistent pain and could run 6 miles. A 55 year old Caucasian man complained of severe pain and fatigue after running a marathon. Within 10 min of topical application of the tincture, he reported nonexistent pain. A 73 year old Caucasian woman had a knee replacement that failed and was done again. She started to use *A. californica* tincture every morning and reported pain relief within 15 min that lasted for about 3 h. Her pain was severe before applying the tincture and became mild after the tincture. She used the tincture daily for 2 months during her recovery. Hip pain was found in one patient. A 55 year old Caucasian man presented with severe tail bone pain due to a fall. The pain made sitting and sleeping very difficult. Within 10 min of topical application of the tincture, he reported mild pain and could sit and sleep normally.

Hand pain was very common. In a crafts workshop in a retirement community, the 26 students, Caucasian and Latino, were having trouble completing the crafts due to severe to moderate arthritis pain in their hands. Both men and women participated and were aged between 70 and 80. All used the tincture topically and reported nonexistent or mild pain within 10 min. The treatment allowed them to work on their projects. A 72 year old Latino man presented with moderate arthritis pain in his hands and shoulders that prevented him from performing his job. Within 10 min of topical application of the tincture, he reported nonexistent pain and could perform his job.

Muscle pain was a common complaint. A 45 year old Caucasian man reported severe pain in his right hand from manual labor. Within 10 min of application of the tincture, he reported his pain was gone and his hand motion was no longer limited. A 67 year old Caucasian woman had severe neck pain from lifting a heavy object. She reported that her pain was gone within 10 min of application of the tincture. Three patients, 45 to 63 years old, complained of severe low back pain from lifting heavy objects. They all reported their pain was gone within 10 min of application of the tincture. A 56 year old Caucasian man reported twisting his ankle while running. He was in severe pain and walked with a cane. Ten minutes after applying the tincture, the pain had decreased to moderate. Another application of the tincture decreased the pain to mild within 10 minutes. The patient was able to sleep normally. A third application of the tincture the next morning allowed the man to walk and climb stairs without a cane and without significant pain.

A 78 year old woman with hepatic cancer was treated with high dose morphine that did not control her pain. She said her pain was extreme. The sagebrush tincture was applied to her chest with a cloth. Within 10 min, she reported her pain was moderate.

DISCUSSION

The genus *Artemisia* with between 200 to 400 species belonging to the family Asteraceae, is very important medicinally and is used throughout the world. Various species have previously been described as possessing antimalarial, antifungal, anti-inflammatory, antibacterial and antiviral agents (Garcia and Adams, 2009). These activities have been attributed to flavonoids (Giangaspero et al., 2009; Yin et al., 2008),

Table 4. Retention times, characteristic ions and base peaks of the *A. californica* compounds found by low resolution HPLC-MS (- ion mode) in the different fractions.

Retention time (min)	Fragment 1	Fragment 2	Fragment 3	Fragment 4	Fragment 5	Fragment 6	Fragment 7	Base peak
6.02	1445.81 Unknown	-	-	-	-	-	-	1445.81
15.1	327.25 (100) Isoorientin (MW= 327)	293.29 (35) M-H2O2	277.27 (15)	265.41 (5)	180.41 (2)	-	-	327.25
15.7	277.42 (100) Tanapartholide A (MW=278)	243.13 (6) M-H2O2	175.10 (4)	-	-	-	-	277.42
15.99	401.15 (33)	355.15 (10)	277.12 (100) Seco-orgonolide (MW= 278)	192.98 (5) M- C5H9O	107.94 (5)	-	-	277.12
18.01	927.08 (20)	615.25 (17) M+152 (pentose)	463.31 (100) Quercetin hexose (MW= 464)	301.31 (11) Quercetin (MW= 302)	285.33 (1)	179.07 (1)	-	463.31
20.97	1332.62 Tamarixetin quadrahexose, dipentose (MW= 1336)	647.62 Tamarixetin hexose, pentose diglycoside	579.42 Tamarixetin diglycoside fragment (578)	445.67 Tamarixetin diglycoside fragment	315.37 (100) Tamarixetin glycoside (MW= 316)	277.37 (65)	-	315.37
21.35	345.40 (35)	315.24 (100) Tamarixetin (glycoside) (MW= 316)	299.43 (16)	285.44 (10)	247.63 (5)	160.40 (3)	-	315.24
22.47	1120.24 (35) Methoxyflavone trihexose, dipentose	1064.53 (59) 763+300 Methoxyflavone hexose quadrapentose	1049.18 (100)	763.87 (64) Methoxyflavone hexose dipentose	749.36 (100)	461.49 (69) Methoxyflavone hexose (MW= 462)	299.36 (23) M-162 Flavone glycoside fragment	1049.18
23.38	359.31 (75)	329.31 (100) Jaceosidin (MW= 330)	314.34 (15)	286.37 (4) M-C3H5O2	263.37	-	-	329.35
23.71	359.42 (100) 6-methoxy-tricin (MW= 360)	329.37	299.34 (15)	286.06 (8)	263.37	202.34 (2)	-	359.42
25.69	373.50 (100) Chrysosplenetin (MW= 374)	343.65 (22) M-CH2O	329.44 (11)	300.49 (8)	257.39 (5)	213.38 (2)	-	373.50

Table 5. UV characteristic peaks of the *A. californica* compounds found by Low Resolution HPLC-MS in the different fractions.

Retention time (min)	Peak 1 (nm)	Peak 2 (nm)	Peak 3 (nm)	Peak 4 (nm)	Peak 5 (nm)	Peak 6 (nm)	Maximum (nm)	Compound
2.01	220	260	286	-	-	-	220	Unknown
6.02	203	-	-	-	-	-	203	Unknown
6.73	200	-	-	-	-	-	200	Luteolin gentiobioside, Quercetin glucoside rhamnoside
14.87	220	-	-	-	-	-	220	Usaramine
14.98	219	-	-	-	-	-	219	Isoorientin
15.67	218	-	-	-	-	-	218	Tanapartholide A
15.69	217	265	-	-	-	-	217	Leucodin
15.75	217	265	378	-	-	-	217	Secogorgonolide
15.78	217	-	-	-	-	-	217	Pestalodiopsolide A
18	216	252	270	313	364	-	216	Quercetin hexose
18.46	217	256	-	-		-	217	Echinolactone B
22.47	205	221	271	294	357	379	221	Methoxyflavone hexose
22.65	205	-	-	-	-	-	205	Marmin
21.09	218	270	330	-	-	-	218	Apigeninidin glucoside
21.2	213	304	335	-	-	-	213	Tamarixetin glycoside
22.69	218	253	341	-	-	-	218	Rupestine
23.21	219	253	272	345	-	-	219	Jaceosidin
23.58	221	257	268	350	-	-	221	Chromonar
23.6	218	255	269	351	-	-	218	6-methoxy-tricin
25.5	217	256	270	350	-	-	217	Chrysosplenetin
27.53	223	-	-	-	-	-	223	unknown
28.31	223	-	-	-	-	-	223	Unknown
40.96	225	274	-	-	-	-	225	Xanthohumol disaccharide

monoterpenes (Reddy et al., 2006) or sesquiterpenes (Ishida, 2005).

