South African Journal of Botany 128 (2020) 200–208

Contents lists available at ScienceDirect

South African Journal of Botany

journal homepage: www.elsevier.com/locate/sajb

Quinic acid derivatives from *Artemisia annua* L. leaves; biological activities and seasonal variation

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ARTICLE INFO

Article History:
Received 7 August 2019
Revised 30 October 2019
Accepted 9 November 2019
Available online 22 November 2019

Edited by B Ncube

Keywords:
Anti-hyperglycemic
Artemisia annua L
Hepatoprotective
HPLC standardization
Quinic acid derivatives
Seasonal variation

ABSTRACT

Genus *Artemisia* is widely known to have various therapeutic applications and chemical constituents. *A. annua* L. (commonly known as sweet wormwood or Qinghao) is an annual herb native to China, traditionally used for treating fever and malaria, and as a source of artemisinin. In the present study, a bio-guided fractionation of the 70% ethanolic extract of the leaves of *A. annua* cultivated in Egypt produced a bioactive polar fraction. Daily doses of this fraction (100 mg/kg b.wt) for 4 weeks substantially reduced the level of the CCl_4-mediated increase in the liver enzymes; AST, ALT and ALP. Similarly, daily doses of the polar fraction (100 mg/kg) significantly reduced the blood glucose level in alloxan-induced diabetic by 32.1% in the second week and 46.9% in the fourth week, relative to that demonstrated by metformin (66.2%), and significantly ($p < 0.01$) restored to normal the blood glutathione level (35.2 ± 1.3 mg/dL), almost identical to that shown by vitamin E. Further purification of the bioactive fraction led to the isolation of 5 quinic acid derivatives; 3-feruloylquinic acid, 3,5-dicaffoeylquinic acid, 4,5-dicaffoeylquinic acid, 3,4-dicaffoeylquinic acid, and 3,4-dicaffoeylquinic acid methyl ester. The isolated compounds are reported here for the first time in *A. annua*, leaves cultivated in Egypt and suggested to be responsible, at least in part, to the biological activities of the polar fraction. Seasonal variation in the content of quinic acid derivatives in the leaves of *A. annua* L. was investigated during four harvest seasons (March, May, July "pre-flowering stage" and August-September "flowering stage") using RP-HPLC. The content of 3-feruloylquinic acid varied greatly throughout the year. It was found to be the lowest (0.036% w/w) in March "leaf stage" but increased through the warmer months from May to June (0.61% w/w), and reached the highest (1.34% w/w) in leaves harvested in July "early summer". Then-after, gradual decline in the content of quinic acid derivatives was evident in leaves collected during flowering stage in late summer "August-September" (0.84% w/w).

1. Introduction

Research interest in genus *Artemisia* is mainly due to its frequent applications in traditional medicine and diversity in chemistry and biological activities (Wright, 2001). Medicinal uses of *A. annua* (commonly known as sweet wormwood or Qinghao) in Chinese medicine is known to have occurred as early as 168 BC. for treating fever, inflammation and malaria (Ferreira et al., 1997; WHO, 2006). In Pakistan, a decoction of the herb is used for the treatment of malaria, while the leaves are used for fever, cough, common cold, and to treat diarrhea, while the oil is used in perfumes (Hayat et al., 2009). The leaves are eaten as part of salads in some Asian countries. In United States, companies currently sell powder and extracts of *A. annua* leaves as dietary supplements. The plant remains the most valuable source for supplying artemisinin and its several derivatives.

The discovery of artemisinin dramatically changes the landscape to combat malaria and leads to a paradigm shift in antimalarial drug development (Reiter et al., 2015). Artemisinin has saved millions of lives and represents one of the significant contributions of China to global health (Reiter et al., 2015). The 2015 Nobel Prize in Physiology or Medicine was awarded to Professor Youyou Tu for her key contributions to the discovery of artemisinin (ART) (Su and Miller, 2015).

ART and its derivatives were found to be effective against cancer, leishmania (Sen et al., 2007; Yang and Liew, 1993), Trypanosoma

Abbreviation: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DMSO, dimethyl sulphoxide; GSH, Glutathione; HPLC/PDA/ESI/MS-MS, high performance liquid chromatography coupled with photodiode array detector and Mass spectroscopy; 1H NMR and 13C NMR, proton and carbon nuclear magnetic resonance; HCT116, human colon carcinoma cell line; HELA, human cervical carcinoma cell line; HePG2, human hepatocellular carcinoma cell line; LD50, Median lethal dose; RP-HPLC, reversed phase-high performance liquid chromatography; SRB, Sulforhodamine B; TMS, trimethyl silane; VLC, Vacuum Liquid Chromatography

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https://doi.org/10.1016/j.sajb.2019.11.008
0254-6299/

(Mishina et al., 2007) and have antiviral activities (Abid Ali Khan et al., 1991; Li et al., 2005), and WHO recommends that the drug to be delivered as part of a combination therapy (ACT, artemisinin combination therapy) for the treatment of chloroquine-resistant malaria (Bhakuni et al., 2001; Ferreira, 2007; Namdeo et al., 2006; Sriram et al., 2004; WHO, 2006). A study on the seasonal variation of ART and its biosynthetic precursors in *A. annua* of different geographical origin was performed by Wallaart et al. (2000). Regardless of the genotype, the plants consistently reach their ART peaks during the vegetation period from May towards its end in August [El-Askary et al. (2004)]. El-Askary et al. (2004) reported a successful field experiment for producing an Egyptian cultivar of the plant with better ART content (1% of dry weight at the pre-flowering stage).

Ferreira, J.F.S., Benedito, V.A., Sandhu, D., J.A., Liu, S. (2018). Seasonal and Differential Sesquiterpene Accumulation in *Artemisia annua* suggest selection based on both artemisinin and dihydroartemisinic acid may increase artemisinin in planta. *Front Plant Sci.* 9: 1096.

Many species of genus *Artemisia* demonstrate antioxidant and hepatoprotective activities. The hydro-alcoholic extract of *Artemisia aucheri* and *A. dracunculus* displayed protective effect against CCl_4-induced hepatotoxicity in rats, which was suggested to be produced as a result of its effect on oxidative stress (Ghavamizadeh and Mirzaee, 2015; Zarezade et al., 2018). High content of flavonoids in the crude alcoholic extract of *A. annua* leaves was presumed to be responsible for its *in vitro* antioxidant activities (Bilia et al., 2006; Cai et al., 2004; Zheng and Wang, 2001). Furthermore, number of secondary metabolites in its polar fraction was tentatively identified using HPLC/PDA/ESI/MS-MS, and their *in vitro* antioxidant and hepatoprotective activities were investigated (El-Askary et al., 2019).

