Scientific Research Publishing

American Journal of Analytical Chemistry, 2023, 14, 1-11
https://www.scirp.org/journal/ajac
ISSN Online: 2156-8278
ISSN Print: 2156-8251

HPLC Profiling for Quality Control of Secondary Metabolites of Aqueous and Hydroethanolic Extract of *Gardenia aqualla Stapf & Hutch*

Claude Bérenger Ngantchouko Ngalemo[1,2], Jean Kisito Kouame[3], Gisele Laurel Tchiengang Tchoua[1], Blondin Gatien Tsawo[1], Patrick Yamen Mbopi[1], Théophille Dimo[4], Christophe N'cho Amin[2], François Nicaise Bony[2]

[1]Higher Institute of Health Sciences, Université des Montagnes, Bangangté, Cameroon
[2]Department of Analytical, Mineral and General Chemistry, Food Technology, Training and Research Unit, Pharmaceutical and Biological Sciences, Félix Houphouet-Boigny University, Abidjan, Côte d'Ivoire
[3]Laboratory of Water and Food Analysis, National Laboratory of Public Health, Abidjan, Côte d'Ivoire
[4]Faculty of Sciences, Department of Animal Biology and Physiology, Laboratory of Animal Physiology, University of Yaoundé 1, Yaoundé, Cameroon
Email: moubeung@gmail.com

How to cite this paper: Ngalemo, C.B.N., Kouame, J.K., Tchoua, G.L.T., Tsawo, B.G., Mbopi, P.Y., Dimo, T., Amin, C.N. and Bony, F.N. (2023) HPLC Profiling for Quality Control of Secondary Metabolites of Aqueous and Hydroethanolic Extract of *Gardenia aqualla Stapf & Hutch*. *American Journal of Analytical Chemistry*, **14**, 1-11.
https://doi.org/10.4236/ajac.2022.141001

Received: December 7, 2022
Accepted: January 13, 2023
Published: January 16, 2023

Abstract

WHO strategy on traditional medicine is based on security and quality of phytomedicine. Commonly adulterated affecting mostly metabolic and sexual dysfunction drugs. Control quality of those phytomedicines requires development of strategy and techniques applicable to them. Among the techniques, Reverse Phase Liquid Chromatography is the most used and has been developed in these studies to assess a protocol to characterize *Gardenia aqualla* leaves extract. The method consists in determining chromatographic conditions using organic and pH gradient models based on water and acetonitrile combined with pH modifiers made up of formic acid (AF) and ammonium hydroxide (NH_3). Results show that extracts contain mainly acidic compounds quickly eluted by NH_3 and more retained by AF. Optimal pH range for separation is 3 - 7 corresponding to 1.59 mM of NH_3 and 6.55 mM of AF. In these conditions, elution of many polar compounds could be effective using a C18 based-deactivated column in a short period of time.

Keywords

Gardenia aqualla Extracts, Quality Control, Method Development, Reverse Phase Liquid Chromatography

1. Introduction

Development of phytomedicines has become over the last 2 decades a real chal-

lenge associated with increase of therapeutic, financial and social crises. Several herbal preparations have been introduced into national formularies, following WHO strategy on traditional medicine (TM) whose requirement is safety and quality of phytomedicine [1]. *Gardenia aqualla* is a specie of the *Rubiaceae* family located in territories stretching from Western Africa to Uganda. The plant roots are described in traditional medicine as an antibacterial and also in sexual disorders [2]. In Cameroon leaves are traditionally used for fertility disorders, especially in man impotence. Very few data on its chemical composition exist, and botanically, leaves are almost the same with other species of the genus, making them subject to many falsifications or contamination (pesticide residues, heavy metals, mycotoxins and microorganisms and recently, pyrrolizidine alkaloids) [3] [4].

