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Full Length Research Paper

The high performance liquid chromatography (HPLC) analysis of ultraviolet (UV) irradiated chlorophyll *a* and secondary plant compounds

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A large number of diseases such as cancer, cardiovascular disease, inflammatory disease, neurodegenerative disorders and ageing are consequences of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Deoxyribonucleic acid (DNA) damage, lipid peroxidation and protein damage are biomarkers for damage in the cell caused by ROS and RNS. Various plant extracts have been tested for anti-oxidant properties. Due to the chlorophyll in the extracts, false negative results from spectrophotometric tests are often observed. A pilot study was done to determine the stability of the secondary compounds with high performance liquid chromatography (HPLC) after the samples were bleached with ultraviolet (UV) radiation and treated with activated charcoal. The chlorophyll *a* was successfully degraded with UV radiation in all the samples leaving a bleached extract suitable for biological assays.

Key words: Chlorophyll *a*, UV radiation, activated charcoal, HPLC, secondary compounds in plant extracts.

INTRODUCTION

Western and eastern cultures have used plants widely for food and medicinal purposes throughout history (Marques and Farah, 2009). The use of medicinal plants has played a significant role in maintaining human health and improving the quality of human life (Marques and Farah, 2009). Well-known vegetables and fruits that are used as food, as well as wild plants that are consumed by local inhabitants are screened for their antioxidant capacity (Karagözler et al., 2008, Marinova et al., 2009). The small molecular weight antioxidants extracted from these plants are considered as possible protective agents to reduce oxidative damage in the human body, after the internal enzymatic mechanisms fail or become inefficient against oxidative damage (Ślusarczyk et al., 2009). These natural antioxidants could be replacement for the synthetic antioxidants, because of their lower toxicity to humans (Abdel-Hameed, 2009). ROS and RNS are products of normal cellular metabolism. Functions of ROS are to act as defence mechanisms against infectious agents and to take part in cellular signalling systems especially when it occurs in low/moderate concentrations. However oxidative- and nitrosative stress occur when there is an overproduction of ROS/RNS on one side and a deficiency in the enzymatic and non-enzymatic antioxidant systems on the other side (Valko et al., 2007; Eggers, 2009). Large quantities of ROS and RNS may be dangerous because of their ability to attack numerous molecules, including proteins, lipids and cellular DNA (Valko et al., 2007; Atmani et al., 2009).

These molecules act as biomarkers for oxidative and nitrosative stress. As a result, a large number of diseases such as cancer, cardiovascular disease, inflammatory disease, neurodegenerative disorders and ageing are consequences of high concentrations of ROS and RNS

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Abbreviations: **HPLC,** High performance liquid chromatography; **ROS,** reactive oxygen species; **RNS,** reactive nitrogen species; **TBA,** thiobarbituric acid; **MDA,** malondialdehyde; **NBT,** nitro-blue tetrazolium; **IR,** infrared; **DNA,** deoxyribonucleic acid; **UV-Vis,** ultraviolet/visible.

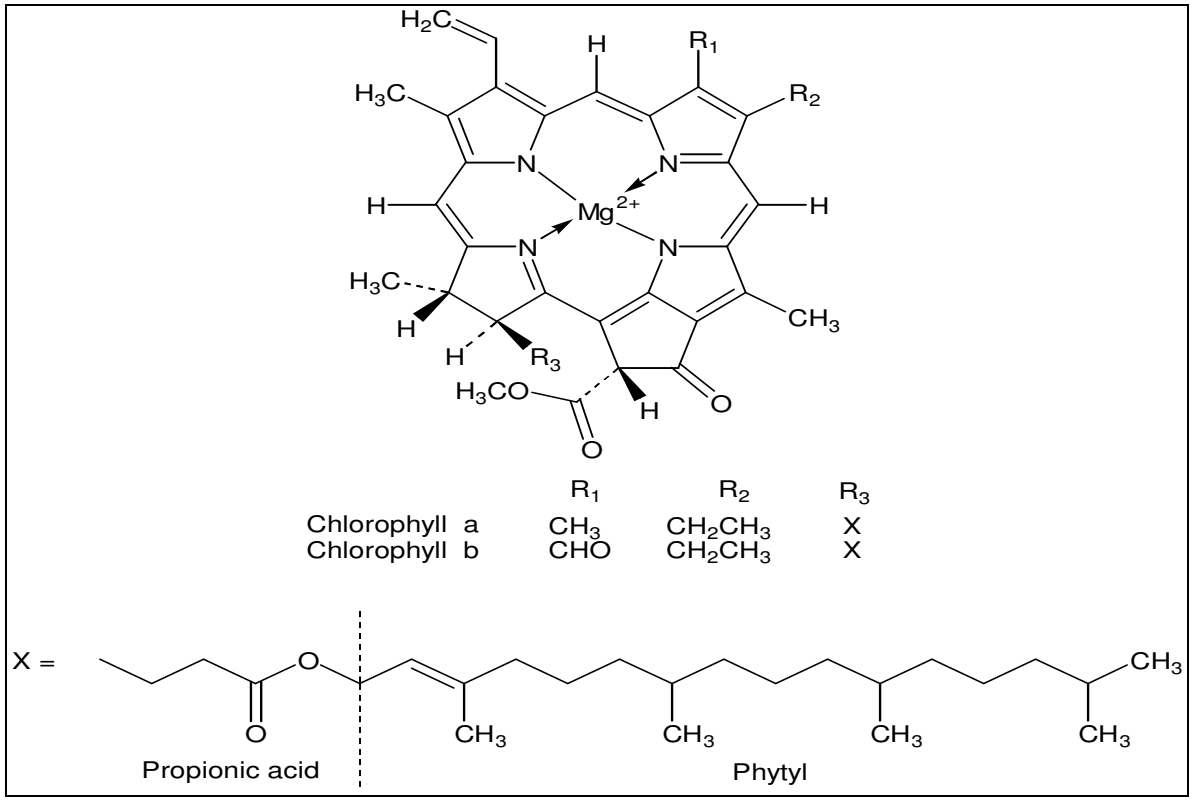

Figure 1. The chlorophyll molecule. (Merck index, 2000).

(Hwang and Kim, 2007; Abdel-Hameed, 2009). One of the most popular spectrometric techniques used for lipid peroxidation measurement in animal material is the thiobarbituric acid (TBA) assay, which is based on the reaction of TBA with malondialdehyde (MDA), a colorless end product of lipid peroxidation, under acidic conditions (Garcia et al., 2005). A pink-colored TBA -MDA adduct forms which are measured at 532 nm after the precipitated protein is removed with centrifugation (Kang et al., 2003). Unfortunately, this method has limitations; the main error of the assay is that the MDA content of many materials of plant and animal origin is inflated by pigments which absorbs in the same region as the TBA - MDA adduct (Anoopkumar-Dukie et al., 2001). Another simple assay, the nitro-blue tetrazolium (NBT) assay, is based on the ability of superoxide and other free radicals to reduce NBT to a blue insoluble diformazan adduct which can be extracted with glacial acetic acid and measured at 560 nm (Ottino and Duncan, 1997).