In a continuation of our work on *A. annua* L. cultivated in Egypt, this study was designed to: 1)- investigate the effect of the polar fraction for its hepatoprotective, antihyperglycemic and antioxidant activities in animal models of hepatotoxicity and diabetes, 2)- isolate and identify major compounds from this fraction, and 3)- investigate the seasonal accumulation of these compounds in the leaves of the plant throughout the year using RP-HPLC technique.

2. Materials and methods

2.1. General

Silica gel RP18 for Vacuum Liquid Chromatography (VLC) and Diaion HP-20 for column Chromatography (Merck, Germany). Bruker NMR Spectrometer, Japan) for ^{1}H NMR (400MH$_Z$) and ^{13}C NMR (100 MHz). Spectra recorded in CD_3OD or DMSO-d6 using TMS as internal standard, and chemical shift values were expressed in δ ppm. Transaminase Kits: (BioMerieux SA, l'Etoile France), Biodiagnostic kits l'Etoile, France) were used for assessment of serum ALT, AST and ALP. Biodiagnostic glutathione kit for the assessment of antioxidant activity and Biomerieux kit for the assessment of blood glucose level (Wak-Chemie Medical, Germany). Silymarin was from Sedico Pharmaceutical Co, (6th of October City, Egypt), metformin was from Chemical Industries Development (C.I.D.) (Giza, Egypt) and Vitamin E (dl α-tocopheryl acetete) was from Pharco Pharmaceutical Co. (Alex., Egypt). Carbon tetrachloride (analar) and Alloxan (Sigma-Aldrich Co., Germany). *O*-Phosphoric acid used was of analytical grade from SD fine Chemlimited (Mumbai, India). Distilled water was further purified using a Milli-Q system (Millipore, MA and USA). Acidulated water was filtered through a 0.45 μm membrane filters (Pall Gelman Laboratory, USA), and degassed in an ultrasonic bath before use in HPLC analysis.

2.2. Plant material

Samples of *Artemisia annua* L. used in this study were collected from the Experimental Station of Medicinal Plants of Faculty of Pharmacy, Cairo University in Giza in July 2011. Plant identity was kindly confirmed by Dr. Ebrahim A. El-Garf, Professor of Botany Department, Faculty of Science, Cairo University, Egypt. A voucher specimen (No. 13-04-2014) was deposited at the Herbarium of the Faculty of Pharmacy, Cairo University, Egypt.

2.3. Extraction and fractionation

The air-dried leaves (2 Kg) of *A. annua* were extracted with 70% ethanol by maceration till exhaustion yielding dark green residue (470 g, 23.5% w/w). The combined polar fraction was prepared as under El-Askary et al. (2019) and used in this study.

2.4. Human cell line for in vitro cytotoxic screening

The following cell lines (supplied by the National Cancer Institute, Cairo, Egypt) were used in this study; human hepatocellular carcinoma cell line (HeP-G2), human colon carcinoma cell line (HCT116) and human cervical carcinoma cell line (HELA). Cytotoxicity of the ethanolic extract, polar fraction and two of the isolated compounds was tested adopting the method proposed by Skehan et al. (1990) using Sulforhodamine B (SRB) stain and ELISA reader at λ_{max} 564 nm.

2.5. In vivo activity

2.5.1. Experimental animals

Thirty male albino mice of 25–30 g body weight and sixty adult male Sprague Dawely albino rats of 130–150 g body weight were used in this study. The animals were kept under the same hygienic conditions and on a standard laboratory diet consisting of vitamin mixture (1%), mineral mixture (4%), corn oil (10%), sucrose (20%), cellulose (0.2%), casein 95% pure (10.5%) and starch (54.3%). The study was performed according to the international rules and guidelines of the Ethical Committee of the National Research Centre for experimental animals use.

2.5.2. Determination of acute toxicity (LD_{50})

Median lethal dose (LD_{50}) of the ethanolic extract was determined according to Andress (1992). LD_{50} of the extract was estimated on mice (n = 30) divided into five groups (6 mice, each) after oral administration of single doses of the extracts (ranging from 1–5 g/kg b.wt.; the maximum soluble dose).

Preliminary experiment was carried out to determine the minimal dose that kills all animals (LD_{100}) and the maximal dose that fails to kill any animals. Several doses at equal logarithmic intervals were chosen in between, each dose was injected in a group of 6 animals by oral administration. The mice were then observed for 24 hrs and symptoms of toxicity and mortality rates in each group were recorded and the LD_{50} was calculated.

2.5.3. In vivo hepatoprotective activity

Liver damage in rats was induced by intraperitoneal injection of 25% carbon tetrachloride (CCl_4) in liquid paraffin (5 ml/kg) (Klassen and Plaa, 1969). Eighteen rats were divided into three groups each of six rats, Group I: control group received a daily oral dose of 1 ml saline for one week before and after liver damage. Group II: liver damaged rats pretreated with daily oral dose of 100 mg/kg body weight of the polar fraction for one week before induction of liver damage by CCl_4. Administration of the fractions was continued after liver damage for another one week. Group III: liver damaged rats pretreated with daily oral dose of silymarin (25 mg/kg). Administration of the drug was continued after liver damage for another one week followed by an overnight fasting. Blood was withdrawn from the retro orbital venous plexus through the eye canthus of anaesthetized rats. Samples were collected at zero time, one week and 72 hr after CCl_4 injection and after one week intervals. Serum was isolated by centrifugation and activity of all serum enzymes; ALT, AST

(Thewfweld, 1974) and ALP were measured according to the method of (Thewfweld, 1974) using commercially available kits (according to the manufacturer's instructions).

2.5.4. In vivo anti-hyperglycemic activity

Induction of diabetes mellitus was made according to the method described by (Eliasson and Samet, 1969) using alloxan. Eighteen male Sprague Dawely albino rats were injected intra-peritoneal with alloxan (150 mg/kg body weight). Only rats with serum glucose levels of more than 250 mg/dL were selected and considered diabetic animals. Animals were divided into 3 groups each of 6 rats, Group I: diabetic rats that served as positive control received a daily oral dose of 1 ml saline. Group II: diabetic rats that received daily oral dose of 100 mg/kg body weight of the polar fraction. Group III: diabetic rats that received daily oral dose of 100 mg/kg body weight of metformin "the reference drug". Hyperglycemia was assessed after 72 h, 2 and 4 weeks by measuring blood glucose level (Tinder, 1969). At the end of each study period, blood samples were collected from the retro orbital venous plexus through the eye canthus of anaesthetized rats after an overnight fast. Serum was isolated by centrifugation and the blood glucose level was measured (Tinder, 1969).