A. californica tincture was found to contain monoterpenoids, lipids, flavonoids, sesquiterpenes and alkaloids. A tincture of the plant has been used by Chumash people as a powerful, topical pain reliever and anti-inflammatory agent, especially as a long term medicine to treat arthritis and other chronic pain problems. Patients (42) suffering from moderate to extreme pain were treated with *A. californica* tincture. All reported pain relief within 10 min. The pain relief lasted several hours and was associated with an anti-inflammatory effect in some cases. Fifteen monoterpenes were discovered in *A. californica*: camphene, menthadiene, β- pinene, eucalyptol, isopropenylmethylcyclohexanol, trimethylheptadienol, isopropylmethylbicyclohexanol, thujanone, thujone, chrysanthenone, camphor, borneol, carene, menthenol, and menthadienol (Figure 1). Along with their antinociceptive use, monoterpenoids are also anxiolytic, anthelmintic, antibiotic and anti-inflammatory (Ishida, 2005).

Monoterpenoids with known pain relieving activity in *A. californica* include camphor (Xu et al., 2005; Martinez et al., 2009a and b), eucalyptol (Liapi et al., 2007; Martinez et al., 2009a and b), camphene (Martinez et al., 2009a and b), β-pinene (Liapi et al., 2007; Martinez et al., 2009a and b), borneol (Martinez et al., 2009a and b; Granger et al., 2005) and thujone (Hold et al., 2000). Many of them penetrate the skin including β-pinene (Schmitt et al., 2009), and are topically active.

Monoterpenes express antinociceptive activity by binding to transient receptor potential cation channel vanilloid (TRPV1), TRPV3 and TRP melastatin 8 (TRPM8) receptors. TRPV1 and 3 are critically involved in nociception and thermosensing (Vriens et al., 2009). They are expressed in sensory neurons (Caterina et al. 1997) in the skin, keratinocytes and other organs, and in pain pathways including the dorsal root ganglia, trigeminal neurons, and spinal cord (Vriens et al., 2009). TRPM8 is expressed in the majority of cold-sensitive afferents of the skin and other organs (Basbaum et al., 2009) and therefore responds to cold. Monoterpenoids

activate these TRP channels, causing momentary pain, then deactivate the TRP channels, causing long term pain relief. Most of the pain relieving monoterpenoids found in *A. californica* are agonists for TRPV3 (heat-sensitive) including camphor (Xu et al., 2005; Vriens et al., 2009; Vogt-Eisele et al., 2007), borneol, thujone and eucalyptol (Vogt-Eisele et al., 2007). Camphor also blocks TRP ankyrin-repeat 1 (TRPA1, cold-sensitive) receptor and activates the TRPV1 (heat-sensitive) receptor (Xu et al., 2005). Eucalyptol has been reported to also be a TRPM8 (cold-sensitive) receptor agonist (Basbaum et al., 2009) and to exhibit an antinociceptive activity comparable to that of morphine. A synergism exists between morphine and eucalyptol that produces much greater than expected pain relief (Liapi et al., 2007). Anti-inflammatory properties have been reported for some monoterpenoids including camphene, β-pinene (Ishida, 2005; Lin et al., 2008) as well as for some sesquiterpenoids (Ishida, 2005). The monoterpene, borneol has been shown to present high anti-inflamma- tory activity (Tung et al., 2008), which results from the inhibition of nitric oxide (NO) and prostaglandin E2 (PGE2) production as well as an increase in the expression of inhibitor of NF-κB kinase (IKK), inducible nitric oxide synthase (iNOS), nuclear factor κB (NF-κB), and a decrease in inhibitor of NF-κBα (IκBα) expression in dose-dependent manners (Lin et al., 2008; Tung et al., 2008).

Oral toxicity of monoterpenoids includes seizures reported for camphor (Farhat et al., 2001; Manoguerra et al., 2006), thujone (Farhat et al., 2001; Hall et al., 2004) and camphene (Farhat et al., 2001). However, anti-convulsant properties have been proved for oral β-pinene, eucalyptol (Sayyah et al., 2002) and borneol (Granger et al., 2005). Topical administration of monoterpenoids, in essential oils can cause skin irritation. However, skin penetration of monoterpenoids in quantities sufficient to cause convulsions and other toxicities has not been reported, except in infants.

Several flavonoids are known to be anti-inflammatory and analgesic. For instance, 6-methoxytricin is anti-inflammatory due to its inhibitory activity on the proliferation and activation of T cells (Yin et al., 2008). Quercetin and quercetin glycoside are anti-inflammatory through suppression of synthesis of TNF-α and NO (Sheu et al., 2009) and analgesic through serotonin 5-HT1A receptor activation (Martinez et al., 2009a and b). Jaceosidin is anti-inflammatory and can penetrate the skin to relieve inflammation through an NFkB induction inhibition mechanism (Clavin et al., 2007). These flavonoids undoubtedly add to the analgesic and anti-inflammatory effects of *A. californica* tincture.

Conclusions

A. californica tincture could be a useful pain reliever since it does not interact with cyclooxygenase (COX) like the non-steroidal Anti-inflammatory Drugs (NSAIDs) and therefore should lack their toxicity such as ulcers (Lin et al., 2008). Eucalyptol antinociceptive activity is comparable to that of morphine, except that morphine can cause constipation, respiratory depression, seizures and coma from stimulation of opioid receptors. Monoterpenoids are not known to interact with opioid receptors. This illustrates the great potential of *A. californica* as an antinociceptive and anti-inflammatory medicine for moderate to extreme pain.

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Full Length Research Paper

Traditional treatment of high blood pressure and diabetes in Souk Ahras District

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This study reports an ethnobotanical survey of the medicinal plants used for the treatment of diabetes and hypertension in six divisions of Souk Ahras District in Algeria. A total of 200 informants, including some healers, were interviewed throughout different divisions of the district. These ethnobotanical investigations allowed the development of an inventory of 59 medicinal plants belonging to 35 families; 28 of the plants are used for diabetes, 15 for hypertension, and 16 for both diseases. In this region, the most frequently used plants to treat diabetes include *Olea europea*, *Ajuga iva*, *Allium cepa*, *A. sativum*, *Myrtus communis* and *Trigonella foenum graecum*. The plants used to treat high blood pressure include *A. cepa*, *A. sativum*, *Artemisia herba-alba*, *Nigella sativa*, *Oleaeuropea*, and *Rosmarinus officinalis*. Ethnomedical documentation and sustainable plant uses can support drug discovery efforts in developing countries.