This assay may be influenced by pigments which are absorbs at 560 nm. Pigments that cause a major problem in plant extracts is the chlorophylls, which can be divided into two forms; one form consists of bluish green chlorophyll *a* and the other form of yellowish green chlorophyll *b* (Levitt, 1969). The empirical formula for chlorophyll *a* is $C_{55}H_{72}O_5N_4Mg$ and is also the same for chlorophyll *b* with a CHO group in the place of the CH_3 (Figure 1) (Levitt, 1969). Chlorophylls are examples of porphyrin ring structures with one double bond reduced, called chlorin (Miessler and Tarr, 2004). At the centre of the chlorin is magnesium chelated and liganded at four sites to pyrrole nitrogen atoms (Prezelin and Nelson, 1990). The chlorophylls also have two major absorption bands in the visible range, due to extended π-delocalization at the edge of the cyclic tetrapyrole (porphyrin) skeleton: "red" (Q) band and "blue" (Soret of B) bands. The Q-band absorption maxima (A_{max}) for chlorophyll *a* and chlorophyll *b* in acetone are located at 662.1 and 645.5 nm (Zvezdanović et al., 2008). Chlorophyll is used to channel the light energy into chemical energy through the process of photosynthesis; the overall reaction can be represented as

$$CO_2 + H_2O \xrightarrow[\text{Chlorophyll}]{\text{Light}} (CH_2O) + O_2$$

Where, (CH_2O) represents sugars, carbohydrates and all cellulose synthesized in the plant (Roberts and Whitehouse, 1976; Miessler and Tarr, 2004). To over-come the problem of interfering colour in samples, the popular technique of decolourisation with activated charcoal is used to adsorb the chlorophyll from the sample (Chapman, 1994; Malek et al., 2008). The sample is stirred with activated charcoal in an organic solvent and then filtered through a wad of cotton in the stem of a funnel to give a decoloured sample. Unfortunately, too

much of the activated charcoal may absorb some of the compounds which is being decoloured (Furniss et al.,1989). A pilot study was done on chlorophyll *a*, known secondary compounds previously isolated from various plants and *Plumbago auriculata* to determine whether the degradation of chlorophyll *a* by UV radiation would affect the secondary compounds in samples negatively. This study was divided into three sections: 1) chlorophyll *a* was used as a control sample and compared to two different treated samples: (i) A sample of chlorophyll *a* that was treated with UV radiation and (ii) A sample of chlorophyll *a* that was treated with activated charcoal; 2) secondary compounds were used as a control sample alongside two separate samples that were treated with UV radiation and treated with activated charcoal; 3) *P. auriculata* extract was used as a control sample and compared to a sample of *P. auriculata* that was treated with UV radiation and a third sample of *P. auriculata* that was treated with activated charcoal. All the above samples were monitored by HPLC for the effect of the UV radiation and the activated charcoal.

MATERIALS AND METHODS

Chemicals

Chlorophyll *a* was purchased from Sigma-Aldrich (Steinheim, Germany) and dissolved in acetone: ddH2O. For the second part of the study, the following compounds were used and referred to as the secondary compounds: nicotine, salicin, strophanthin-G, rutin, atropine sulphate and dienoestrol. These secondary compounds were previously isolated from various plants in our laboratories and the structures were confirmed with infrared (IR) spectrometry and compared to the spectrums of the Sigma Library of RT-IR spectra (Keller, 1986). The secondary compounds were dissolved in methanol and milliQ water. Methanol (HPLC grade), acetonitrile (HPLC grade) and ethyl acetate (HPLC grade) were purchased from Merck Chemicals (Pty) Limited South Africa; acetone (HPLC grade) was from Anatech Instruments (Pty) Limited, South Africa and acetic acid from Merck Chemicals (Pty) Limited, South Africa. The activated charcoal was from Merck Chemicals (Pty) Limited South Africa. All the solutions were filtered through 0.45 µm Acrodisc GHP Syringe filters prior to HPLC separations.

Preparation of chlorophyll *a*

The chlorophyll *a* was dissolved in acetone: ddH_2O (1:1) stock solution and stored at -20°C. For the experiments, 100 µl of the stock solution diluted with 900 µl ddH_2O was used as the control sample; the same dilutions were used for the samples for treatment with UV radiation and treatment with activated charcoal. These samples were used in section one of the study.

Preparation of secondary compounds

All the secondary compounds were dissolved in methanol and diluted with ddH_2O, except for the Dienoestrol which was only dissolved in methanol. These samples were used in section two of the study.

Plant material and the extraction of plant pigments

P. auriculata was obtained from the Botanical Garden of the North-West University (Potchefstroom). The leaves were air dried and powdered with a mortar and pestle. Plant compounds were extracted from the powdered leaves using the soxhlet extraction method. The leaves were extracted with four organic solvents in order from less polar to the most polar; the first solvent used was petroleum ether, followed by dichloromethane, ethyl acetate and lastly ethanol. The ethanol extract was concentrated and dissolved in methanol and used in the third section of the study.

Sample preparation with activated charcoal

The prepared samples were stirred for 1 h with activated charcoal, filtered to remove the charcoal and the clear samples were filtered once more through 0.45 µm Teflon membrane filters.

Sample preparation with UV radiation

Continuous irradiation of the various prepared samples was performed in a cylindrical photochemical reactor (A/C 220/230 V, 50 Hz, 22 W) for 1 h. The samples were irradiated in Pyrex tubes placed on a circular holder at a distance of 8 cm from the lamps for 1 h.

HPLC analysis

An Agilent 1100 series HPLC equipped with a vacuum degasser, gradient pump, auto sampler and a ultraviolet/visible (UV-VIS) diode array detector was used to analyse the samples. ChemStation software (Rev. A.08.03) was used for data acquisition and analysis. Chromatograms were registered at 254 and 665 nm with 4 and 8 nm bandwidth. UV spectra were recorded from 190 to 600nm. The plant samples were separated on a reversed-phase Eclipse XBD C18 column (150 x 4.6 mm, 5 µm particle sizes, Agilent Technologies, Palo Alto, USA) using gradient elution. The differences in the structure of chlorophyll *a*, secondary compounds and the *P. auriculata* made it difficult to use a single method for all of the samples on the HPLC. Therefore method A was used to monitor the different chlorophyll *a* samples and method B was used to monitor the secondary compounds and the *P. auriculata* samples. Method A used an isocratic system of ethyl acetate and methanol in the ratio 32:68, the flow rate was 1 mL/min and the injection volume was 5 µl. Method B was based upon a gradient (Table I); solvent A consisted of milliQ water with 1% acetic acid and solvent B consisted of acetonitrile with 1% acetic acid, the flow rate was 1 mL/min and the injection volume was 5 µl.