2.5.5. In vivo antioxidant activity

Induction of diabetes mellitus was made according to the method described by (Eliasson and Samet, 1969) using alloxan. Thirty male Sprague Dawely albino rats (130–140 g) were injected intraperitoneally with alloxan (150 mg/kg body weight). Hyperglycemia was assessed after 72 h by measuring blood glucose level (Tinder, 1969). Twenty-four rats were divided into four groups each of 6 rats: **Group I**: normal rats that served as negative control received 1 ml saline. **Group II**: diabetic rats received 1 ml saline (positive control). **Group III**: diabetic rats received 7.5 mg/kg body weight of vitamin E seven days after induction of diabetes mellitus. **Group IV:** diabetic rats received oral dose of the polar fraction (100 mg/kg body weight). After seven days, blood samples were collected, heparinized and used for determination of blood glutathione level using bio-diagnostic kit (absorbance at 405 nm), relative to vitamin E (reference drug) (Buetler et al., 1963).

2.6. Isolation of the major compounds from the polar fraction

Part of the polar fraction (50 g) was chromatographed on Diaion HP-20 column (45 × 3 cm i.d, 500 g) and elution was started with water and then with a mixture of water and methanol with gradual increase of methanol in water till 100% percentage. Fractions (250 ml each) were collected and monitored by HPLC to give three major fractions; **I-III**. VLC (RP-18, 6.7 cm L x 4 cm D, 23 g) of **fraction I** [eluted with 25% MeOH in H_2O, 7.5 g] gave **compound 1** (80 mg), while VLC (RP-18, 6.7 cm L x 4 cm D, 23 g) of Fr. **III** (eluted with 50–75% MeOH in H_2O, 1.5 g) gave 4 sub-fractions (**A, B, C** and **D**). VLC (RP-18, 29% MeOH/H_2O) of **sub-fr A** [eluted with 25–29% MeOH/H_2O, 1200 ml] gave **compound 2** (R_t = 10.2 in., 120 mg). Similarly, VLC of **sub-fr B** [eluted with 29–30% MeOH in water, 1800 ml) gave **compound 3** (62 mg), **sub-fr C** (eluted with 31% MeOH in water, 1500 ml) gave **compound 4** (30 mg), and **sub-fr D** [eluted with 32% MeOH in water, 1800 ml) gave **compound 5** (25 mg).

2.7. Spectroscopic data of the isolated compounds

Compound (1)

UV spectral data (MeOH) λ_{max}: 323–240 nm. ^{1}H NMR δ ppm (400 MHz, DMSO-d_6): Quinic acid moiety: 1.93, 2.10 (2H, m, H-6 axe, eq), 2.22 (2H, m, H-2 axe, eq), 5.37 (1H, m, H-3), 3.65 (1H, m, H-4), 4.17 (1H, m, H-5). Ferulic acid moiety: 3.76 (3H, s, OCH_3), 6.38 (1H, d, *J* = 15.6 Hz, H-8′), 6.79 (1H, d, *J* = 7.6 Hz, H-5′), 7.08 (1H, m, H-6′), 7.27 (1H, bs, H-2′), 7.61 (1H, d, *J* = 15.6 Hz, H-7′). ^{13}C NMR δ ppm (75 MHz, DMSO-d_6) Quinic and ferulic acid moieties: 35.88 (C-6), 44.13 (C-2), 56.10 (C 3′-CH_3), 69.8 (C-5), 73.0 (C-3), 74.02 (C-4), 76.35 (C-1), 115.50 (C2′), 115.67 (C8′), 115.99 (C5′), 123.49 (C6′), 126.04 (C1′), 145.20 (C4′), 145.23 (C7′), 148.4 (C3′), 166.8 (C9′), 179.33 (COO-).

Compound (2)

UV spectral data (MeOH) λ_{max}: 244–327 nm; ^{1}H NMR, δ ppm (400 MHz, DMSO-d_6): Quinic acid moiety: 2.1 (2H, m, H-6 axe, eq), 2.49 (2H, m, H-2 axe, eq), 3.78 (1H, m, H-4), 5.2, 5.25 (2H, m, H-5, H-3). Two caffeic acid moieties: 6.35 (1H, d, *J* = 15.9 Hz, H-8″), 6.29 (1H, d, *J* = 15.9 Hz, H8′), 6.79 (2H, d, *J* = 7 Hz, H-5″, 5′), 6.99 (2H, m, H6″, 6′), 7.03 (2H,brs,H-2″, 2′), 7.56 (1H,d,*J* = 15.9 Hz, H-7″), 7.6 (1H, d, *J* = 15.9 Hz, H-7′).

Compound (3)

UV spectral data (MeOH) λ_{max}: 245–327 nm, ^{1}H NMR δ ppm (400 MHz, DMSO-d_6): Quinic acid moiety: 2.12 (1H, m, H-6 axe), 2.17 (2H, m, H-2 axe, eq), 2.28 (1H, m, H-6 eq), 4.37 (1H, m, H-3), 5.11 (1H, m, H-4), 5.63 (1H, m, H-5). Two caffeic acid moieties: 6.21 (1H, d, *J* = 16 Hz, H-8″), 6.31 (1H, d, *J* = 16 Hz, H-8′), 6.76 (2H, d, *J* = 8.1 Hz, H-5″,5′), 6.93 (2H, m, H-6″,6′), 7.04 (1H, brs, H2″,2′), 7.47 (1H, d, *J* = 16 Hz, H-7″), 7.62 (1H, d, *J* = 16.2 Hz, H-7′).