Quality of phytomedicine is one of the main requirements of WHO as they should guarantee safety and effectiveness before been register [1] [3]. Many authors have developed techniques for characterization of plant metabolites using either TLC [5], HPTLC [6] or column chromatography [7]. Among chromatography techniques, Liquid chromatography has been widely used [8] for phytochemical substances assessment. More recent procedures coupled with liquid chromatography are being developed for a better identification of metabolites including high resolution-liquid chromatography using UV coupled or not with mass detector [9] [10]. Using such techniques, many organized society in developed countries have introduced a list of plants targeting specific activity [11].

In Africa, some organized societies like the "West African Health Organization" have published the list of plants of the region used in TM in the "Pharmacopoeia of West Africa" [12] [13]. These lists have been drawn up according to uses, transmitted for several years, thus responding to the definition of improved traditional medicines of category 1 [14]. Hydroethanolic and aqueous extract of the fresh leaves have been realized to serve as polar matrix in respect with traditionally uses such as in Cameroon. Bony *et al.* [15] used chromatographic techniques to define tradimedicine analytical strategy.

This work aimed to establish a characterization profile of *Gardenia aqualla Stapf & Hutch* by liquid chromatography.

2. Material and Methods

2.1. Plant Material

The drug consists of the dry aqueous extract and the dry hydroethanolic extract of fresh leaves of *Gardenia aqualla* harvested during the month of February in the locality of Bangoua—Western region of Cameroon, identified in the National Herbarium of Cameroon under reference 35,933 HNC.

2.2. Reagents

For this analysis, we used reagents for analysis made up of 98˚ ethanol, and 25% ammonia acquired from Carlo Erba and 99% formic acid from Merck. HPLC

grade Acetonitrile was acquired from VWR and ultra-pure water extemporaneously produced by a Milli Q brand ultra-purifier.

Equipment used consisted of a Waters high-performance liquid chromatography chain consisting of a 1525 series binary pump, a 717 Plus autosampler equipped with an automatic injector, and a Waters 2487 dual λ absorbance detector. The chain is controlled by Breeze software for data acquisition and processing.

2.3. Extraction of Fresh Leaves

Thoroughly washed with distilled water, fresh leaves were used in this part. For that purpose, leaves were cut into small pieces and used for extraction. Three hundred grams were macerated in the appropriate solvent (water-ethanol mixture (30/70) or distilled water). The solvent was renewed every 24 hours during three days. Macerates were filtered through Whatman paper No. 3. Each resulting filtrate was concentrated under reduced pressure with a BÜCHI R-201 rotary evaporator at 38˚C and subsequently dried in an oven at 40˚C. The yield was the assessed. We founded 18.34 g (yield of 6.11%) of dark green, hygroscopic powder representing the hydro-ethanolic extract (EHE) or 12.97 g (yield of 4.33%) of dark brown colour corresponding to aqueous extract (EA).

2.4. Chromatographic Analyses

2.4.1. Sample Preparation

In a 10 ml vial, 70 mg of dry extract was dissolved in 5 mls of distilled water. The solution is brought to the ultrasound tank for 10 minutes and the volume is reduced to 10 ml with distilled water. The solution obtained was filtered through a filter membrane of 0.45 µm pore diameter before injection.

2.4.2. Method Development

The stationary phase selected was a C18 possessing deactivated silanol groups, thus requiring molecules made highly non-polar. The extracts are of aqueous and hydroethanolic type, therefore they contain a large proportion of polar compounds. The chemical nature of said compounds should undergo modulation by modifying the mobile phase. For this purpose, acid pH of mobile phase was ensured by formic acid (AF) 0.1% v/v and alkaline pH based on NH_3 (25%) at 0.1% v/v were prepared and a third system mixing the two (AF (0.1%) and NH_3 (0.1%)) was constituted. Eluate is detected at 210 nm for 45 minutes following the elution gradient showed in Table 1 below.

The table presents the elution program at 1 ml/min of flowrate as the organic phase proportion (%B) varies.

2.4.3. Method Optimization

Optimization of the method was done with the aim of obtaining the maximum number of peaks with the best possible resolution on a BDS Hypersil 250 × 4.6 mm column with a particle size of 5 µm. Actions will aim to determine optimal

Table 1. Basic elution gradient.