Key words: Diabetes, hypertension, ethnobotanical, traditional medicines.

INTRODUCTION

Currently, the world population is confronted with the rapid emergence of several chronic diseases, including diabetes and high blood pressure, which present economic, as well as a serious current and long-term health problems (Tra Bi et al., 2008). Firstly, diabetes, mainly type 2, has become a truly global problem for humanity, since projections estimate as many as 380 million diabetics globally by 2025, representing a staggering 7.1% of the world population (International Diabetes Federation, 2006).

According to the investigation of the National Institute of Public Health (INSP), and according to the classification Global Burden of Disease (GBD), diabetes occupies the 4th place among the top ten causes of death (INSP, 2005). Secondly, according to the World Health Organization (WHO) (1985) more than 20% of the world's population is affected by high arterial blood pressure (Eddouks et al., 2009). In view of the expansion of these diseases, the resolution of WHOAFR/RC50/R3 in August 31, 2000 encouraged African countries to elaborate regional strategies on traditional medicine in order to begin research on medicinal plants and to improve their optimal uses in the healthcare systems(Eddouks et al., 2009). Finally, the United States Food and Drug Administration (FDA) showed that natural products have a significant place in the discovery of new therapeutic agents (Cordell, 1995; Newman and Cragg, 2007). Ethnopharmacologic knowledge is a holistic system approach that can serve as an innovative and powerful discovery engine for newer, safer, and more accessible medicines (Cordell, 1995; Nanyingi et al., 2008; Patwardhan, 2005).

In Southern Algeria, several ethnobotanical surveys have been carried in the Central Sahara region (Maiza et al., 1990, 1992, 1993a, 1993b, 1995, 2006). However, in Northern Algeria ethnobtanical studies still remain unexplored, especially in the North-Eastern part of the country. The purpose of the present investigation was to establish an inventory of medicinal plants which grow and/or are available in the Souk Ahras province, andwhich are used traditionally to treat diabetes and high blood pressure.

MATERIALS AND METHODS

Study area

Geographically, Souk Ahras District is situated in the north Eastern part of Algeria, bordering on Tunisia, and covers approximately

4,541 km² (estimate of 2007). It is divided into 10 sectors and includes 26 villages. It is bordered by the Republic of Tunisia in the east, Guelma in northwest, Oum El Bouaghi in the southwest, Tebessa in the southeast and El Tarf, Annabain in the northeast. Souk Ahras district is also crossed by the principal wadi in North Africa, the Medjerda. It lies between latitudes north 36° 14′ 00″ N and longitudes east 8° 10′ 00″ E of the Prime Meridian. The geomorphological configuration of the Souk Ahras region reveals two important areas. The study area is characterized by a Mediterranean climate in the North and a continental climate in the far South of this region.

Ethnobotanical survey

Questionnaire

A questionnaire was developed and modeled according to various surveys (El-Hilaly et al., 2003 ; Tahraoui et al., 2007). The questions were focused on the names of the most commonly-used plants, the reasons for using the plants, the part of the plant used, the method of medicinal plant preparation, the route of administration, and the possible adverse effects of plants, and the accessibility of the population to health services.

Based on the information gathered, the plants mentioned as being used for the treatment of diabetes and high blood pressure were selected from the synoptic table. The questionnaire was addressed to two groups of people: those who knew the use of medicinal purposes, the local herbalist, and those who used medicinal plants, the patients.

Local herbalists

Eighteen local herbalists having a practical knowledge of the use plants in medicine were interviewed in six villages: M'daourouch, Sedrata, Drea, Machrouha, Taoura and Souk Ahras during the six-month period from January to June, 2010. Local herbalists were selected based on their knowledge of medicinal plants, either for self-medication or for treating patients.

Study population

A total of 200 patients from public health institutions were selected based on their socio-economic level, knowledge, attitude, and pathology (Höft et al., 1999) The study population included patients suffering from high blood pressure and diabetes of both sexes.

Data analyses

Informants were asked to be present at the local field collection sites, and indicate the medicinal plants being used with the local name. The species mentioned by the informants were then taxonomically identified. The botanical identification and the nomenclature of the listed plants with their different vernacular and scientific names were based on the morphological descriptions presented in the Flora of Algeria (Quezel and Santa, 1963).

RESULTS

Medicinal plants used by the local population

A total of 200 patients (112 women and 88 men) ranging in age from 10 to 90 years old, were included in the study. Fifty-three (53) people were listed who requested plants for use in partnership with synthetic drugs against arterial hypertension and diabetes for a percentage of 26.5%. Of these 53 patients, 31 requested plants against diabetes (15.5%), while 22 requested plants against hypertension (11% of the population) (Figure 1). As noted, of these two pathologies, diabetes is that which is known better by traditional medicine in Souk Ahras Province.

However, in order to propose a treatment for any patient presenting one or the other of the diseases, all the herbalists who operate as tradi-therapeutists also require the diagnosis of a doctor. It is an effective collaboration between western and traditional medicine practitioners. In order to ascertain the medicinal species used by these patients, as delivered by the herbalists, interviews were conducted with the herbalists. This approach demonstrates that diabetic patients use medicinal plants in addition to pharmaceutical drugs. Furthermore, the local knowledge encompasses historical and present beliefs, traditions, practices, and views developed by the local human communities over time (Vandebroek et al., 2011).