RESULTS AND DISCUSSION

Figure 2 shows the peak of chlorophyll *a* eluted at time 3.355 min, while Figure 3 shows the total degradation of the chlorophyll *a* after 1 h of UV-irradiation. The sample treated with activated charcoal in Figure 4 also shows no peaks on the chromatogram. Both these methods for chlorophyll *a* elimination were successful and could be used to decolour the samples effectively. A chromatogram of secondary compounds were obtained with HPLC analysis in Figure 5; the compounds that were used were nicotine, salicin, strophanthin-G, rutin and dienoestrol.

Table 1. The solvent gradient that was applied for chromatography (Solvent A: milliQ water, buffered with 1% acetic acid, solvent; B, acetonitrile, buffered with 1% acetic acid).

Time (min)	% A	% B
0	100	0
5	85	15
15	75	25
25	50	50
35	25	75

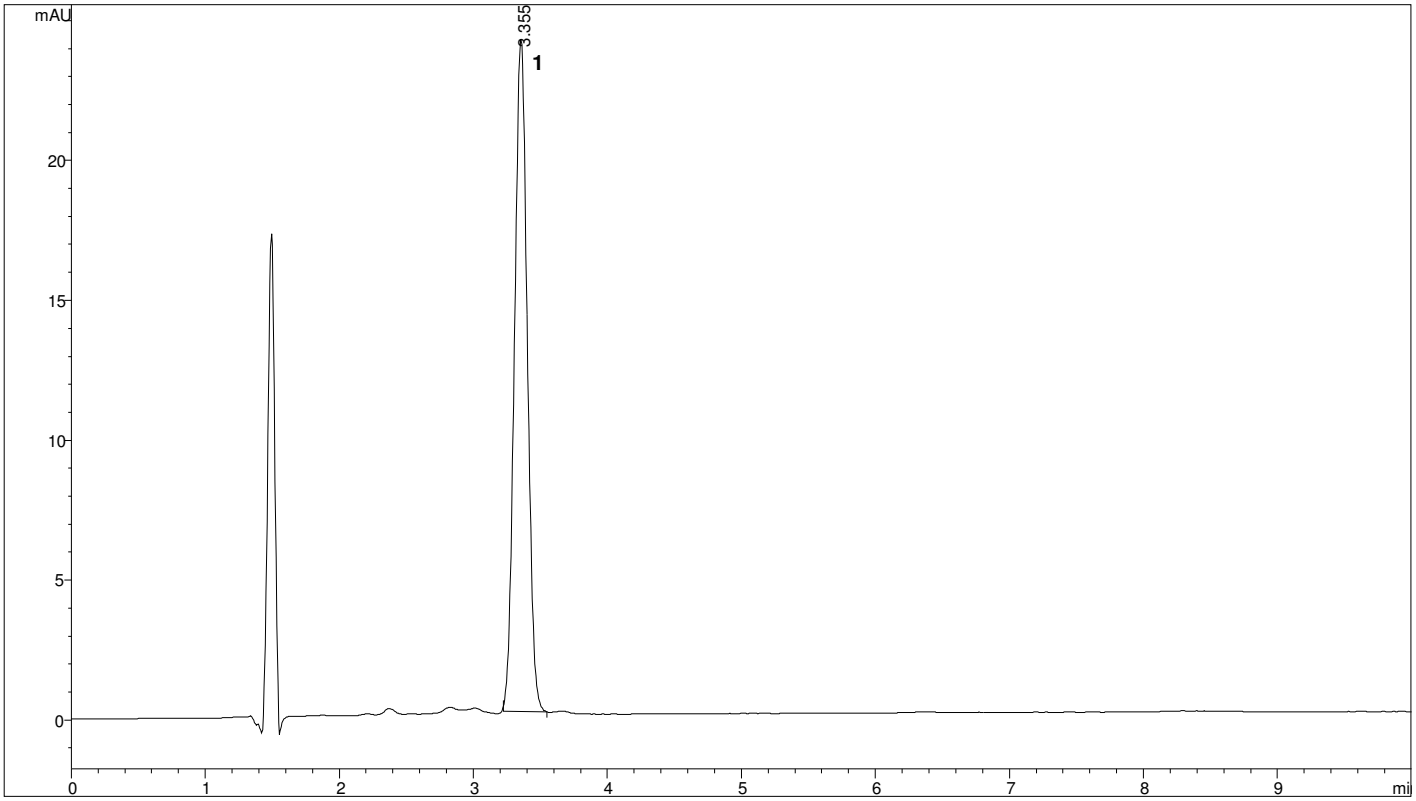

Figure 2. The peak of chlorophyll *a* eluted at time 3.355 min.

Figure 6 shows the UV-irradiated secondary compounds and Figure 7 shows the activated charcoal treated secondary compounds; the UV-irradiated compounds were all significantly higher in concentration than the activated charcoal treated compounds after 1 h (Table 2). The UV irradiated sample showed about 4% decrease in concentration whilst the concentration of the activated charcoal treated sample decreased by 18%. The results show that the UV irradiation is a more successful treatment for the decolouring of samples and the percentage of the secondary compounds that remained was far more significant than the activated charcoal after 1 h of treatment (Table 3). It can be concluded that the UV irradiation is a more effective discoloration procedure than the activated charcoal treatment. Figure 8 shows the chromatogram of *P. auriculata*, Figure 9 is the UV-irradiated sample and Figure 10 is the activated charcoal treated sample. With both the treatments, the solutions were successfully discoloured by removal of chlorophyll *a*. However, UV treatment caused an average reduction of 19% in the concentration of compounds while activated charcoal caused a reduction of 66%; low concentration compounds will be lost after charcoal treatment as witnessed by the disappearance of peak no. 6 in Figure 10. As a result, the UV irradiation method gave better results with the known secondary compounds and also with the *P. auriculata* than the activated charcoal treatment. It is recommended to use UV irradiation with