	Time (min)	Flow (ml/min)	%A	%B
1	0.01	1.00	97.0	3.0
2	30.00	1.00	0.0	100.0
3	40.00	1.00	0.0	100.0
4	41.00	1.00	97.0	3.0
5	45.00	1.00	97.0	3.0

proportions of combined PH modifiers, optimal pH range and organic gradient leading to the best peak resolution.

3. Results and Discussion

The aim of this work was to define an HPLC analytical condition to characterize *Gardenia aqualla Stqpf* & *Hutch*. Two extraction using aqueous and (30 - 70) hydroethanolic solvents were performed to cope with traditional use of the plant as aphrodisiac [16], through infusion into palm wine.

3.1. Method Development

Liquid chromatography is a widely used method for analysis of complex mixtures including phytochemicals whose identification, quantification and purity can be determined [8]. As such, reverse phase analysis modality has been mainly used in analysis of secondary plant metabolites and according to Ganzera and Sturm [17], it is the main technique that would allow analysis of compounds of multiple polarity and/or molecular weight [18].

Quality of an analysis requires adequate sample preparation (extracts) and an elution system that ensures interaction with analytes and stationary phase without altering it [19]. Indeed, palm wine is made up of 93.3% of water and a lot of sugar whose fermentation process could turn it into a 20% alcohol [20] [21] [22]. Furthermore, Petko Penchev *et al.* [23] described that 30 - 70 hydroethanol mixture would optimally extract polar compounds with a better extractive effect on polyphenols and particularly flavonoids [23] [24]. Separation is then done by modulating the retention of ionogenic analytes based on a dual mode of organic gradient (choice of solvent), and pH (use of pH modifiers) [25] [26].

3.2. Effects of pH Modifiers

The chromatographic conditions used in this work was developed according to Sheumack and Burley [27], Dulac *et al.* [28], who describe use of a 0.1% - 0.3% aqueous solution of formic acid with an organic solvent, Yaling lu *et al.* [6] who used ammonium hydroxide in phenolic plant analysis while Cremin and Zeng [29], Dobrev *et al.* [30] et Bony *et al.* [31] used the mixture of formic acid and ammonium hydroxide simultaneously in a binary solvent system. These pH modifiers are indicated in liquid chromatography coupled with mass spectrometric

detector. According to Agilent booklet on pH modifiers, ammonium hydroxide is dedicated mostly for LC-MS analyses while triethylamine is more suitable for LC-UV as pH modifiers [32]. Applied to our development, results of analyses realized using each of the systems has showed different behavior using hydroethanolic extract (HE) as matrix.

Chromatograms of **Figure 1** show that formic acid (**Figure 1(b)**) has increased retention of compounds, describing their potential acidic state, while 0.1% ammonium hydroxide (**Figure 1(c)**) also known as silanol-blockings agents reduced the retention. Law B [33] showed that high amount of protonated amine in eluent reduces interaction with basic analytes by masking residual silanols. This blocking effect decreases with the order of alkylamine from Primary < Secondary < Tertiary ≤ Quaternary. The higher the substitution by an alkyl radical group, the lower will be the blockage effect on silanol group meaning that compounds shaping like $(CH_3)_3N^+R$ or $(CH_3)_2N^+RH$ possessing a long alkyl chain will be less silanol-blocking agents, inducing such a chromatogram as presented in **Figure 2** using 0.1% trimethylamine (TEA) (**Figure 2(a)**) and 0.1%NH_3 (**Figure 2(b)**).

