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

H.I. El-Askarya, S.S. Mohamedb, H.M.A. El-Goharib, S.M. Ezzata,c,*, M.R. Meselhyd

a Department of Pharmacognosy, Faculty of Pharmacy, Cairo University, Cairo 11562, Egypt
b Department of Pharmacognosy, Faculty of Pharmacy, Misr University for Science and Technology (MUST), 6th of October, Egypt
c Department of Pharmacognosy, Faculty of Pharmacy, October University for Modern Sciences and Arts (MSA), 6th of October 12611, Egypt

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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 CCl4-mediated increase in the liver enzymes; AST, ALT and ALP. Similarily, 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 "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).

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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 eaten 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, leishmaniasis (Sen et al., 2007; Yang and Liew, 1993), Trypanosoma...
(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; Sriman 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).

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Many species of genus Artemisia demonstrate antioxidant and hepatoprotective activities. The hydro-alcoholic extract of Artemisia aucheri and A. dracunculus displayed protective effect against CCl4-induced hepatotoxicity in rats, which was suggested to be produced as a result of its effect on oxidative stress (Ghavamizadeh and Mirzae, 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 Diasion HP-20 for column Chromatography (Merck, Germany). Bruker NMR Spectrometer, (Japan) for 1H NMR (400MHz) and 13C NMR (100 MHz). Spectra recorded in CD3OD or DMSO-d6 using TMS as internal standard, and chemical shift values were expressed in 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 Biorientek 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 (CILD), (Giza, Egypt) and Vitamin E (dl-α-tocopheryl acetate) 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 MilliQ 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 λmax 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 (LD50)

Median lethal dose (LD50) of the ethanolic extract was determined according to Andress (1992). LD50 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 (LD100) 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 LD50 was calculated.

2.5.3. In vivo hepatoprotective activity

Liver damage in rats was induced by intraperitoneal injection of 25% carbon tetrachloride (CCl4) 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 CCl4. 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 CCl4 injection and after one week intervals. Serum was isolated by centrifugation and activity of all serum enzymes; ALT, AST
2.5.4. In vivo anti-hyperglycemic activity

Induction of diabetes mellitus was made according to the method described by (Elisson and Samet, 1969) using alloxan. Eighteen male Sprague Dawley 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 served as positive control received a daily oral dose of 100 mg/kg body weight of metformin (Thewfikeld, 1974) and ALP were measured according to the manufacturer's instructions.

After seven days, blood samples were collected, heparinized and received oral dose of the polar fraction (100 mg/kg body weight). Group IV: diabetic rats that received daily oral dose of metformin (400 mg, DMSO-d$_6$): Quinic acid moiety: 1.9 (1H, m, H-6 ax, eq), 2.12 (1H, m, H-6, 6′), 7.01 (1H, brs, H2-2′, 2′), 7.56 (1H, d,d = 15.9 Hz, H-7′), 7.6 (1H, d, J = 15.9 Hz, H-7′).

Compound (2)

UV spectral data (MeOH) $\lambda_{max}$: 244–327 nm; $\text{1H NMR}$ 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 (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, H-8′), 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,d = 15.9 Hz, H-7′), 7.6 (1H, d, J = 15.9 Hz, H-7′).

Compound (3)

UV spectral data (MeOH) $\lambda_{max}$: 245–327 nm; $\text{1H NMR}$ 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) $\lambda_{max}$: 244–327 nm; $\text{1H NMR}$ 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) $\lambda_{max}$: 244–327 nm; $\text{1H NMR}$ 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: 6.18 (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 mmL x 4 mm ID, 5 μm, Merck, Germany), preceded by a C$_{18}$ guard column (10 mm L x 4 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$_3$PO$_4$ in H$_2$O “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 x 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 significance at p < 0.01.