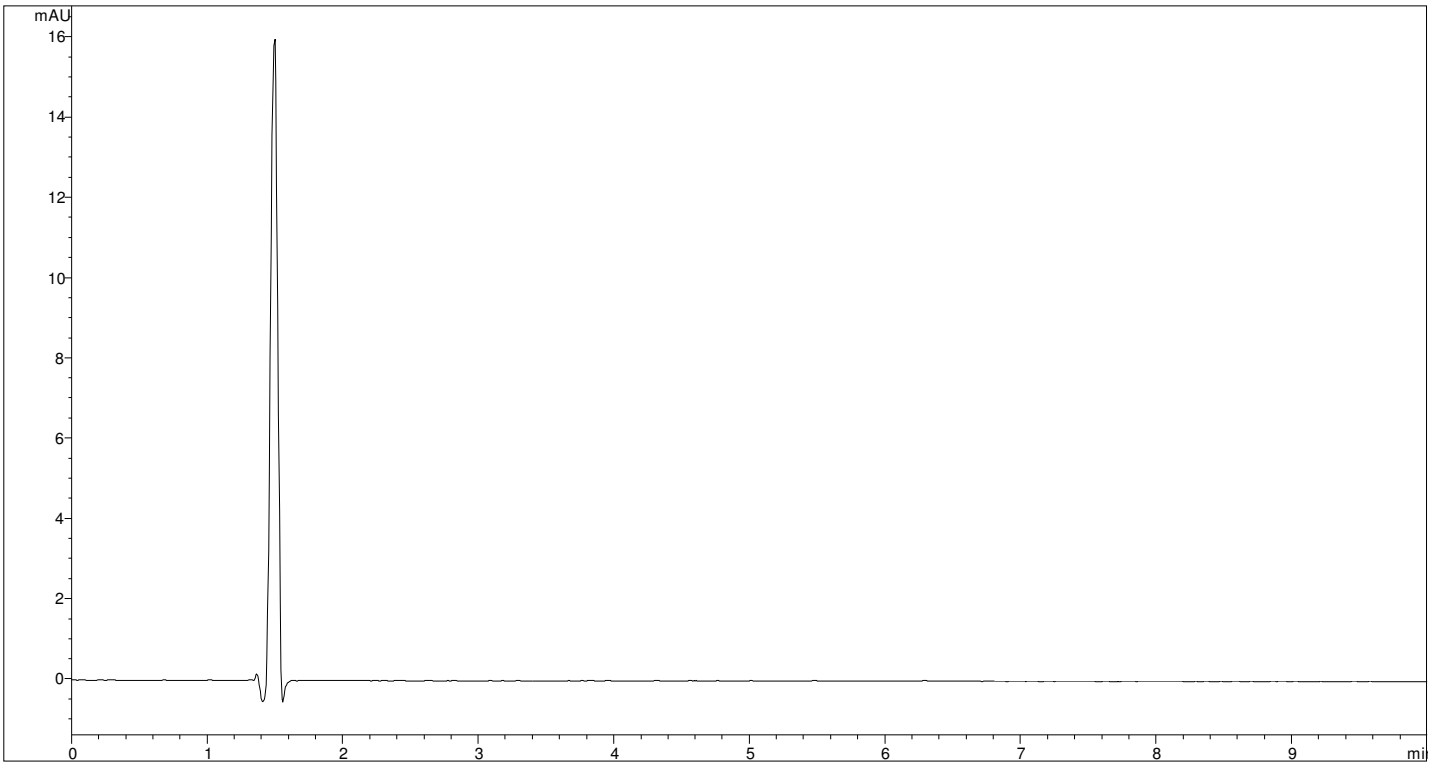

Figure 3. The chromatogram of the chlorophyll *a* sample that was treated with UV radiation showing that chlorophyll *a* was completely degraded after 1 h of treatment in the photochemical reactor.

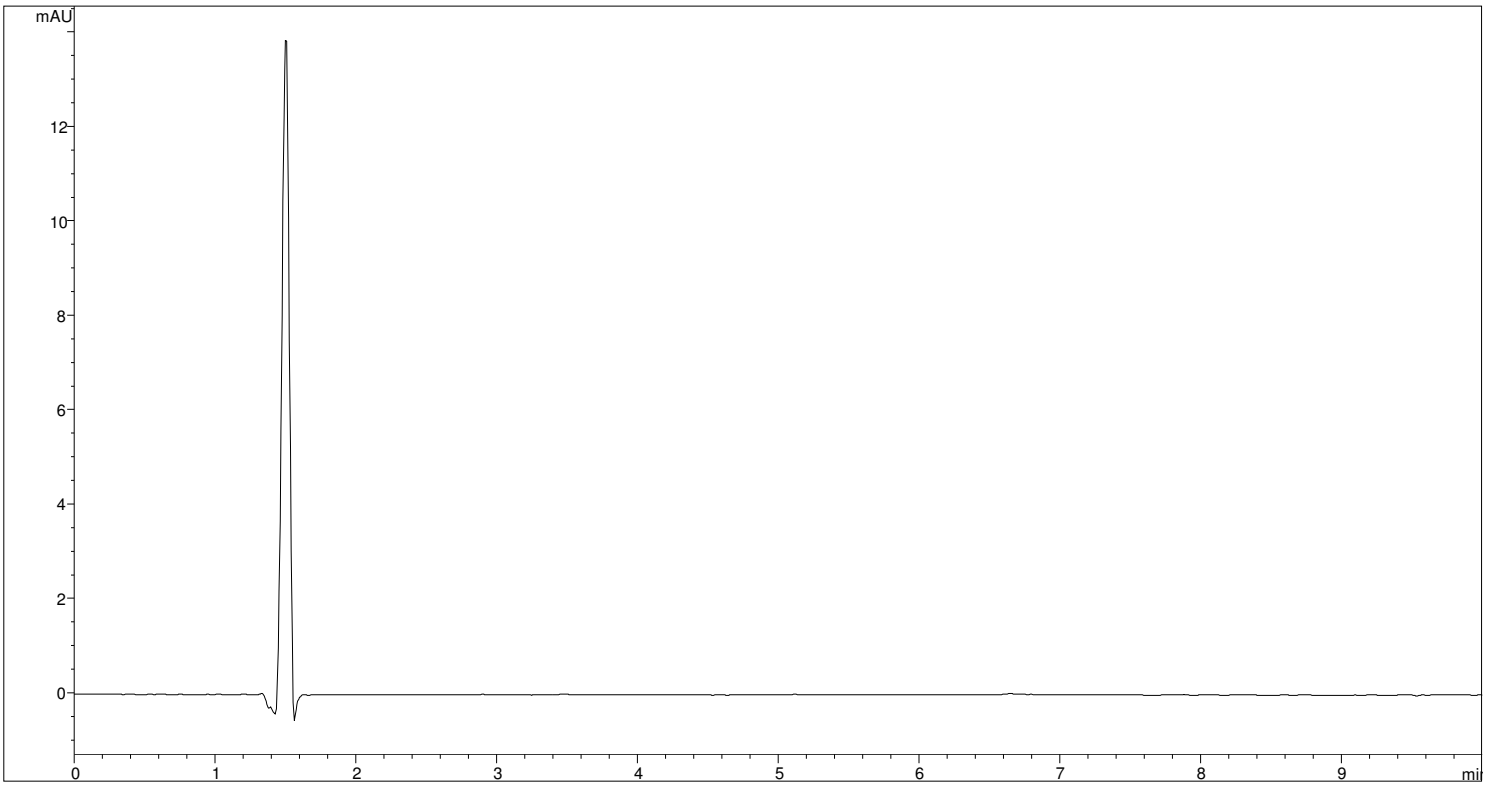

Figure 4. A typical chromatogram of chlorophyll *a* after treatment activated charcoal. The chromatogram shows that all the chlorophyll *a* was absorbed by the activated charcoal from the sample.