3.3. Optimization of the Method

3.3.1. pH Optimization

For better separation effect, pH of the eluent was measured and optimized. The

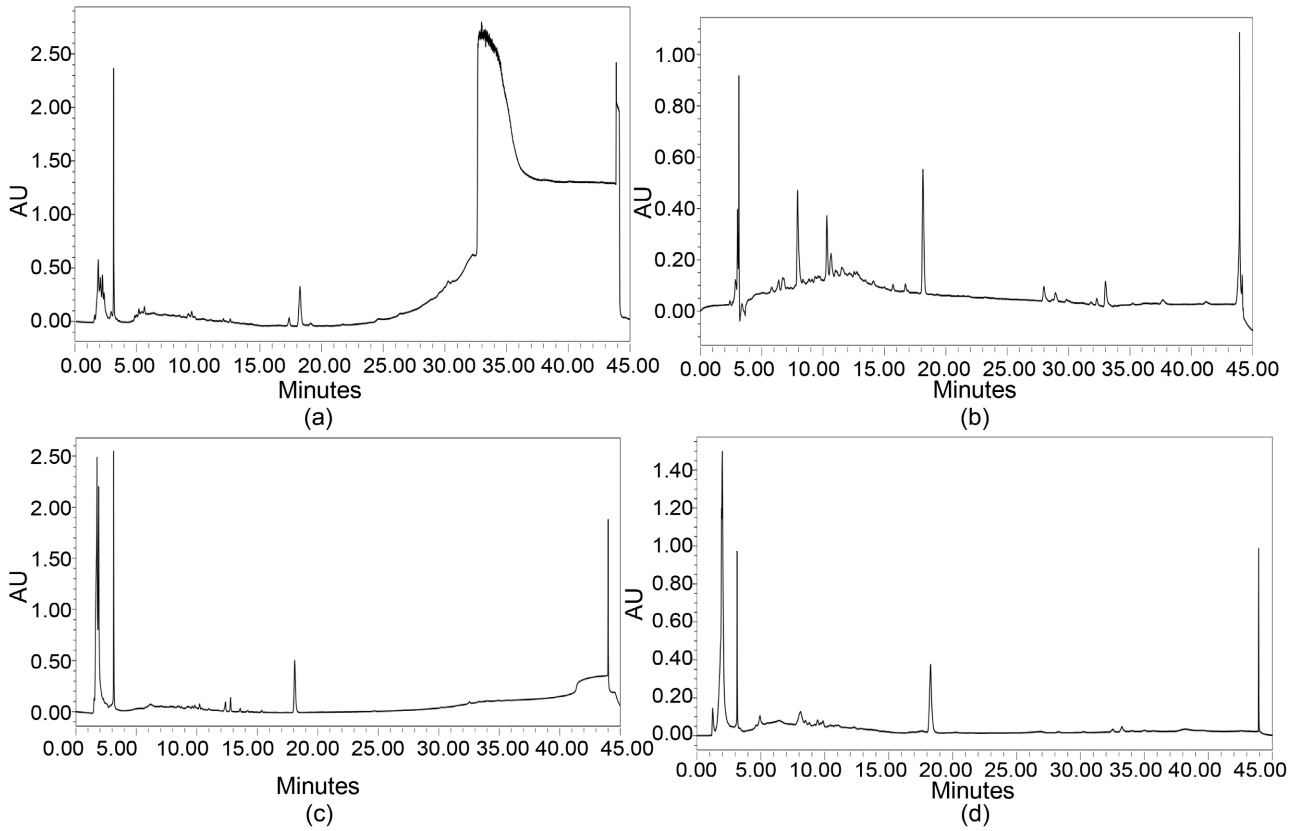

Figure 1. Chromatograms of Hydroethanolic extract using 0.1% formic acid (b) and 0.1% Ammonium (c) and with a AF 0.1%/NH_3 0.1% mixture of pH modifiers (d) compare to no modifier (a).