3. Results

3.1. LD₅₀

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 CCl₄; 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 CCl₄-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

| Group/ Time (days) | Group I (Diabetic rats) Control group | Group II (Diabetic rats + Polar fr) % of change | Group III (Diabetic rats + Metformin) % of change |
|-------------------|--------------------------------------|-----------------------------------------------|-----------------------------------------------|
| Zero              | 254.9 ± 6.4                          | 267.3 ± 8.2                                   | 259.7 ± 6.1                                   |
| 2 Weeks           | 262.3 ± 7.9                          | 181.6 ± 5.9                                   | 146.3 ± 5.2                                   |
| 4 Weeks           | 238.9 ± 7.7                          | 141.7 ± 4.1                                   | 87.8 ± 2.4                                   |
| % Relative potency| 70.9                                 | 100                                           | 66.2                                         |

*Statistically significant difference from control group at p < 0.01.
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; 1H NMR, 13C NMR and 1H–1H COZY (see experimental), the chemical structures of these compounds (Fig. 1) were identified as 3-feruloylquinic acid (1) (Chen et al., 2014; Lim and Harnly, 2010), 3,5-dicaffeoylquinic acid (2); 4,5-dicaffeoylquinic acid (3) (Lee et al., 2010; Li et al., 2011), 3,4-dicaffeoylquinic acid (4) (Iwai et al., 2004; Nakatani et al., 2000; Yoshioka et al., 2004), and 3,4-dicaffeoylquinic 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-caf-feoylquinic acid (2), 4,5 di-caf-feoylquinic acid (3), 3,4 di-caf-feoylquinic acid (4) and 3,4 di-caf-feoylquinic 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 suggests 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.](image)

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, antihyperglycemic 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 CCl4-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 CCl4 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 CCl4 administered rats is only a confirmation of previous reports on the hepatotoxicity of CCl4 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 gives 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 Ighodoro 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 type I diabetes mellitus in cells, and oxidative stress, which is involved in the development and progression of diabetes-associated...
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 drosarifolia (El-Askary et al., 2019), Moringa oleifera (Inbathamizh

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-dicaffeoylquinic acid (4) and 3,4-dicaffeoylquinic acid methyl ester (5).
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 ethanol 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% ethanol 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).
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 artemisinin.

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-September 2014 Late summer (flowering stage) | 0.84% |
Declaration of Competing Interest

None.

References

Abdi Ali Khan, M.; Jain, D.; Bhakuni, R.; Zain, M.; Thakur, R., 1991. Occurrence of some antiviral sterols in: – Artemisia annua. Plant Science 75, 161–165.

Bhakuni, R.; Jain, D.; Sharma, R.; Kumar, S., 2001. Secondary metabolites of Artemisia annua and their biological activity. Current Science 80, 35–48.

Bilia, A.R.; Melillo de Malgalhaes, P.; Bergonzi, M.C.; Vincieri, F.F., 2006. Simultaneous quantification of the phytochemicals of Artemisia annua and cleome droserifolia using HPLC/PDA/ESI/MS. Revista Brasileira de Farmacognosia 29, 213–220.

Ela, M.A.A.; El Buetler, E.; Duron, O.; Kelly, B., 1963. Improved method for determination of blood glucose. Journal of Biological Chemistry 238, 4757–4763.

Farah, A.; Donangelo, C.M., 2006. Phenolic compounds in coffee. Brazilian Journal of Plant Physiology 18, 23–30.

Ferreira, J.F.S.; Simon, J.; Janick, J., 1997. Artemisia annua: botany, horticulture, pharmacology (a review). Horticultural Reviews 19, 319–371.

Ferreira, J.F., 2007. Nutrient deficiency in the production of artemisinin, dihydroartemisinin, artemisinin and artemisinic acid in artemisia annua L. Journal of Agricultural and Food Chemistry 55, 1686–1694.

Lee, H.J.; Lee, J.Y.; Kim, S.M.; Nho, C.W.; Jung, S.H.; Song, D.-G.; Kim, C.Y.; Pan, C.-H., 2010. Inhibitory effects of dicafeoylquinic acids from artemisia duba on aldo-keto reductase family 1B10. Journal of the Korean Society for Applied Biological Chemistry 53, 826–830.

Lin, L.-Z.; Hamly, J.M., 2010. Identification of the phenolic components of chrysanthemum flower (Chrysanthemum morifolium ramar). Food Chemistry 120, 319–326.