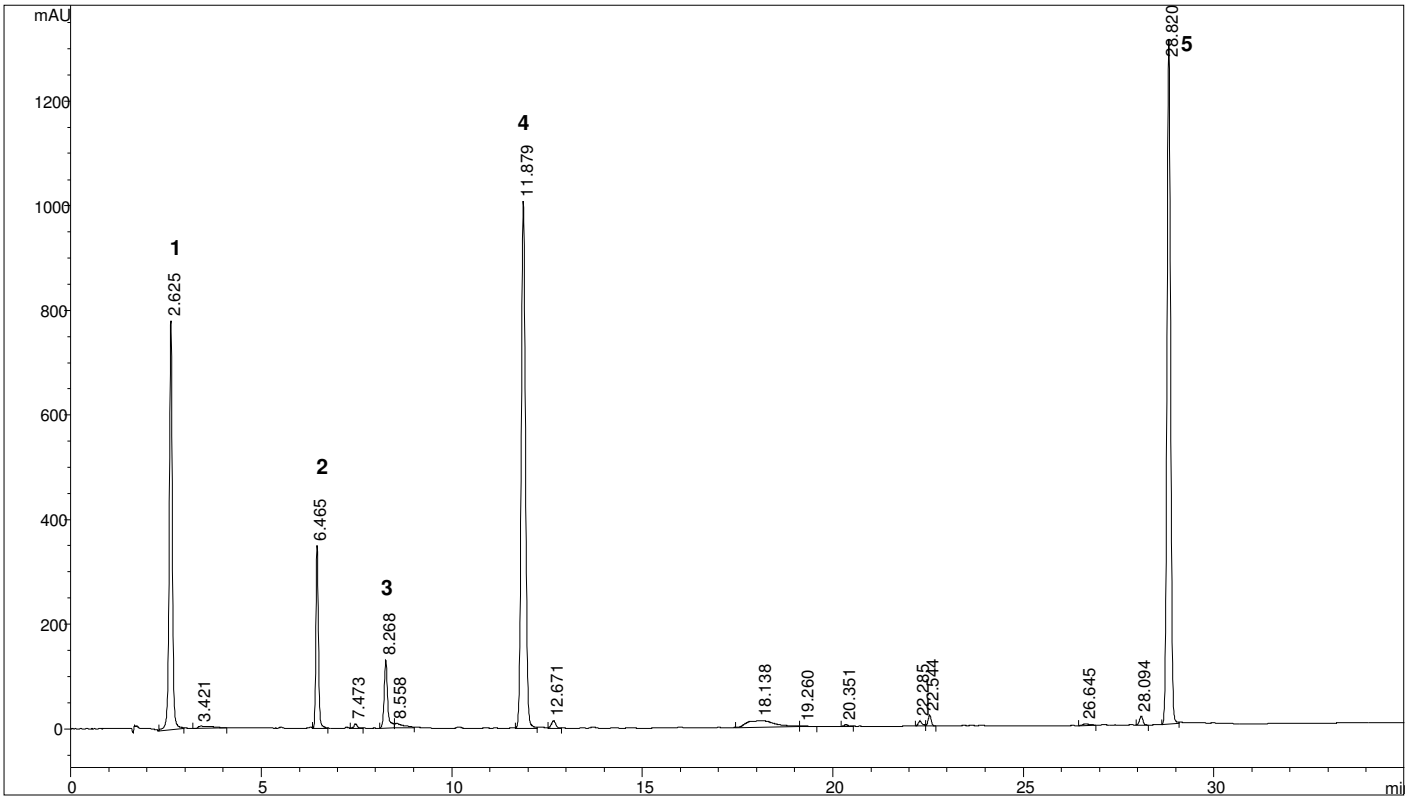

Figure 5. HPLC chromatogram of secondary compounds isolated from various plants recorded at 254 nm. Solvent gradient was applied to obtain the results. The peaks were identified as: 1, nicotine; 2, salicin; 3, strophanthin-g; 4, rutin; 5, dienoestrol.

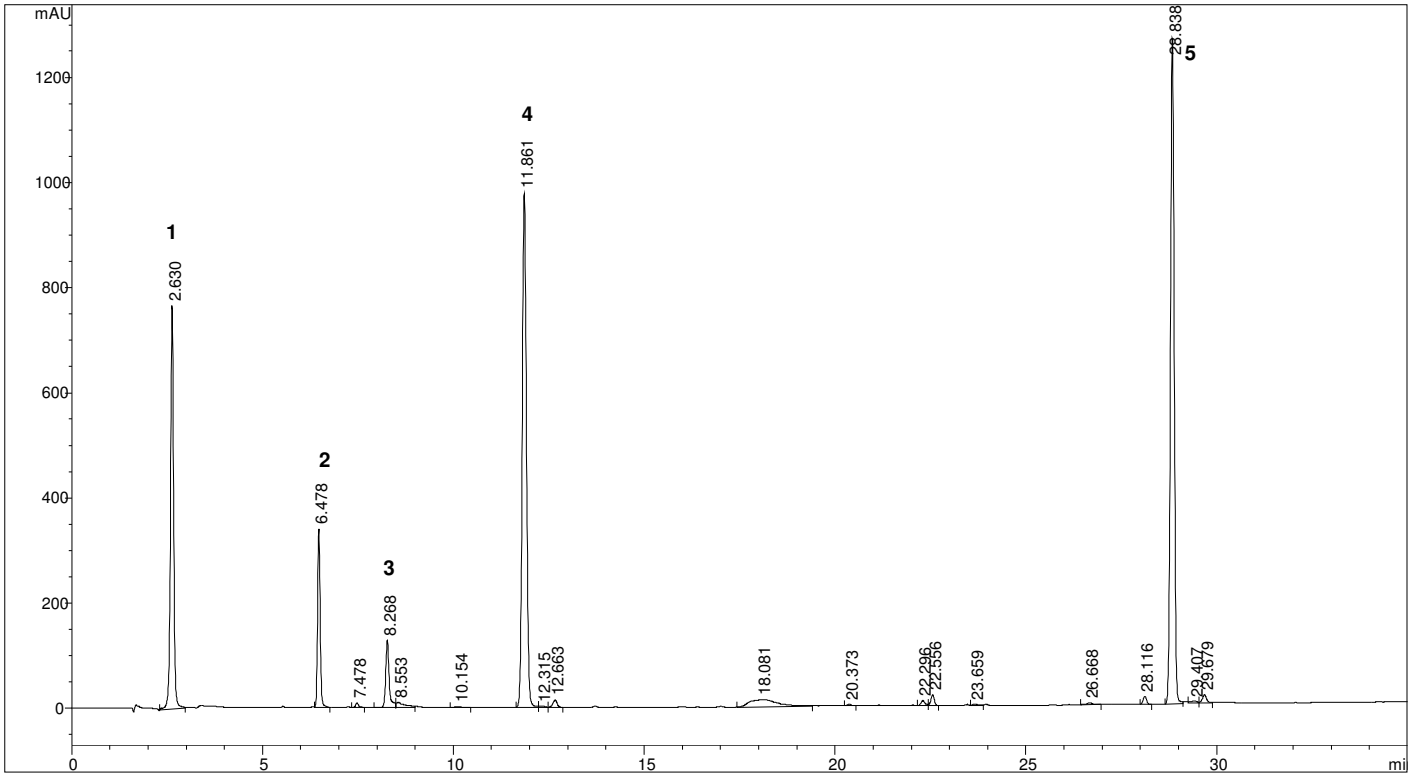

Figure 6. HPLC chromatogram of secondary compounds from various plants after 1 h of UV radiation. Solvent gradient was applied to obtain the results. The peaks were identified as: as: 1, nicotine; 2, salicin; 3, strophanthin-G; 4, rutin; 5, dienoestrol.