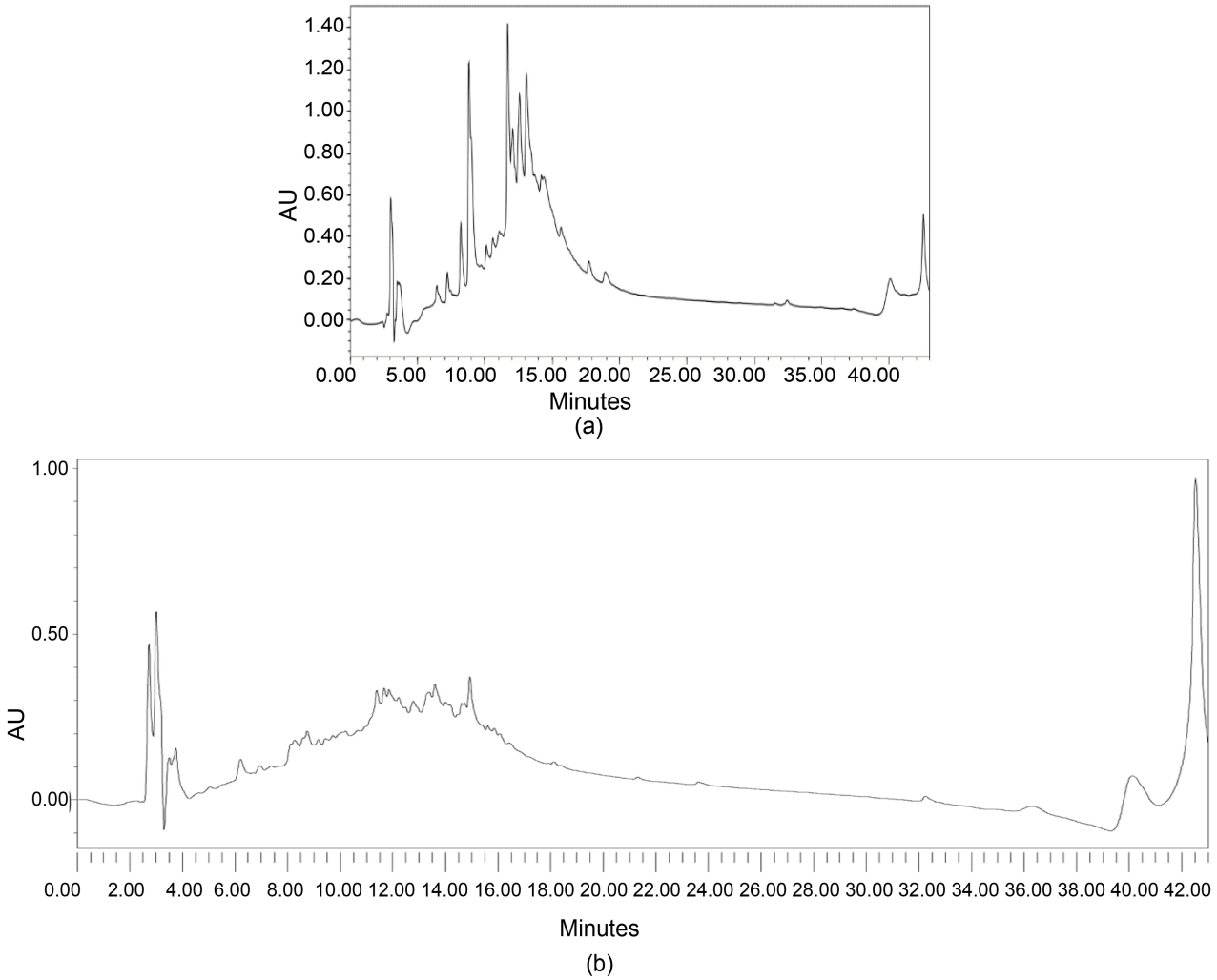

Figure 2. Chromatograms resulting from analysis of hydroethanolic extract using triehtylamine (a) and 0.1% NH_3 (b).

gradient mode was developed from low pH in 2.06 with 0.1% formic acid in water phase to 9.3 with 0.1% of NH_3 in acetonitrile phase. Modification of pH had been done by reducing the proportion of modifiers to 0.025% FA and 0.012% NH_3. Measurement of mixture's pH showed 3.18 à pH 22.4˚C for Phase A (Eau + 0.025% FA + NH_3 0.012%) and 6.94 à 21.1˚C for Phase B (ACN + 0.025% FA+ NH_3 0.012%), corresponding to 6.55 mM of AF and 1.59 mM of ammonium hydroxide giving almost better separation power as showed in chromatograms of **Figure 3** below. According to Schoenmakers *et al.* [34], variation of pH plays an essential role in developing a method as it may act on chemical effect of stationary phase either by affecting the silanol dissociation, or by affecting the interaction between ionic or ionogenic compounds with the stationary phase considering here the dissociation constant of the compounds.