Compound (4)

UV spectral data (MeOH) λ_{max}: 244–327 nm, ^{1}H NMR δ ppm (400 MHz, DMSO-d_6): Quinic acid moiety: 1.9 (1H, m, H-6 axe), 2.08 (2H, m, H_2-2 axe, eq), 2.17 (1H, m, H-6 eq), 4.2 (1H, m, H-5), 4.97 (1H, m, H-4), 5.37 (1H, m, H-3). Two caffeic acid moieties: 6.11 (1H, d, *J* = 16 Hz, H-8″), 6.25 (1H, d, *J* = 16 Hz, H-8′), 6.74 (2H, d, *J* = 8.1 Hz, H-5″,5′), 6.94 (2H, m, H-6″,6′), 7.01 (1H, brs, H2″,2′), 7.42 (1H, d, *J* = 16 Hz, H-7″), 7.48 (1H, d, *J* = 16.2 Hz, H-7′)

Compound (5)

UV spectral data (MeOH) λ_{max}: 244–327 nm, ^{1}H NMR δ ppm (400 MHz, DMSO-d_6): Quinic acid moiety: 1.9 (1H, m, H-6 ax, eq), 2.08 (2H, m, H_2-2 ax, eq), 2.17 (1H, m, H-6 eq), 3.75 (3H, s, OCH_3), 4.2 (1H, m, H-5), 4.89 (1H, m, H-4), 5.55 (1H, m, H-3). Two caffeic acid moieties: 6.18 (1H, d, *J* = 15 Hz, H-8″), 6.35 (1H, d, *J* = 15 Hz, H-8′), 6.72 (2H, d, *J* = 8.1 Hz, H-5″,5′), 6.96 (2H, m, H-6″,6′), 7.01 (2H, brs, H2″,2′), 7.42 (1H, d, *J* = 16 Hz, H-7″), 7.48 (1H, d, *J* = 16.2 Hz, H-7′).

2.8. Seasonal variation of quinic acid derivatives in the extract of A. annua leaves

2.8.1. HPLC apparatus

Agilent Technologies 1100 series, HPLC system (Agilent Technologies, Palo Alto, CA), equipped with a quaternary pump and degasser G1322A series 1200 was used. Agilent Chem Station software was used for data acquisition and processing. Separation was carried out on Lichrospher RP-C_{18} column (250 mmLx 4 mm ID, 5 μm, Merk, Germany), preceded by a C_{18} guard column (10 mm L x4 mm ID, 5 μm).

2.8.2. HPLC conditions

Quaternary pump (series 1200) and degasser (G 1322A). Lichro-Chart (250–4 mm) Lichrosphere RP-18 (5 μm) Merck column together with a Guard column (10–4 mm). UV detector at 325 nm. Mobile phase: methanol “solvent A” and 0.3% H_3PO_4 in H_2O “solvent B” applying gradient elution: 32% A/B to 72% A/B in 20 min. flow rate 1 ml/min, injection volume 20 μl, and UV detection: 325 nm.

2.8.3. Establishment of the calibration curve

An aliquot (5 mg) of **compound 1** was dissolved in 25 ml methanol, and the solution (200 μg/ml) was kept at 4 °C until use. Five serial concentrations of **compound 1** in methanol; 40, 80, 120, 160, and 200 μg/ml, were prepared, 20 μl of each were injected in triplicates, and calibration curve was constructed by plotting mean peak areas versus concentration. Linearity was assessed by linear regression method, calculated by the least square method. The correlation coefficient (r^2) for the standard calibration curve was 0.999; and

linearity of the peak area of **compound 1** was in the range of 40–200 μg/ml.

2.8.4. Effect of seasonal variation on the concentration of quinic acid derivatives in A. annua leaves

Samples (200 mg each) of the air-dried powder of *A. annua* leaves (collected at different seasons, were separately extracted with 70% EtOH (6 × 10 ml) in test tubes by sonication for 5 min till exhaustion. The combined extracts were transferred to a measuring flask (50 ml capacity), completed to the mark with 70% EtOH and mixed well by sonication. Five ml of the extract were purified by elution over Lichrolut®-RP-18 cartridge (500 mg), followed by washing with water (1 ml x 5) till complete elution of the active compounds (as monitored by HPLC). The eluate was transferred to a measuring flask (10 ml capacity), completed to the mark with water, mixed well and an aliquot (20 μl) was analyzed by HPLC. For comparison, content of quinic acid derivatives was determined in the water extract of the leaves.

2.8.4.1. Statistical analysis. GraphPad prism 7 software was used in statistical analyses of different biochemical markers. Results are expressed as mean±SEM (n = 6) and the analyses were performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test with a statistical significant difference at $p < 0.01$.

3. Results

3.1. LD_{50}

The ethanolic extract was found to be safe up to 4 g/kg b. wt.

3.2. Hepatoprotective activity

No significant differences in liver enzymes; AST, ALT and ALP (47.2 ± 2.4 IU/L, 36.7 ± 2.8 IU/L, 7.3 ± 0.1 KAU, respectively) in healthy rats (before liver damage; **Group I**) were observed during the study course after administration of either the polar fraction (100 mg/kg b.wt) or silymarin (25 mg/kg b.wt), as can be seen in **Groups II and III**, respectively (Table 1). However, liver damage was evident in the animals in **Group I** 7 days after daily administration of CCl_4; as indicated by significant increase in the activity of the liver enzymes (163.4 ± 6.2 IU/L, 149.6 ± 5.7 IU/L, and 52.1 ± 1.9 KAU, respectively) when compared to that of the rats before liver damage.

On the other hand, treatment of the liver damaged rats with silymarin (25 mg/kg b.wt) led to significant reduction in the level of the elevated enzymes as shown in **group III** (35.7 ± 1.2 IU/L, 33.9 ± 0.8 IU/L, 7.6 ± 0.1 KAU, respectively). Similarly, treatment with the polar fraction (daily dose of 100 mg/kg b.wt) (**Group II**) substantially reduced the level of the CCl_4-mediated increase in the liver enzymes; AST, ALT and ALP (51.9 ± 2.6 IU/L, 49.8 ± 1.6 IU/L, and 13.6 ± 0.7 KAU, respectively), relative to that shown in the rats in **group I**.

3.3. Antihyperglycemic activity

The effect of polar fraction of the ethanolic extract of *A. annua* leaves on blood glucose level was investigated on alloxan-induced diabetic rats and the results were presented in Table 2. It revealed that *i.p.* administration of alloxan significantly (p <0.01) increased the level of blood glucose in rats as shown in **group I** (254.9 ± 6.4 mg/dl). However, oral administration of the polar fraction **(**daily doses of 100 mg/kg b. wt) up to 4 weeks significantly reduced blood glucose level in diabetic rats. From the second week onwards, a significant antihyperglycemic effect was marked (32.1% reduction in blood glucose) and a maximum reduction in **Group II** was reached on the fourth week (46.9%), relative to that demonstrated by metformin (66.2%) (**Group III**) used as reference drug.