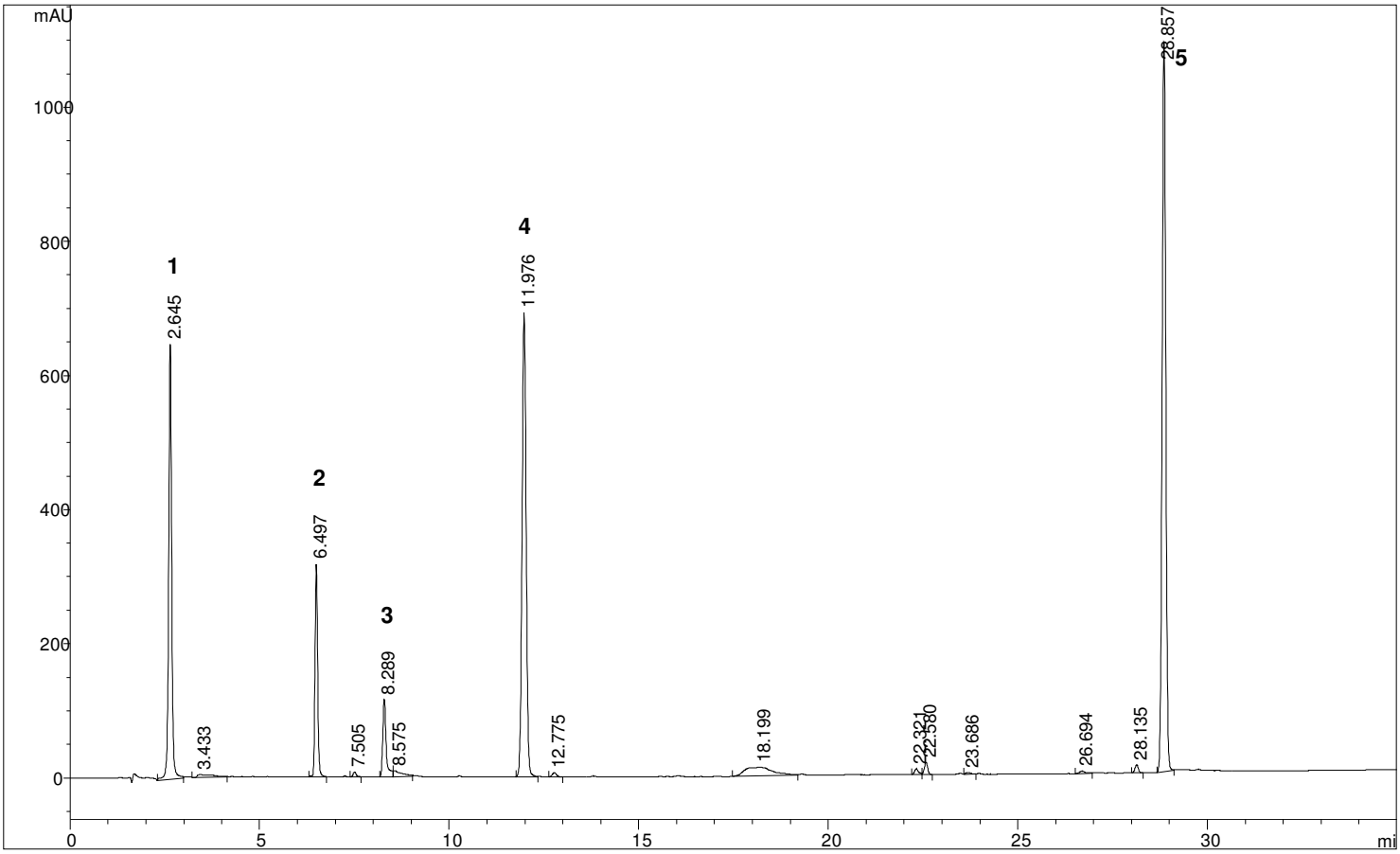

Figure 7. HPLC chromatogram of secondary compounds treated with activated charcoal. The peaks were identified as: 1, nicotine; 2, salicin; 3, strophanthin-G; 4, rutin; 5, dienoestrol.

Table 2. The percentage secondary compounds that remaining after treatment with UV-irradiation and activated charcoal.

Secondary compound	% Remaining after UV-irradiation	% Remaining after activated charcoal
Nicotine	97.7	83.0
Salicin	97.5	91.6
Strophanthin-G	89.3	86.0
Rutin	98.6	68.5
Dienosterol	97.4	82.8
Average remaining	96.1	82.4

Table 3. The percentage of compounds remaining from *P. auriculata* after treatment with UV-irradiation and activated charcoal.

P. auriculata compound	% Remaining after UV-irradiation	% Remaining after activated charcoal
1	93.4	41.8
2	78.1	33.4
3	79.6	47.1
4	82.1	43.8
5	83.4	41.7
6	67.1	0
Average remaining/degradation/elimination	80.6	34.6