Medicinal plant diversity and ethnobotanical knowledge

The results of the survey indicate that there are 59 medicinal plant species in use in Souk Ahras (Table 1). Most of these species grow naturally in the different local regions and their properties are important in traditional Arabic medicine. They are distributed in 35 plant families. The families most represented are Lamiaceae, Apiaceae, Liliaceae, Brassicaceae, Cupressaceae and Myrtaceae. Among the plants listed in Table 1, 44 species (74.6% of the total plants) are used for arterial hypertension and diabetes, 28 species (47%) are used for treating diabetes, and 15 species (25%) are used for treating high blood pressure. The most frequently used plants to treat diabetes include *Ajuga iva*, *Allium cepa*, *Myrtus communis*, *Olea europea*, and *Ptychotis verticillata*, and those to treat hypertension include *A. cepa*, *A. sativum*, *O. europea*, and *P. verticillata*. The plant part which is mostly used in medicinal preparations is the leaves. They are available throughout the year are of easy access and are a sustainable resource. They are followed by the stem bark, roots and the floral parts (Figure 2). The stem bark and root parts may or may not be sustainable, depending on the plant source. All of these plant parts are prepared mainly in the form of a decoction (47%). This is the mode of plant preparation most commonly used by the large majority of the herbalists. This is followed by 40% of infusions and 13% of macerations which are also prepared. These preparations are all prepared and used practically as a drink. According to the survey, the practitioners administer their remedies in the form of a standard decoction prepared by boiling the plant parts in hot water, an infusion in water or oil, or by

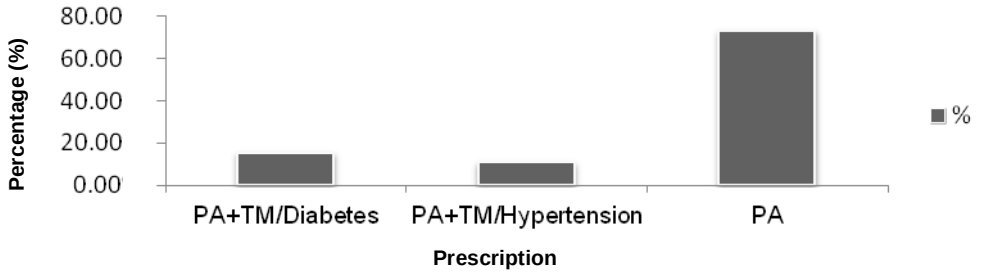

Figure 1. Distribution of the prescription according to mode of treatment. PA: Pharmaceutical agents, TM: traditional medicine.

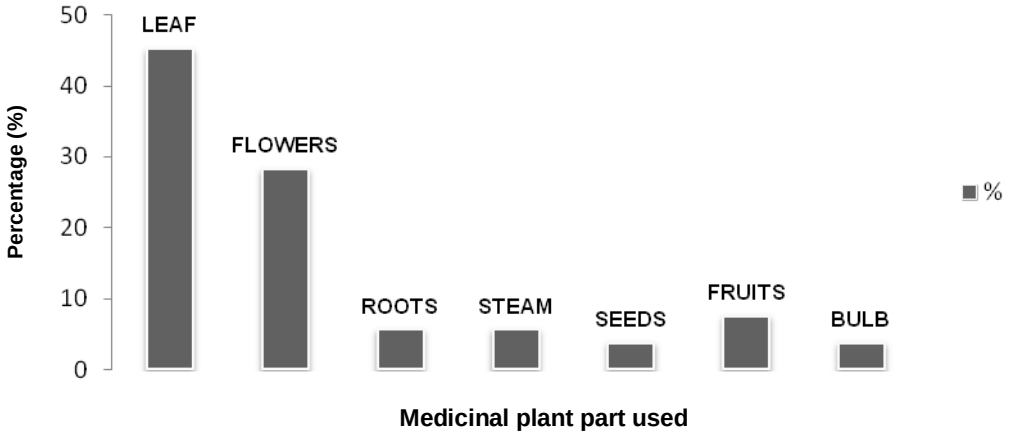

Figure 2. Distribution of the use of medicinal plants according to the plant part used.

macerating the plant parts in oil. The remedies were administered orally or externally according to the disease being treated and the method of preparation (Table 1).

DISCUSSION

The claimed therapeutic indications of some of these plants have been validated by studies in experimental animals. In patients with diabetes, for example *Artemisia herba-alba* (Al-Waili,1986,1988a), *Marrubium vulgare*, *O. europaea* var. *oleaster* (Circosta et al., 1986), *Trigonella foenum-graecum*, and for patients with hypertension *A. sativum*, *Art. herba-alba* (Al-waili et al., 1986; 1988; Eddouks et al., 2002; Jouad et al., 2001; Twaij et al.,1988) and *O. europea* var. *oleaster* (Komaki et al., 2003; Gonzalez et al., 1992; Sedef et al., 2009). Some species, such as *Hordeum vulgare* and *Zygophyllum cornutum* are described for the first time for treating diabetes and *Borago officinalis, Centaurea benedicta* and *Arbutus unedo* are indicated for treating hypertension in this survey.

The Islamization of Algeria played a paramount role in the cultural development of plant as medicinal agents. Among the plants quoted as anti-hyperglycemic, some are drawn directly from Qurrun and other religious manuscripts. This is the case in particular for *M. communis* L. and *Nigella sativa* L. (Eddouks et al., 2007). Among the cited plants, some are mentioned in other traditional pharmacopeias, especially those of the Mediterranean region. Cultural mixing supports the exchange of knowledge relating to the traditional systems of care (Eddouks et al., 2007). The food practices and the nutritional factors are regarded as the foundation in the treatment and the prevention of diabetes (Eddouks et al., 2007; Srivastava and Mehdi, 2005). However, some of the plants identified are toxic, although fortunately, most of the prescribers/users are aware of the toxic plants found in Souk Ahras province. The main toxic plants toxic plants are *Citrullus colocynthis* (Abdel-Hassan et al., 2000; Al-Ghaithi et al., 2004; Nmila et al., 2000), *Nerium oleander* (Eddouks et al., 2007), and *N. sativa* (Al-Hader et al., 1993; El Tahir et al., 1993; Murli et al., 2011; Labhal et al., 1999; Zaoui et al., 2000) which are still used in the treatment of diabetes and/or hypertension.

The importance of the conservation and preservation of medicinal plants, including the preservation of the ethnobotanical knowledge is being increasingly recognized. However, there are often significant problems with accessing and interpreting this knowledge (Huntington,

Table 1. List of medicinal plants used in traditional medicine in Souk Ahras Province (United States Department of Agriculture (USDA), 2010).

Family	Plant name	Local name	Part used	Therapeutic indications
Anacardiaceae	*Pistacia lentiscus* L.	Dhrou	LF	Expectorant: cough and bronchitis
			FR	Healing wounds and burns
			RS: Mastic	Irritations, stomach ulcers
Apiaceae	*Ammi visnaga* Lam.	Khlellal, Siouak en'bi	SE	Urethral lithiasis and nephretic colic
				Sedative
				Vasodilator
	Ptychotis verticillata Duby	Nûnkha	AP	Hypoglycemic
				Hypotensive
	Coriandrum sativum L.	Kosbar	LF	Carminative
			SE	Antispasmodic
	Apium graveolens L.	Krafs	RT, SE, LF	Antispasmodic
				Carminative
				Diuretic
	Pimpinella anisum L.	Habet h'lawa	SE, LF	Carminative
				Antispasmodic
				Stomachic
	Petroselinum sativum L.	Maâdanous	LF, RO, SE	Diuretic
				Hypotensive
Apocynaceae	*Nerium oleander* L.	Defla	LF	Cardiotonic
Capparidaceae	*Capparis spinosa* L.	Kebbar	FR,SE	Hypoglycemic, diuretic
Boraginaceae	*Borrago officinalis* L.	Boukhrich	LF	Diuretic
			FL	Sudorific
				Hypotensive
Brassicaceae	*Artemisia absinthium* L.	Chadjaret Merièm	LF	Hypoglycemic
	Artemisia herba alba Asso	Chih	LF, FL	Hypoglycemic
	Centaurea benedicta L.	Khirriya, chouk el-djamel	PL	Hypotensive

Table 1. Contd.