3.3.2. Optimization of Gradient

On regard of these conditions, gradient was optimized to modify retention parameters. By increasing the initial organic phase à the beginning of the analyses,

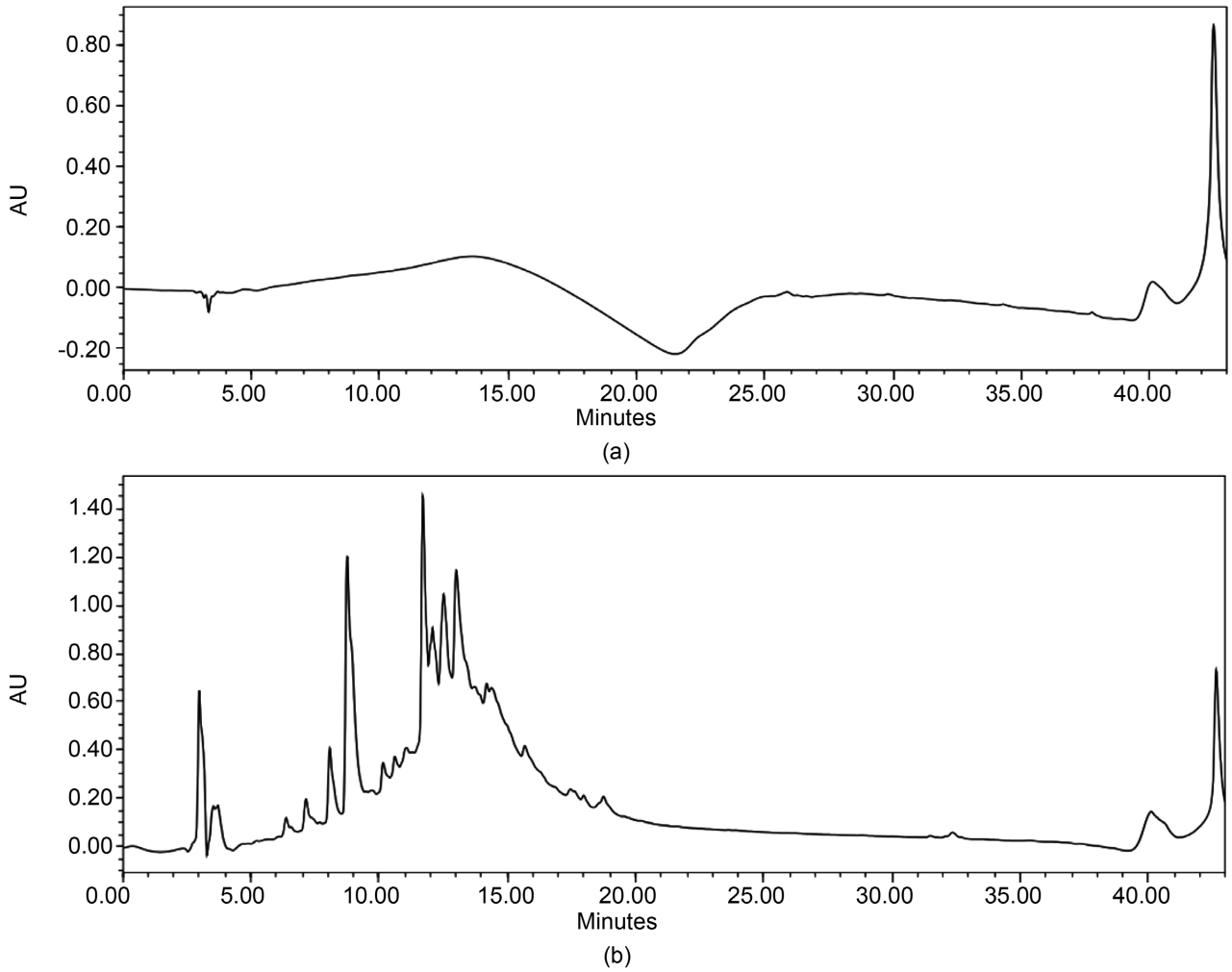

Figure 3. Chromatograms of Hydroethanolic extract (b) and solvent of reconstitution (a).

the retention power was complete reduced even with a reduce flowrate (**Figure 4**).