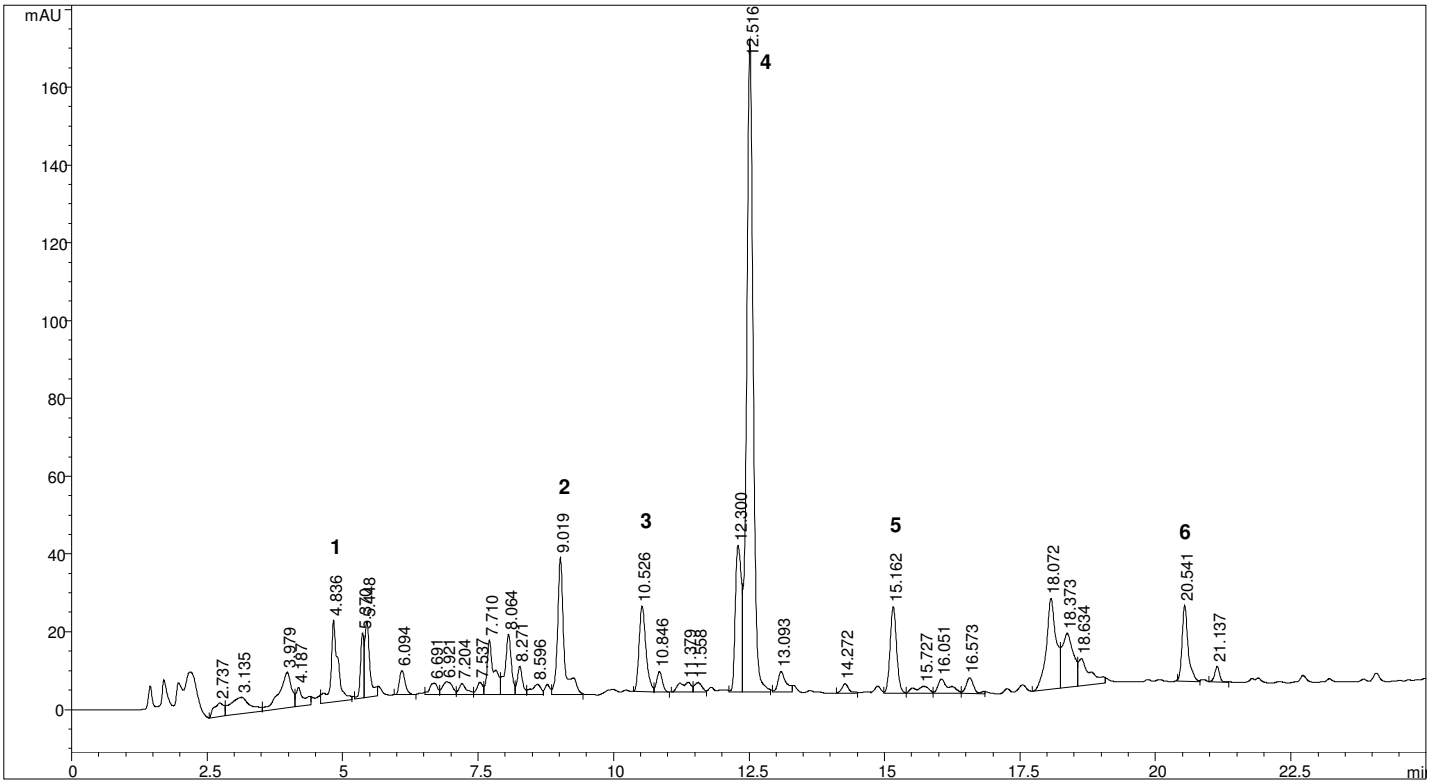

Figure 8. The chromatogram of *P. auriculata* compounds.

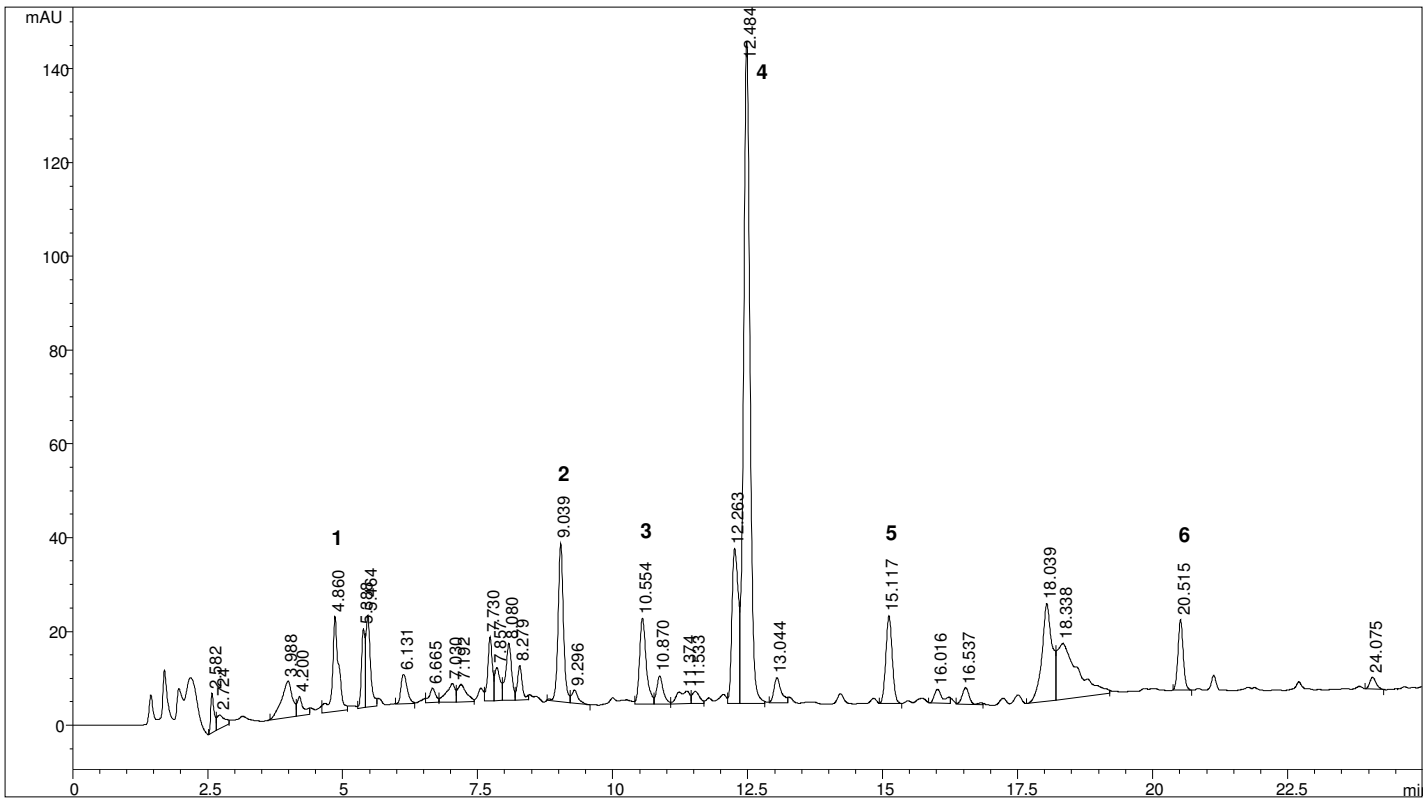

Figure 9. The chromatogram of *P. auriculata* compounds treated with UV-irradiation.