	Cynara scolymus L.	Quarnoun	LF	Hypoglycemic, Depurative
	Lepidium sativum L.	Habb errachad	SE	Hypoglycemic
Cucurbitaceae	*Citrullus colocynthis* (L.) Schrad.	Handal	FR	Hypoglycemic
Cupressaceae	*Juniperus phoenicea* L.	Aâr-âar	LF	Hypoglycemic
			LF	Diuretic
			FR	Antirhematic , Antiseptic
	Tetraclinis articulata Mast.		LF	Hypoglycemic, Hypotensive
			SE	Hypoglycemic
Ericaceae	*Arbutus unedo* L.	Lenj	LF	Hypoglycemic, Hypotensive
			RT	Antiinflammatory, antidiarrheal
Euphorbiaceae	*Ricinus communis* L.	Kharowâ	LF	Purgative
			SE	Laxative
Fumariaceae	*Fumaria officinalis* L.	Soltan el bouqoul	AP (wihout RT)	Hypotensive, diuretic
Globulariaceae	*Globularia alypum* L.	Tasselgha	LF, FL	Purgative, depurative
				Hypoglycemic
Poaceae	*Hordeum vulgare* L.	Chair	SE	Diuretic
				Hypoglycemic
Hypericaceae	*Hypericum perforatum* L.	Mesmoun	FL	Healing
Lauraceae	*Laurus nobilis* L.	Rand	LF	Tonic
				Antiseptic
				Hypoglycemic
Liliaceae	*Allium cepa* L.	Bsel	BL	Hypotensive
				Hypoglycemic
	Allium sativum L.	Tthoum	BL	Hypotensive
	Tanacetum parthenium Sch. Bip.	Baboundj	FL, LF	Anti-inflammatory
				Antispasmodic

Table 1. Contd.

Lamiaceae	*Ajuga iva* Schreb.	Chendgoura	FL, LF	hypoglycemic
	*Marrubiumvulgare*L.	Marriouret	FL	Hypoglycemic
	Mentha piperata L.	Nânâ har	AP	Analgesic
				Carminative
	Mentha viridis L.	Nânâ	LF	Diuretic
	Origanum majorana L.	Mardqouch	FL	Stomachic
	Origanum vulgare L.	Zaatar	FL	Stomachic
	Rosmarinus officinalis L.	Klil	FL, LF	Hypoglycemic
				Diuretic
				Stimulant
	Salvia officinalis L.	Souak en'nbi	LF, FL	Antisudorale
				Antispasmodic
				Hypoglycemic
	Thymus vulgaris L.	Zaitra	LF	Stomachic
				Antiseptic
				Antispasmodic
Loranthaceae	*Viscum album* L.	Loussiq	LF	Hypotensive
Malvaceae	*Malva sylvestris* L.	Khoubeiza	FL, LF	Laxative
Moraceae	*Morus nigra* L.	Toute	LF	Hypoglycemic
			SE	Laxative
				Diuretic
Myrtaceae	*Eucalyptus globulus* Labill.	Kalitus	LF, FL	Hypoglycemic , hypotensive
	Myrtus communis L.	Raihan	LF, FR	Hypoglycemic
				Anti-inflammatory
Oleaceae	*Olea europea* L.	Zaitoun	LF, FR	Hypoglycemic
				Hypotensive
Papaveraceae	*Papaver rhoeas* L.	Bbenaâman	FL	Soothing
				Sudorific, Emollient

Table 1. Contd.

Papilionaceae	*Trigonella foenum graecum* L.	Besbas	SE	Hypoglycemic
Polygonaceae	*Rumex patientia* L.	Houmeida	RT	Laxative
Ranuculaceae	*Nigella sativa* L.	Sanouj	SE	Hypoglycemic , Hypotensive
Rhamnaceae	*Zizyphus lotus* (L.) Lam.	Sadra	LE	Hypoglycemic, urinary infections
Rosaceae	*Rosa canina* L.	Nesrine	LF, FL	Calmantes (palpitations)
			FR	Astringent, anti diarrhéique
	Crataegus oxyacanta L.	Boumkherri	LF, FR, FL	Antispasmodic Hypotensive
Rutaceae	*Ruta graveolens* L.	Fidjel	AP	Emmenagogue Antihelminthic
Tiliaceae	*Tilia cordata* L.	Zaizafoun	FL	Antispasmodic Sedative
Urticaceae	*Urtica dioica* L.	Horaigua	LF	Hypoglycemic
Valerianaceae	*Valeriana tuberosa* L.	Sounboul	RT	Hypotensive
Verbenaceae	*Verbena officinalis* L.	Louiza	AP	Antispasmodic Anti-inflammatory
Zingiberaceae	*Zingiber officinale* L.	Zanjabil	RH	Tonic Analgesic
Zygophyllaceae	*Zygophyllum cornutum* Coss.	Bougriba	AP	Hypoglycemic

AP: areal part, BL: bulb, FL: flowers, FR**:** fruit, LF: leaf, PL: whole plant, RH: Rhizome, RS: resin, RT: roots, SE: seeds

2011; Saslis-Lagoudakis and Clarke, 2012). Recently, Saslis-Lagoudakis and Clarke. (2012) reportedthat the closer interaction between local practitioners and ethnobiologists who can study the relation-ship between humans and the natural world will enable local knowledge to be better applied in ecological and evolutionary biological research. The collaboration between local practitio- ners, ecologists, evolutionary biologists and ethnobio- logists is one of the most effective ways to incorporate local knowledge into biodiversity-related research (Saslis-Lagoudakis and Clarke, 2012).