3.4. Antioxidant activity

Administration of alloxan significantly ($p < 0.01$) reduced the blood level of the endogenous antioxidant glutathione (21.7 ± 0.3 mg/dL), when compared to that of control group (36.5 ± 1.4 mg/dL) (Table 3).

Table 1
Effect of polar fraction (polar fr) of alcoholic extract of *A. annua* leaves on liver enzymes in CCl_4-damaged rats (n = 6).

Group	Levels of liver enzymes											
	Before liver damage						After liver damage					
	Zero			7d			72h			7d		
	AST(U/L)	ALT (U/L)	ALP (KAU)	AST(U/L)	ALT (U/L)	ALP (KAU)	AST(U/L)	ALT (U/L)	ALP (KAU)	AST(U/L)	ALT (U/L)	ALP (KAU)
Group I (+ saline)	47.2 ± 2.4	36.7 ± 2.8	7.3 ± 0.1	46.4 ± 1.2	37.2 ± 1.3	7.2 ± 0.1	152.3 ± 4.9	144.7 ± 5.3*	48.6 ± 1.4*	163.4 ± 6.2*	149.6 ± 5.7*	52.1 ± 1.9*
Group II (liver damaged) (+ Polar fr 100 mg/kg b. wt)	45.1 ± 1.8	38.9 ± 1.4	7.2 ± 0.1	43.6 ± 2.1	36.7 ± 1.6	7.1 ± 0.1	76.4 ± 2.8	62.3 ± 3.2*	22.7 ± 0.8*	51.9 ± 2.6•*	49.8 ± 1.6•*	13.6 ± 0.7•*
Group III (liver damaged + Silymarin 25 mg/kg)	42.6 ± 1.4	36.2 ± 1.4	7.4 ± 0.1	39.7 ± 1.3	34.7 ± 1.3	7.2 ± 0.1	51.3 ± 2.1	56.8 ± 1.7	18.3 ± 0.8*	35.7 ± 1.2•*	33.9 ± 0.8	7.6 ± 0.1

•Statistically significant from zero time at $p < 0.01$.
*Statistically significant from 72 hr after CCl4 at $p < 0.01$.

Table 2
Effect of polar fraction (Polar fr) of the alcoholic extract of *A. annua* leaves on blood glucose level in alloxan-induced diabetic rats.

Group/ Time (days)	Group I (Diabetic rats) Control group Mean ± S.E (mg/dL)	Group II (Diabetic rats + Polar fr)		Group III (Diabetic rats + Metformin)	
		M ± S.E (mg/dL)	% of change	M ± S.E (mg/dL)	% of change
Zero	254.9 ± 6.4	267.3 ± 8.2	–	259.7 ± 8.1	–
2 Weeks	262.3 ± 7.9	181.6 ± 5.9	32.1	146.3 ± 5.2*	43.7
4 Weeks	258.9 ± 7.7	141.7 ± 4.1	46.9	87.8 ± 2.4*	66.2
% Relative potency	–	70.9	100		

*Statistically significant difference from control group at $p < 0.01$.

Table 3
Effect of polar fraction (Polar fr) of the alcoholic extract of *A. annua* leaves on blood glutathione level in alloxan-induced diabetic rats.

Group	Blood glutathione level Mean ± *S.E* (mg/dl)	% of change	% Relative potency
Group I (control)	36.5 ± 1.4	–	–
Group II (Diabetic rats)	21.7 ± 0.3*	–	–
Group III (Diabetic + vitaminE)	35.9 ± 1.2	65.4	100
Group IV (Diabetic + Polar fr)	35.2 ± 1.3	62.2	95.1

*Statistically significant difference from control group at $p < 0.01$.

However, treatment with the polar fraction (100 mg/kg b.wt, *p.o.*) significantly ($p < 0.01$) restored to near normal the level of glutathione in the blood of diabetic rats (35.2 ± 1.3 mg/dL), almost identical to that shown by vitamin E used as a reference drug (35.9 ± 1.2 mg/dl, relative antioxidant potency of 91–95%).

3.5. Identification of the isolated compounds

Five major compounds (**1**–**5**) were isolated from the polar fraction using different chromatographic techniques. Based on their spectroscopic data; ^{1}H NMR, ^{13}C NMR and ^{1}H–^{1}H COZY (see experimental), the chemical structures of these compounds **(Fig. 1)** were identified as 3-feruloeylquinic acid (**1**) (Chen et al., 2014; Lin and Harnly, 2010), 3,5-dicaffoeylquinic acid (**2**); 4,5-dicaffoeylquinic acid (**3**), (Lee et al., 2010; Li et al., 2011), 3,4-dicaffoeylquinic acid (**4**) (Iwai et al., 2004; Nakatani et al., 2000; Yoshioka et al., 2004), and 3,4-dicaffoeylquinic acid methyl ester (**5**) (Iwai et al., 2004; Nakatani et al., 2000; Yoshioka et al., 2004), when compared with those reported. To the best of our knowledge, this is the first report for isolation of **1**–**5** from *A. annua* leaves, though they were previously isolated from other *Artemisia* species.

3.6. HPLC fingerprint of the ethanolic extract

The presence of quinic acid derivatives; 3-feruloylquinic acid (**1**), 3,5 di-caffeoylquinic acid (**2**), 4,5 di-caffeoylquinic acid (**3**), 3,4 di-caffeoylquinic acid (**4**) and 3,4 di-caffeoylquinic acid methyl ester (**5**) was used as a fingerprint for the 70% ethanolic extract as verified by HPLC (**Fig. 2**). The retention time values of each compound before and after co-chromatography with the extract were determined, and an HPLC profile of the extract was constructed.

On the other hand, water extract of the leaves of *A. annua* collected in summer showed an HPLC chromatogram (**Fig. 3a**) quite similar to that of the 70% ethanolic extract. However, the water extract of the leaves powder showed a slightly higher concentration of 3-feruloylquinic acid (**1**); 1.47% against 1.34% in 70% ethanolic extract. This observation suggest that water is a good solvent for extraction of quinic acid derivatives and optimum exploitation of these natural products from *A. annua* leaves is possible if the harvest of plant material occurs during the appropriate stage of plant growth; early summer, in order to obtain extracts rich in these derivatives.