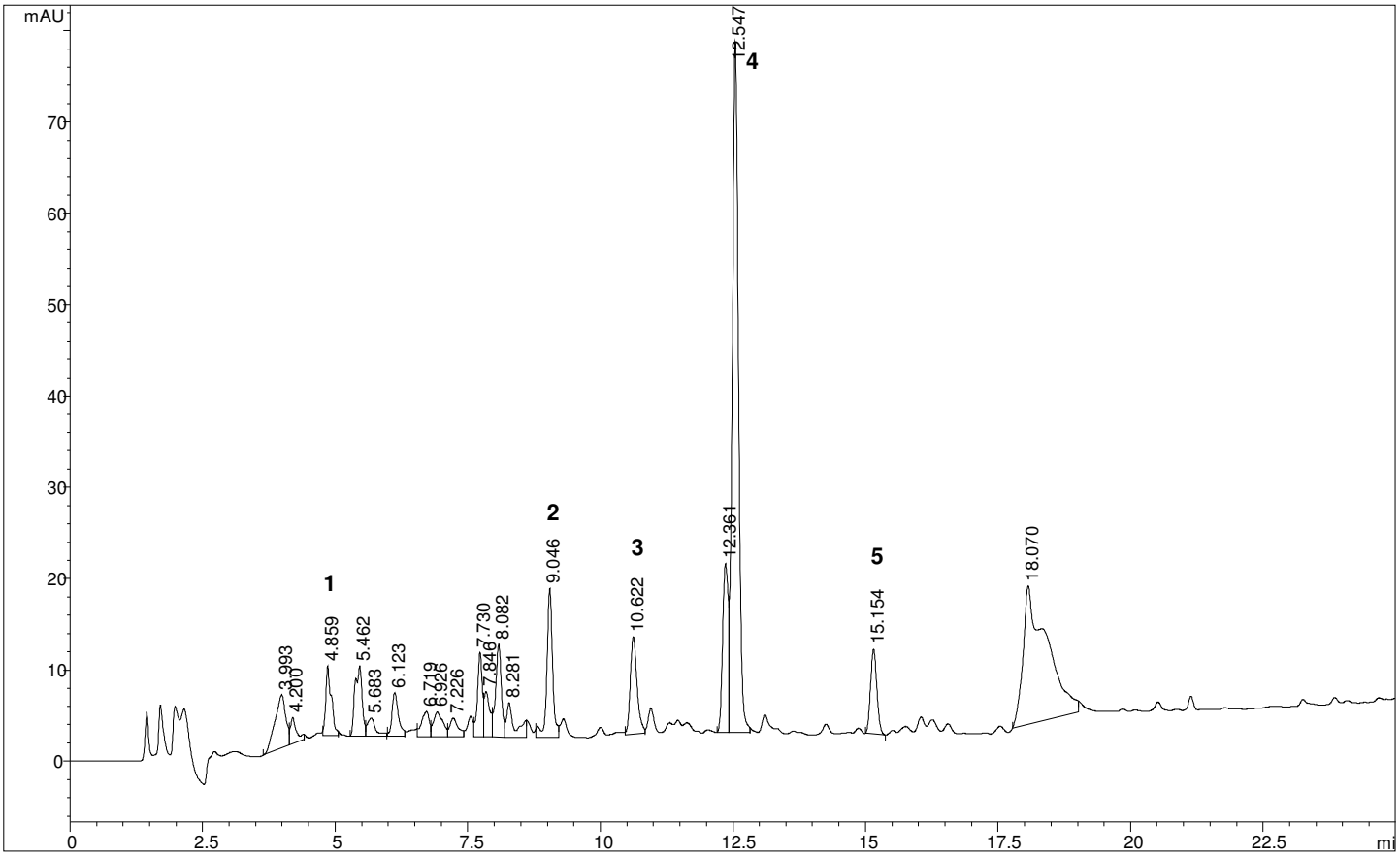

Figure 10. The HPLC chromatogram of *P. auriculata* compounds treated with activated charcoal.

HPLC to monitor the stability of the compounds in future experiments of decolouring.

REFERENCES

Abdel-Hameed ES (2009). Total phenolic contents and free radical scavenging activity of certain Egyptian *Ficus* species leaf samples. Food Chem. 114: 1271-1277.

Anoopkumar-Dukie S, Walker RB, Daya S (2001). A sensitive and reliable method for the detection of lipid peroxidation in biological tissues. J Pharm. Pharmacol. 53: 263-266.

Atmani D, Chaher N, Berboucha M, Ayouni, Lounis H, Boudaoud H, Debbache N, Atmani D (2009). Antioxidant capacity and phenol content of selected Algerian medicinal plants. Food Chem. 112: 303-309.

Chapman DM (1994). Benefits and limitations of a novel chlorophyll adsorbent. JAOCS, 71(4): 397-400.

Eggers AE (2009). Why do Alzheimer's disease and Parkinson's disease target the same neurons? Med. Hypotheses, pp. 698-700.

Furniss BS, Hannaford AJ, Smith PWG, Tatchell AR (1989). Vogel's. Textbook of practical organic chemistry: Pearson Education Limited, Edinburg Gate, Harlow, pp. 26-253.

Garcia YJ, Rodríguez-Malaver A, Peñaloza N (2005). Lipid peroxidation measurement by thiobarbituric acid assay in rat cerebellar slices. J. Neurosci. Meth. 144: 127-135.

Hwang E, Kim G (2007). Biomarkers for oxidative stress status of DNA, lipids, and proteins in vitro and in vivo cancer research. Toxicology, 229:1-10.

Kang DG, Yun C, Lee HS (2003). Screening and comparison of antioxidant activity of solvent extracts of herbal medicines used in Korea. J Ethnopharmacol. 87: 231-236.

Karagözler AA, Erdağ B, Emek YC, Uygun DA (2008). Antioxidant activity and praline content of leaf extracts from *Dorystoechas hastate*. Food Chem. 111: 400-407.

Keller RJ (1986). Chemotherapeutic Agents. The Sigma library of FT-IR spectra. Volume I. Ed. I. Sigma Chemical Company, St. Louis, Missouri. pp. 607-736.

Levitt J (1969). Introduction to plant physiology. Saint Louis, The C.V. Mosby Company, pp. 169-190.

Malek SNA, Wahab NA, Yaacob H, Shin SK, Lai HS, Serm LG, Rahman SNSA (2008). Cytotocix activity of *Pereskia bleo* (Cactaceae) against selected human cell lines. Int. J. Cancer Res. 4(1): 20-27.

Marinova EM, Toneva A, Yanishlieva N (2009). Comparison of the antioxid ative properties of caffeic and chlorogenic acids. Food Chem. 114: 1498-1502.

Marques V, Farah A (2009). Chlorogenic acids and related compounds in medical plants and infusions. Food Chem. 113(4): 1370-1376.

Merck Index CD-ROM Version 12.3 (2000). Chapman & Hall/CRC.

Miessler GL, Tarr DA (2004). Inorganic Chemistry, third edition. Pearson Education, Inc., pp. 594-636.

Ottino P, Duncan JR (1997). Effect of vitamin E succinate on free radical formation, lipid peroxidation levels and cyclooxygenase activity in murine melanoma cells. Nutr. Res. 17(4): 661-676.

Prezelin BB, Nelson NB (1990). The formation of ATP and reducing power in the light. In: Dennis DT & Turpin DH. (eds). Plant Physiology, Biochemistry and Molecular Biology. Longman, Scientific, Technical, pp. 212-222.

Roberts J, Whitehouse DG (1976). Pratical plant physiology. London: Longman, p 161.

Ślusarczyk S, Hajnos M, Skalicka-Woźniak K, Matkowski A (2009). Antioxidant activity of polyphenols from *Lycopus lucidus* Turcz. Food Chem. 113: 134-138.

Valko M, Leibfritz, D, Moncol J, Cronin MTD, Mazur M, Telser J (2007). Free radicals and antioxidants in normal physiological functions and human disease. Int. J. Biochem. Cell B. 39:44-84.

Zvezdanović J, Cvetić T, Veljović-Jovanović S & Marković D (2008). Chlorophyll bleaching by UV-irradiation *in vitro* and *in situ:* Absorption and fluorescence studies. Radiat. Phys. Chem. in press.

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