Conclusion

To preserve the ethnobotanical knowledge, it is

important to document and restore the remains of ancient medical practices that still exist in Algeria. For instance, traditional medicine can usefully and cost-effectively be integrated into the treatment of type II diabetes and hypertension using an optimized strategy for the patient. Scientific collaboration between local practitioners, ecologists, evolutionary biologists and ethnobiologists should be encouraged in Algeria, to access local knowledge and incorporate it into biodiversity research. The preservation of the traditional knowledge is an essential requirement for prioritizing and conducting research on natural products and drug development as a way to provide and enhance local cost-effective local health care practices (Saslis-Lagoudakis and Clarke, 2012).

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Full Length Research Paper

The quantitation of hydroxymethylfurfural in Australian *Leptospermum* honeys

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This research extends the rapid high-performance liquid chromatographic (HPLC) analysis of *O*-(2, 3, 4, 5, 6-pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA) derivatives of methylglyoxal and dihydroxyacetone in Australian *Leptospermum* (L.) honeys to quantify hydroxymethylfurfural (HMF) content. Results showed that among the studied honeys stored at 4°C, all six *L. polygalifolium* and one *L. liversidgei* honeys exceeded the HMF upper limit (40 mg/kg) of the International Honey Commission (IHC), while four *L. liversidgei*, one *L. semibaccatum* and one *L. laevigatum* honeys satisfied this IHC regulation. It was found that all of the 13 heat treated (37°C for 60 days) *Leptospermum* honeys exceeded the IHC limit.

Key words: Hydroxymethylfurfural, *Leptospermum*, honey.

INTRODUCTION

Honey is prone to sugar crystallization. This rate of crystallization increases with increasing supersaturation ratio of glucose and viscosity. The former of these factors dominate at temperatures below 15°C, whilst the latter factor dominates at temperatures above 15°C (Venir et al., 2010). Commercial honey processing methods such as heating (significantly above 15°C), decrease viscosity and thus successfully decrease the rate of crystallization of honey (Turhan et al., 2008), and are also responsible for the production of undesirable compounds that reduce honey quality (Ajlouni and Sujirapinyokul, 2010). Hydroxymethylfurfural (HMF), a cyclic aldehyde, is one of such unfavourable compounds, which is virtually absent in fresh and untreated foods (Teixido et al., 2011). Although HMF is found in a variety of processed foods; honey is the only food for which there exists a recommendation on the allowable content of HMF (Arribas-Lorenzo and Morales, 2010). HMF is high in honeys that have been heat treated, stored in non-adequate conditions or adulterated with invert syrup (Ajlouni and Sujirapinyokul, 2010), thus HMF is a recognised parameter related to the quality of honey (Spano et al., 2006). The International Honey Commission (IHC) has stated that after processing and/or blending, HMF levels shall not exceed 40 mg/kg, unless the honey originates from regions with tropical ambient temperatures, in which case levels shall not exceed 80 mg/kg (Ajlouni and Sujirapinyokul, 2010).

The main concern surrounding HMF intake by humans is that, in both *in-vivo* and *in-vitro* experiments (Capuano and Fogliano, 2011), sulfotransferases (SULTs) metabolise HMF to its mutagenic derivative sulfomethylfurfural (SMF) by sulfonation of the allylic hydroxyl functional group (Arribas-Lorenzo and Morales, 2010; Husoy et al., 2008; Teixido et al., 2011). Differing views persist about the risk HMF and its metabolite SMF pose to human health (Spano et al., 2009). The cytotoxicity of HMF to humans has been demonstrated by reduced granulocyte metabolism (Nassberger, 1990).

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Epidemiological studies identified HMF in caramelised sugar as a possible dietary factor associated with the risk of colorectal cancer (Bruce et al., 1993). Single subcutaneous injections showed both HMF and SMF to be weak intestinal carcinogens in multiple intestinal neoplasia mice (Svendsen et al., 2009). There is the concern that humans may be more sensitive to HMF than rats and mice, because humans express SULTs in their extrahepatic tissue more extensively compared to rats and mice (Capuano and Fogliano, 2011; Husoy et al., 2008). In contrast, genotoxic and mutagenic effects were not observed in *in vitro* experiments of mammalian cells, except at high (≥ 1500 mg/kg) HMF concentrations (Janzowski et al., 2000).

Since consumer protection and quality control gained importance, the presence of potentially toxic compounds in foods has been attracting more attention (Spano et al., 2009). This attention has been directed to the determination of HMF in several food products (including honey), because HMF occurrence is an indication of quality deterioration (Schultheiss et al., 2000). The IHC has recommended three methods for determination of HMF (Zappala et al., 2005). Method 1 involved the measurement of ultraviolet (UV) absorbance of clarified aqueous honey solutions with and without bisulphite (White, 1979). Method 2 involved the measurement of UV absorbance of honey solutions with added barbituric acid and *p*-toluidine (Winkler, 1955). Method 3 involved the dissolution of honey in water and after filtration, HMF determination on a reversed phase-high performance liquid chromatography (RP-HPLC) column by isocratic elution with water and methanol mobile phases (Jeuring and Kuppers, 1980). Methods 1 and 3 usually gave similar values for HMF, but Method 2 consistently gave HMF values higher than the other methods (Zappala et al., 2005). Sulphuric acid was added to the water/methanol mobile phase of Method 3 in a new gradient method to completely resolve the peaks of HMF and homogentisic acid (the marker of strawberry tree origin of honey) (Spano et al., 2006). This adapted method has since been applied to concurrently determine HMF, 2-furfural, 3-furfural, 2-furoic acid and 3-furoic acid in strawberry tree, thistle cardoon and *Eucalyptus* honeys (Spano et al., 2009). No previous methods have concurrently determined potentially toxic and potentially beneficial compounds in honey.

This study extends previous PFBHA derivatisation and RP-HPLC determination of the beneficial antibacterial compound MGO and its precursor DHA in Australian *Leptospermum* (L.) honeys (Windsor et al., 2012) to the determination of the potentially toxic HMF in Australian *Leptospermum* honeys (a genus that has not previously been studied in relation to HMF concentrations). This study investigates the concentrations of HMF in Australian *Leptospermum* honeys and the effect of heating on these concentrations. This technique provides a sensitive and selective means for the simultaneous determination of potentially toxic HMF and also potentially beneficial bioactive compounds present in honey.

MATERIALS AND METHODS

The honey derivatisation and HPLC conditions were adapted from Windsor et al. (2012), a brief summary follows. Honeys were obtained from Tyagarah Apiaries, Tyagarah, New South Wales, Australia, 2841. Unprocessed honey samples were stored at 4°C until HMF analysis via HPLC, while a duplicate of each sample was heated at 37°C for 60 days prior to HMF analysis via HPLC. HPLC MilliQ grade water was used in all analyses. HPLC Chromasolv grade acetonitrile (ACN) was obtained from Merck, Kilsyth, Victoria, Australia, 3137. PFBHA (99%) and hydroxyacetone (HA) (90%) were purchased from Sigma-Aldrich, Castle Hill, New South Wales 1765, Australia.