Gradient was then modulated to reduce organic occurrence while slowing the flowrate to 0.8 ml/min in between an increase gradient of pH going from 3 to 7. This led to a better distribution of peaks all over the chromatogram and shortening of the analysis period to 30 min. The technique was applied to ethanolic and hydroethanolic extract of *G. aqualla* using standard C18 based-deactivated silanol Hypersil stationary phase. Results are presented in **Figure 5** using this final gradient protocole presented in **Table 2** below.

The table presents the increase of proportion of the organic phase from 2% to 100% a 0.8 ml/min flowrate.

4. Conclusion

The aim of this work was to establish liquid chromatography method for characterization of aqueous and hydroethanolic extract of *Gardenia aqualla*. After optimization of the procedure concerning optimal proportions of combined PH

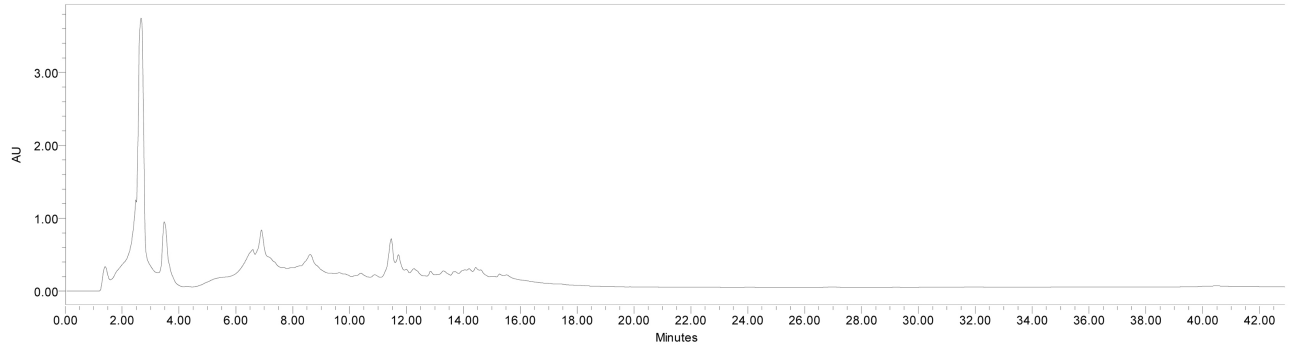

Figure 4. Chromatograms of hydroethanolic extract of *Gardenia aqualla*. Eluent is H_2O/acetonitrile containing each 0.025% FA & 0.012% NH_3 following a gradient of organic phase from 5% to 100% in 20 min of organic phase.