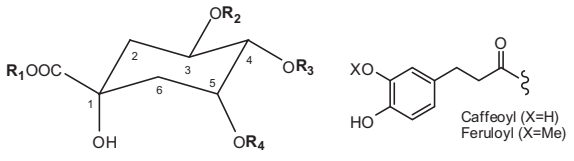

Fig. 1. Chemical structure of compounds isolated from the polar fraction of the 70% ethanolic extract of *A. annua* leaves.

3.7. The effect of seasonal variation on the concentration of quinic acid derivatives in A. annua leaves

The results in **Fig. 3b and Table 4** revealed that concentration of quinic acid derivatives (3-feruloylquinic acid (**1**), used as marker) was variable in the leaves harvested in different seasons. As deduced from the standard calibration curve, the concentration of 3-feruloylquinic acid (**1**) in *A. annua* leaves varied greatly throughout the year; from the leaf stage in March to the flowering stage in August-September "late summer". The lowest content of 3-feruloylquinic acid (0.036% w/w) was evident in samples collected during spring (March-April), but gradually increased through the warmer months from May-June "early summer" to reach 0.61%. The highest content (1.34%) of **1** was evident in the leaves harvested in June-July "summer", but gradual decline in the content of **1** (0.84% w/w) was seen in the leaves collected in late summer (August-September) **(Fig. 3b and Table 4)**.

4. Discussion

We report here the *in vivo* hepatoprotection, antihyperlycemic and antioxidant activities of the polar fraction of the 70% ethanolic extract of *A. annua* leaves in model rats. For the first time, 5 major quinic acid derivatives were isolated from its polar fraction and their chemical structures were elucidated. Besides, a versatile HPLC method was developed and utilized to: a)- study the influence of seasonal variation on the content of the quinic acid derivatives in *A. annua* leaves, b)- produce a fingerprint profile of *A. annua* leaves, and c)- conduct routine quality control analysis of *Artemisia* extracts or preparations containing it.

The 70% ethanolic extract of *A. annua* leaves was reported to protect HePG2 cells from CCl_4-induced hepatotoxicity (El-Askary et al., 2019), reduce hepatic dysfunction and fat accumulation in high fat diet-fed mice, reduce serum hepatic enzymes and TG levels (Kim et al., 2016), and inhibit lipid peroxidation (Chukwurah et al., 2014).

Hepatotoxic chemicals like CCl_4 induce hepatic injury mainly by inducing lipid peroxidation and generation of highly reactive oxidative intermediates in liver (Weber et al., 2003). However, hepatoprotective drugs are either capable of reducing the harmful effects or maintaining the normal hepatic physiological mechanism which have been imbalanced by a hepatotoxin.

In this study, elevation of the marker enzymes; AST, ALT and APT in CCl_4 administered rats is only a confirmation of previous reports on the hepatotoxicity of CCl_4 and is a direct reflection of alterations in the hepatic structural integrity of rat liver. However, treatment of the animals with the polar fraction of the 70% ethanolic extract of *A. annua* leaves maintained to normal the levels of these markers.

Besides, extraction of *A. annua* leaves with polar solvents such as water and alcohol give extracts rich in various polyphenols and flavonoids with a remarkable antioxidant effect (El-Askary et al., 2019; Iqbal et al., 2012; Skowyra et al., 2014) and protect against oxidative stress (Kim et al., 2014).

Alloxan is a diabetogenic agent that selectively destroys insulin secreting pancreatic beta cells and causes kidney damage, which is however reversible when administered intraperitoneal to experimental animals.

Alloxan causes diabetes by a mechanism that involves partial degradation of the β-cells of pancreatic islets and subsequent compromise in the quality and quantity of insulin produced by these cells (Macdonald Ighodaro et al., 2017). The model employs two distinct pathological effects, which include selective inhibition of glucose-stimulated insulin secretion, and induced formation of reactive oxygen species (ROS) which promotes selective necrosis of β-cells and changes in the activities of antioxidant enzymes in various tissues. Both effects collectively result in a pathophysiological state of insulin-dependent diabetes or type1-like diabetes mellitus in cells, and oxidative stress, which is involved in the development and progression of diabetes-associated