Analyses were performed on a Perkin Elmer Series 200 Pump and Autosampler with a Flexar photo diode array detector (λ = 263 nm). HPLC separations were performed on a Synergi Fusion column (75 × 4.6 mm, 4 μm particle size). Mobile phase A was water:ACN (70/30, v/v) and mobile phase B was 100% ACN. The following 23 min gradient elution was employed: A:B = 90:10 (isocratic 2.5 min), graded to 50:50 (8.0 min), graded to 0:100 (1.0 min), 0:100 (isocratic 7.0 min), graded to 90:10 (1.0 min), 90:10 (isocratic 4.0 min), and detection at 263 nm.

The PFBHA derivatising reagent was 19.8 mg/ml in citrate buffer (0.1 M) adjusted to pH 4 with NaOH (4 M). The HA internal standard solution was prepared by dissolving HA (152.5 mg) in 50.0 ml of water. Honey samples (0.1 to 0.15 g) were weighed into 16 × 75 mm test tubes. HA standard solution (250 µl) and PFBHA derivatising solution (1500 µl) were added to each of these test tubes. Each of the test tubes was thoroughly mixed and was allowed to stand for 1 h for complete derivatisation. ACN (6 ml) was added to each test tube and mixed until all crystals dissolved. Water (2 ml) was added to each test tube and was mixed. A 1.5 ml aliquot of each sample was placed in an HPLC vial for analysis. Calibration was against a series of HMF standards with the same addition of HA internal standard.

RESULTS AND DISCUSSION

An HPLC method has previously been used to simultaneously separate a standard mixture of potentially toxic compounds (HMF, 2-furfural, 3-furfural, 2-furoic acid and 3-furoic acid) and successfully been applied to real samples of strawberry tree, thistle cardoon and *Eucalyptus* honeys (Spano et al., 2009). This work's HPLC method concurrently determines the potentially toxic HMF, and also the beneficial antibacterial compound MGO and its precursor DHA in Australian *Leptospermum* honeys. The PFBHA derivative of HMF has both a cis and trans isomer around its C=N double bond. These two isomeric forms of this potentially toxic compound were observed as two distinct peaks in the HPLC trace eluting at 10.87 and 11.25 min (Figure 1). The PFBHA derivatives of the beneficial antibacterial compound MGO (eluting at 16.4 min) and its precursor DHA (eluting at 4.7 min) also elute as clear peaks (Figure 1). Therefore, this technique could be used as a quantitation method for all of these three compounds. The HPLC trace also showed derivatised sugars (eluting at 1 to 2 min), internal standard, hydroxyacetone (eluting

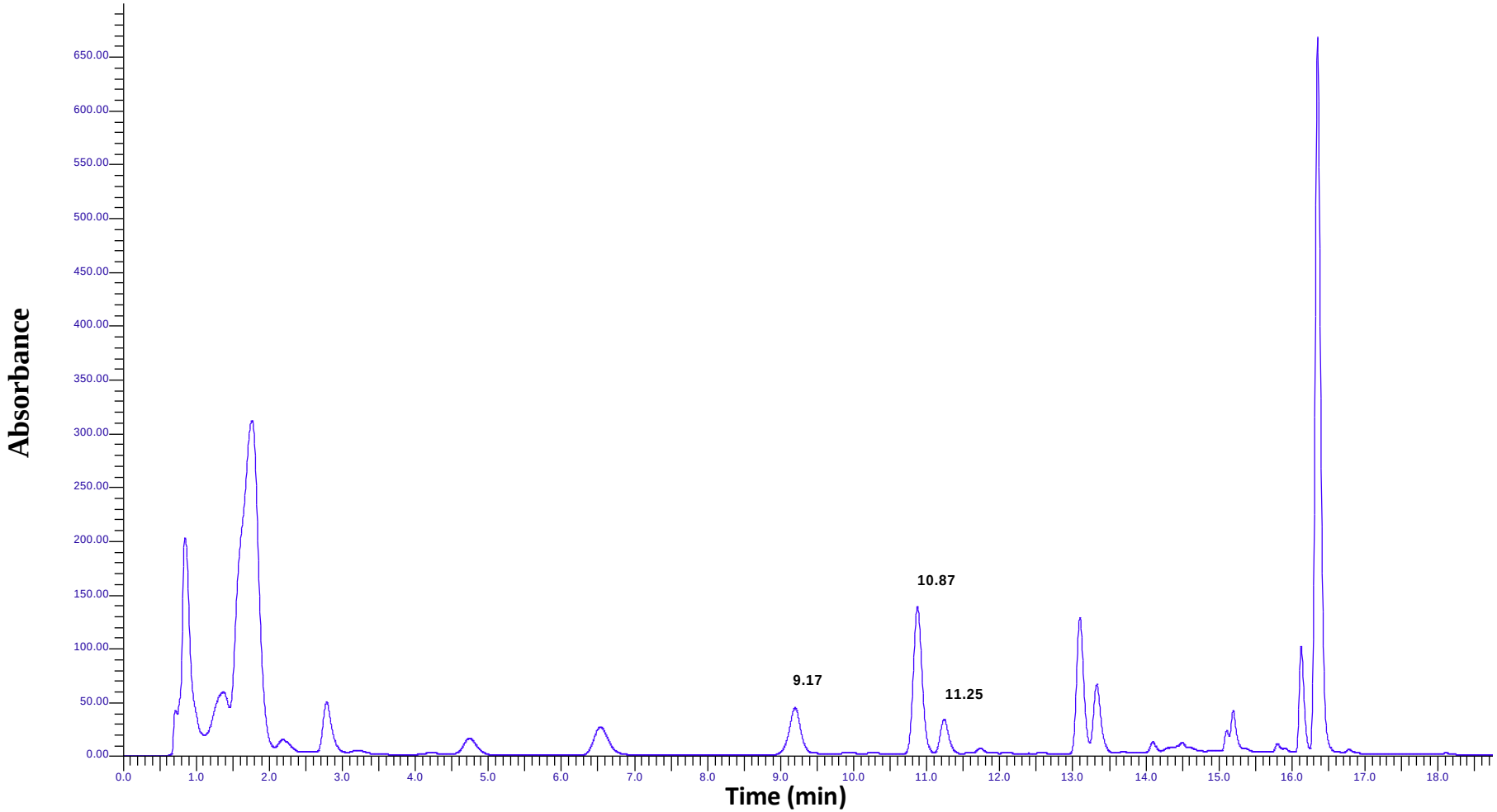

Figure 1. HPLC trace of heat treated sample number 1.