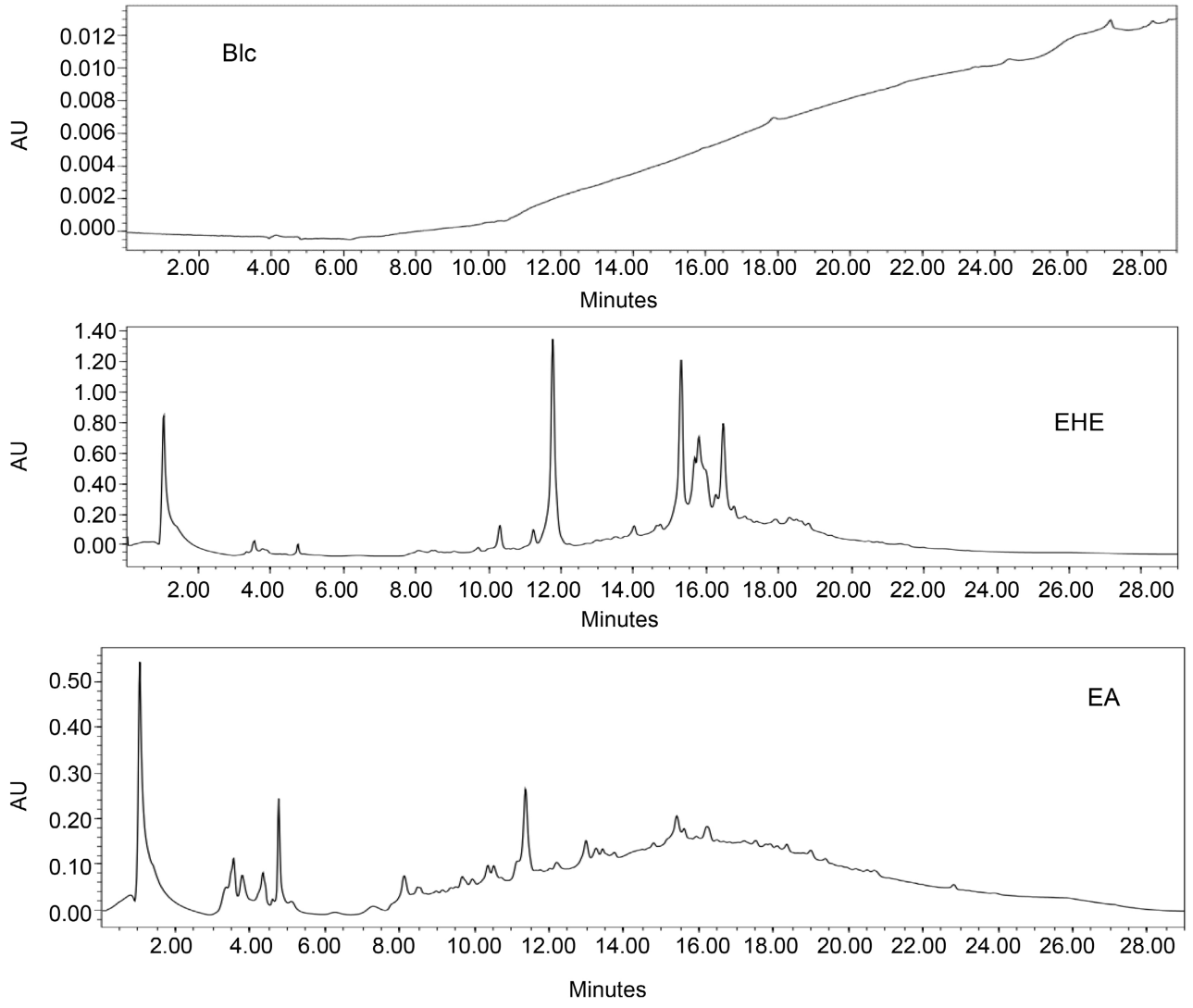

Figure 5. Chromatogram profile of hydroethanolic extract (EHE) and aqueous extract (EA).

modifiers, optimal pH range and organic gradient. Results show that analytes contained in the extracts are mainly acidic compounds quickly eluted by high pH and retained by formic acid. Optimal pH range for separation was 3 - 7

Table 2. Final elution gradient.

Time (min)	H_2O/ 0.025% FA/0.012% NH_3 (%)	ACN/ 0.025% FA/0.012% NH_3 (%)	Flow (ml/min)
0	98	2	0.80
20	50	50	0.80
25	0	100	0.80
28	0	100	0.80
30	98	2	0.80

corresponding to 1.59 mM ammonia and 6.55 mM of formic acid. In these conditions, elution of many polar compounds could be effective using a C18 based-deactivated column. Further analysis like High Resolution liquid chromatography or GCMS could complete to specify a family describing fully the nature of the extract as well as the genus.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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