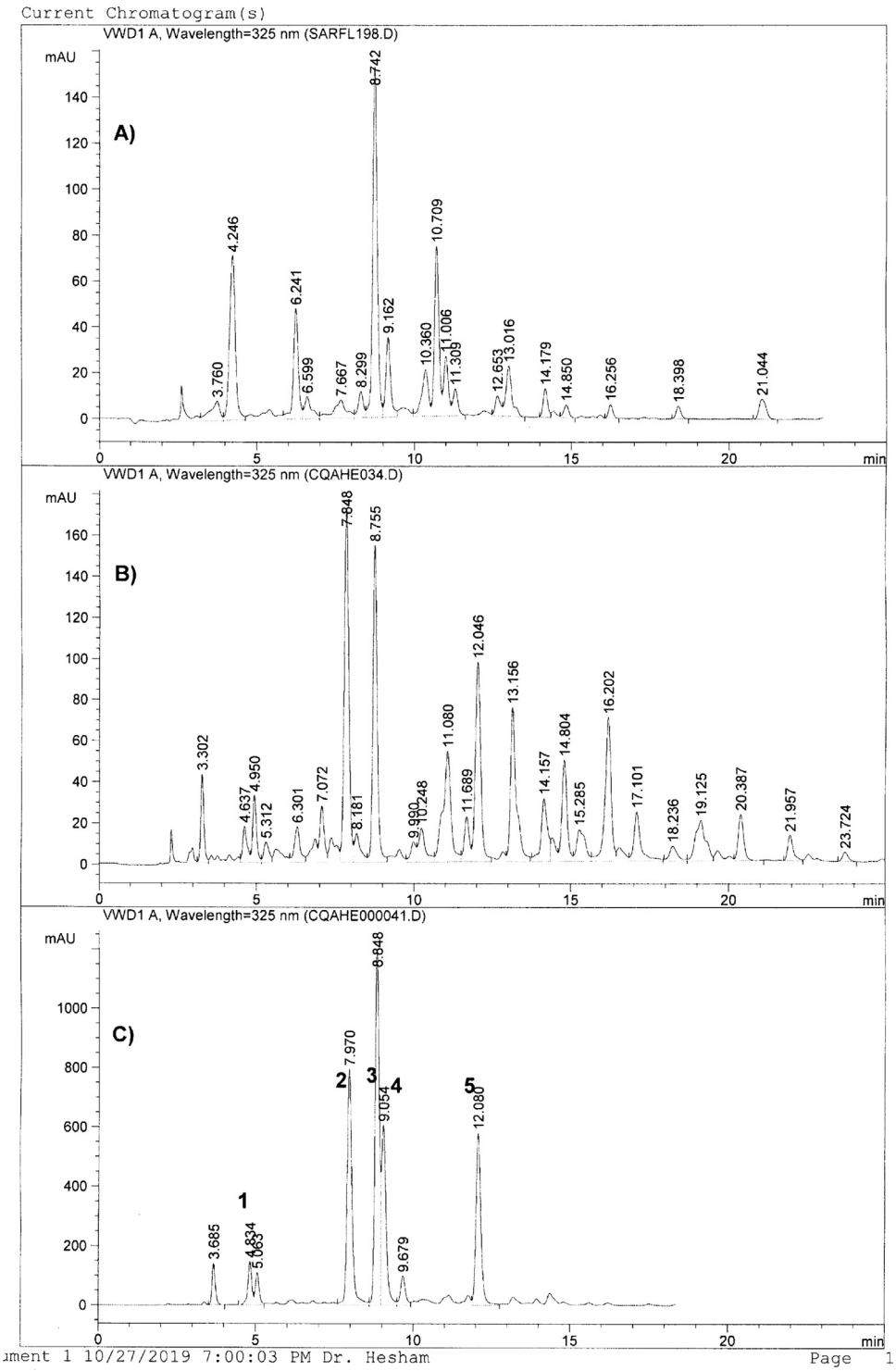

Fig. 2. HPLC fingerprint profile of (A): 70% ethanolic extract of *A. annua* leaves, (B): the bioactive fraction, and (C): admixture of the isolated compounds; 3-Feruloylquinic acid (1), 3,5-dicaffeoylquinic acid (2), 4,5-dicaffeoylquinic acid (3), 3,4-dicafeoylquinic acid (4) and 3,4-dicafeoylquinic acid methyl ester (5).

complications and triggers liver morphological and ultrastructural changes that closely resemble human disease (Lucchesi et al., 2015).

Antioxidants play an important role in scavenging the free radicals and protect the human body from oxidative stress (Eurich et al., 2007; RG, 2005). Hence, drug with both antioxidant and antidiabetic property would be useful for supporting liver and treating diabetes mellitus.

Glutathione (GSH) is a part of first line of defense against free radical induced damage and maintains low level of lipid peroxides (Kakis, 1980), and used to prevent oxidative stress in most cells. The results obtained here clearly demonstrated that the polar fraction is able to augments the antioxidant status, and restore to normal the overall antioxidant capacity in diabetic condition. Kim et al. (2016) reported that the 80% ethanolic extract of *A. annua* leaves reduces obesity and insulin resistance, and dependently inhibit α-glucosidase activity, and that phenolics are responsible, at least in part for such activities.

Phenolic acids such as quinic acid derivatives were identified as major constituents in extracts of other medicinal plants including *Cleome droserifolia* (El-Askary et al., 2019), *Moringa oleifera* (Inbathamizh

and Padmini, 2012) and green coffee beans (Farah and Donangelo, 2006), and were found responsible for most of the biological activities of their extracts. These derivatives were found to decrease the level of the biochemical markers AST, ALT, ALP and bilirubin (Ela et al., 2012), and modify plasma insulin, liver protein and DNA in Zucker (fa/fa) rats (De Sotillo et al., 2006).

In this study, we found that the polar fraction "bioactive fraction" of the ethanolic extract of *A. annua* is rich in quinic acid derivatives, from which we isolated 5 major derivatives.

Our present data suggest that the relation between this fraction and its biological activities is mediated, at least in part, by the antioxidant potential of quinic acid derivatives and its ability to enhance