at 9.17 min) and the PFBHA derivatising agent at 6.5 min PFBHA peak shows that the derivatisation reaction of HMF and the other carbonyl compounds present in the honey went to completion. Unprocessed honeys stored at 4°C until HMF analysis via HPLC have HMF content largely determined by the botanical origin of the honey (Spano et al., 2009; Turhan et al., 2008). Eleven out of twelve strawberry tree honeys had HMF to honey concentrations greater than the IHC recommended limit of 40 mg/kg (Spano et al., 2009); whilst honeydew, thistle, *Eucalyptus*, cistus and chestnut honeys had HMF to honey concentrations less than the IHC recommended limit of 40 mg/kg (Spano et al., 2009; Turhan et al., 2008). This work's analysis of unprocessed Australian *Leptospermum* honeys stored at 4°C until HMF analysis via HPLC showed a genus average and

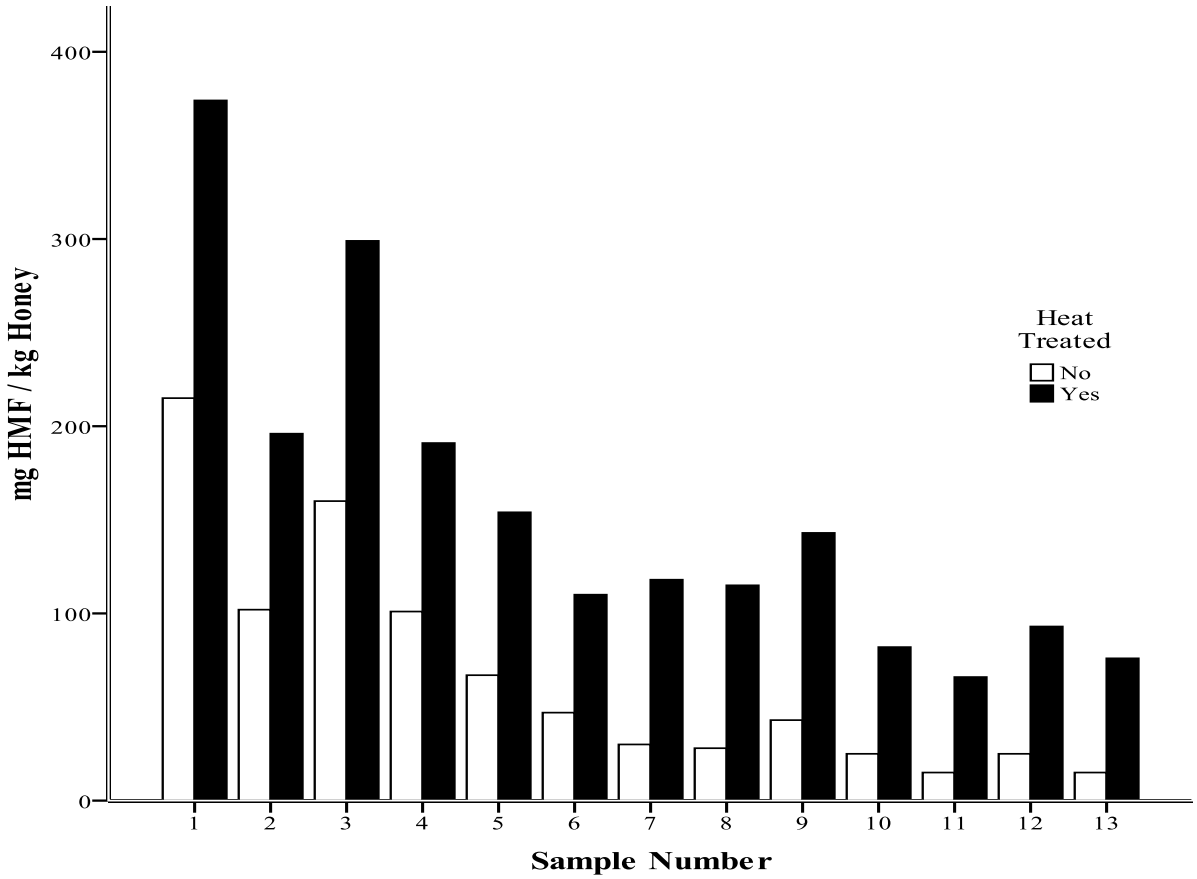

Figure 2. HMF content in untreated and heat treated honey samples.

standard deviation of HMF to honey concentrations of 67 ± 62 mg/kg, but also revealed a species specific determination of HMF content. All *Leptospermum polygalifolium* honey samples (sample numbers 1 to 6) and one (sample number 9) out of five *Leptospermum liversidgei* honey samples (sample numbers 7 to 11) had HMF to honey concentrations greater than the IHC recommended limit of 40 mg/kg; whilst *Leptospermum semibaccatum* and *Leptospermum laevigatum* honey samples (sample numbers 12 and 13, respectively) had HMF to honey concentrations less than the IHC recommended limit of 40 mg/kg (Figure 2).

The honeydew honey stored at 4°C until initial HPLC analysis mentioned earlier (Turhan et al., 2008), was then heated at 75°C for 90 min, but its HMF to honey concentration was still less than the IHC recommended limit of 40mg/kg (Turhan et al., 2008). Unprocessed Australian honeys from *Banksia* and *Eucalyptus* botanical origin stored at room temperature until initial HPLC analysis, then heated at 65°C for 2 min, had HMF to honey concentrations less than the IHC recommended limit of 40 mg/kg prior and post heat treatment (Ajlouni and Sujirapinyokul, 2010). Twenty samples of commercially available honeys in Spain stored at room temperature until initial HPLC analysis, then heated at 35°C for 29 days, had HMF to honey concentrations less than the IHC recommended limit of 40 mg/kg prior to heat treatment, but some samples showed levels greater than this limit towards the end of the heating period (Escriche et al., 2008). It is believed that the temperatures honey would be exposed to during collection and storage, range between 35 and 45°C (Escriche et al., 2008). Therefore, the temperature at which samples were heated in this study (37°C for 60 days) are realistic conditions for temperatures honey may be exposed to. Australian *Leptospermum* honeys subjected to this heat treatment showed an average and standard deviation of HMF to honey concentrations of 155 ± 91 mg/kg. A paired samples t-test revealed a statistically significant increase of HMF to honey concentrations of 88 ± 32 mg/kg (paired t = 10.071; one-tail P < 0.001; df = 12) post heat treatment. Figure 2 shows all heat treated Australian *Leptospermum* honeys had HMF to honey concentrations greater than the IHC recommended limit of 40 mg/kg.

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