Lucchesi, A.N.; Cassettari, L.L.; Spadella, C.T., 2015. Alloxan-induced diabetes causes morphological and ultrastructural changes in rat liver that resemble the natural history of chronic fatty liver disease in humans. Journal of Diabetes Research 2015, 1–11.

Macdonald Ighodaro, O.; Mohammed Aedeosun, A.; Adeboye Akinloye, O., 2017. Alloxan-induced diabetes, a common model for evaluating the glycemic-control potential of therapeutic compounds and plants extracts in experimental studies. Medicina 53, 365–374.

Mishina, Y.V.; Krishna, S.; Haynes, R.K.; Meade, J.C., 2007. Artemisinins inhibit trypano soma cruzi and trypanosoma brucei rhodesiense in vitro growth. Antimicrobial Agents and Chemotherapy 51, 1852–1854.

Nakatani, N.; Kayano, S.-i.; Kikuzuki, H.; Sumino, K.; Katagiri, K.; Mitani, T., 2000. Identification, quantitative determination, and antioxidative activities of chlorogenic acid isomers in prune (Prunus d orientalis L.). Journal of Agricultural and Food Chemistry 48, 5512–5516.

Namdeo, A.G.; Mahadik, K.; Kadam, S., 2006. Antimalarial drug-artemisinin. Pharmacognosy Magazine 2, 106.

Reiter, C.; Führlich, T.; Zeino, M.; Marschall, M.; Bahls, H.; Leidenberger, M.; Friedrich, O.; Kappes, B.; Hambé, I.; Ellenfëth, T., 2015. New efficient artemisinin derived agents against human leukaemia cells, human cytomegalovirus and plasmodium falciparum: 2nd generation 1, 2, 4-trioxane-furoic hybrids. European Journal of Medicinal Chemistry 97, 164–172.

RG, A., 2005. The physiological and biochemical effects of diabetes on the balance between oxidative stress and antioxidant defense system. Medical Journal of Islamic World Academy of Sciences 15, 31–42.

Sen, R.; Bandypadhyay, S.; Dutta, A.; Mandal, G.; Ganguly, S.; Saha, P.; Chatterjee, M., 2007. Artemisinins triggers induction of cell-cycle arrest and apoptosis in leishmania donovani promastigotes. Journal of Medical Microbiology 56, 1213–1218.

Skehan, P.; Storeng, R.; Scudiero, D.; Monks, A.; McMahon, J.; Vistica, D.; Bokov, J.; Bohec, K.; Kenney, S.; Boyd, M.R., 1990. New colorimetric cyto toxicity assay for anticancer-drug screening. Journal of the National Cancer Institute 82, 1107–1112.

Skowyra, M.; Gallego, M.; Segovia, F.; Almajano, M., 2014. Antioxidant properties of artemisia annua extracts in model food emulsions. Antioxidants 3, 116–128.

Siriram, D.; Rao, V.S.; Chandrasekar, K.V.C.; Yogeewarai, P., 2004. Progress in the history of chronic fatty liver disease in humans. Journal of Diabetes Research 2015, 1–11.

Tindel, P., 1969. Estimation of serum glucose and triglycerides by enzymatic method. American Clinical Biochemistry 6.

Wallaart, E.; Pras, N.; Beekman, A.C.; Quax, W., 2000. Seasonal variation of artemisinin and its biosynthetic precursors in plants of artemisia annua of different geographic origin: Proof for the existence of chemotypes. Planta Medica 66, 57–62.

Webber, L.W.; Boll, M.; Stampf, A., 2003. Hepatotoxicity and mechanism of action of hal- oalkanes: carbon tetrachloride as a toxicological model. Critical Reviews in Toxicology 33, 105–136.

WHO, 2006. WHO Monograph On Good Agricultural and Collection Practices (GACP) For Artemisia Annua L.W.H.O., Geneva, Switzerland.

Wright, C.W., 2001. Artemisia. CRC Press.

Yang, D.; Liew, F., 1993. Effects of qinghaosu (artemisinin) and its derivatives on experimental cataracts in rats treated with CCl4 and CHCl3. Toxicology and Applied Pharmacology 18, 2019.