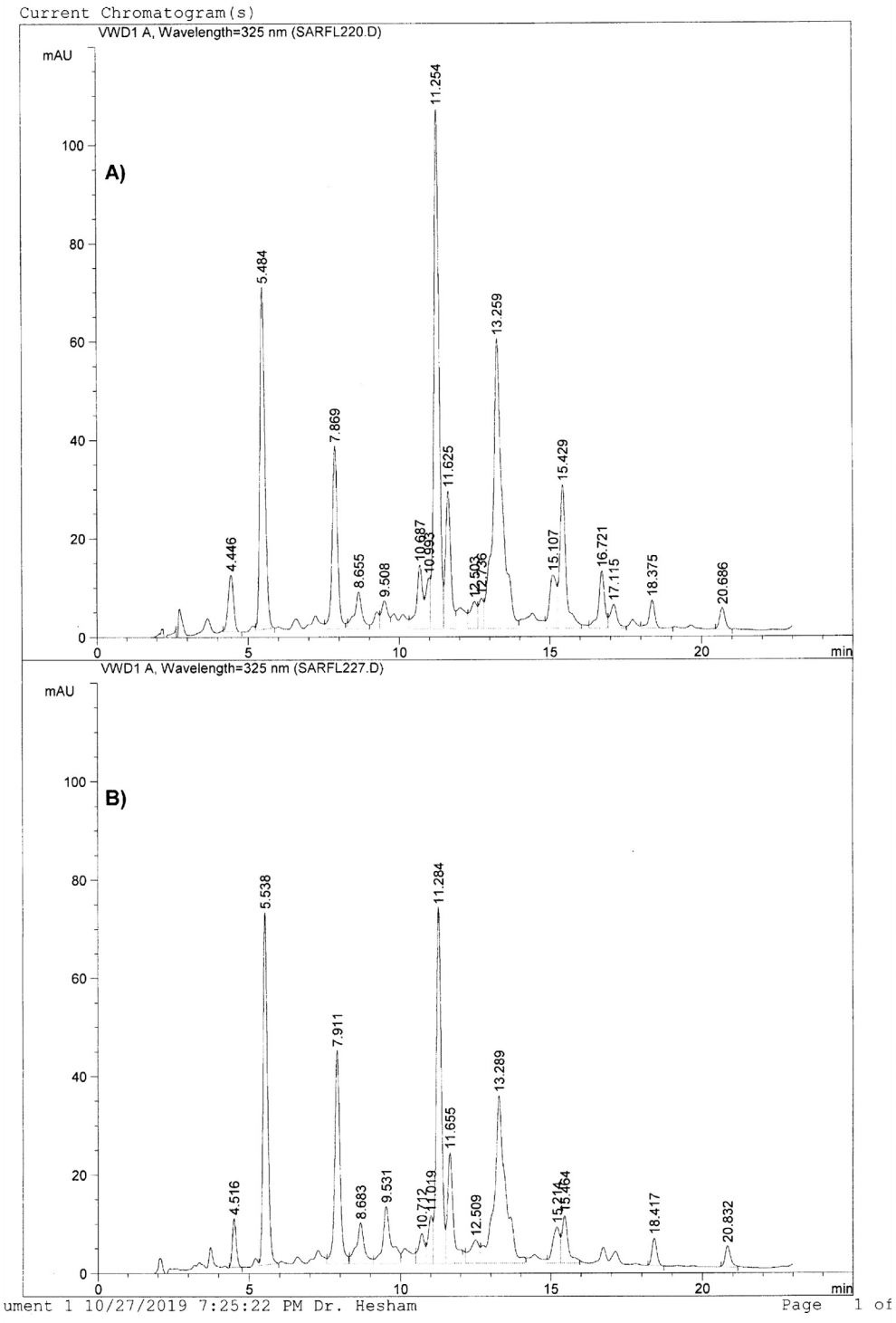

Fig. 3. (a) HPLC profile of quinic acid derivatives in the 70% ethanolic extract (A) and water extract of *A. annua* leaves (B) during summer. (b) HPLC chromatogram of the 70% ethanolic extract of *A. annua* leaves at different seasons; in Spring (during March-April) (A), in early Summer (during May-June) (B), in Summer (during July) (C), in late Summer (during August-Sept.) (D).

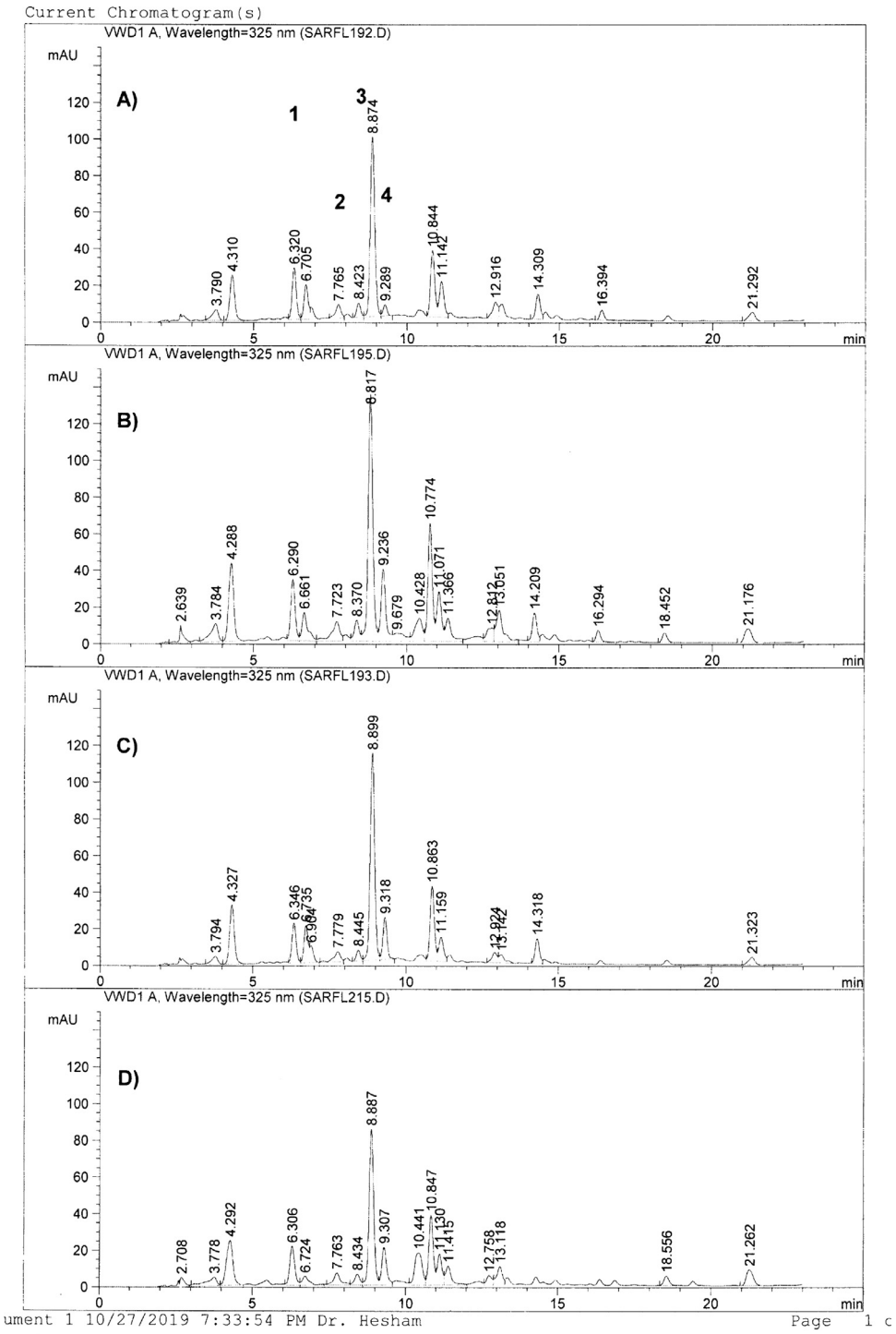

Fig. 3 Continued.

Table 4
Content of 3-feruloyl quinic acid (1) in *A. annua* leaves during different growth stages as determined by HPLC.

Time of collection	3-Feruloylquinic acid (**1**) (%w/w)
March-April 2014 Spring(leaf stage)	0.036%
May-June 2014 Early summer (leaf stage)	0.61%
July 2014 Summer (pre-flowering stage)	1.34%
August-september2014 Late summer (flowering stage)	0.84%

secretion of glutathione and normalize liver markers. Investigation of the biological activities of the isolated compounds in animal models is currently in progress in our lab.

It is worth to mention that the highest content of quinic acid derivatives is in the leaves of *A. annua* harvested in summer, at the pre-flowering stage of the plant; the same stage where content of artemisinin is the highest (El-Askary et al., 2004). Accordingly, a commercially viable process for scale-up to obtain fractions rich in these derivatives could be approached using residues of *A. annua* leaves left after extracting artmesinin.

Declaration of Competing Interest

None.

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