Anti- Ehrlichia properties of the dichloromethane extract of Ageratum conyzoides

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Abstract

Ehrlichia canis is an intracellular bacterium that infects hematopoietic cells. It is the causative agent of canine monocytic ehrlichiosis (CME). The disease may be acute, subclinical, or chronic, and is treated with tetracyclines including doxycycline. However, this class of tetracyclines may cause several side effects due to prolonged treatment. Bacterial resistance to antimicrobials has been extensively reported.

The present study aimed to assess the anti- Ehrlichia activity of the dichloromethane extract (DCM) of Ageratum conyzoides L. on Ehrlichia- infected DH82 cells. For this purpose, the DCM extract of A. conyzoides collected in the municipality of São Luís, State of Maranhão (MA), northeast Brazil, was obtained from the aerial parts of the plant by exhaustive percolation in H 2 O- CH 2 Cl 2 (2:8) and subsequent extraction of the chemical compound. The chemical composition of these samples was investigated. The anti- Ehrlichia properties of A. conyzoides were confirmed in Ehrlichia- infected DH82 cells at a concentration of 200 µg.mL -1 of its DCM extract. The results of the treatments were evaluated at 18h and 36h after the insertion of the treatments evaluated with A. conyzoides.

Based on the results of the chemical analysis of the samples, we may attribute these antirickettsial properties to the compounds from the lignan family that are found in this medicinal plant .

Introduction

Ehrlichia spp. is an obligate intracellular bacterial pathogen that parasitizes hematopoietic cells (leukocytes). This rickettsial organism infect a wide range of mammalian hosts including dogs, cats, cattle, horses, and humans (Bogićević et al. 2017). Canine monocytic ehrlichiosis (CME) is caused by the bacterium Ehrlichia canis and has the ixodid tick Rhipicephalus sanguineus (common name: brown dog tick) as its main vector (Dagnone et al. 2001). The disease may be acute, subclinical, or chronic.

In the acute phase of the disease, clinical signs include depression, lethargy, anorexia, pyrexia, lymphadeno - and splenomegaly, and weight loss. Affected animals may exhibit bleeding especially petechiae and ecchymoses on the skin and mucous membranes and occasional epistaxis (Waner and Harrus 2000; Sousa et al. 2010). The subclinical phase of the disease consists of variable, persistent thrombocytopenia, leukopenia, and anemia in the absence of clinical signs (Varela 2003; Breitschwerdt 2004; Sousa et al. 2010). The chronic phase of canine monocytic ehrlichiosis may be severe and present as a fatal hemorrhagic syndrome due to bone marrow failure (Mylonakis et al. 2003).

The drug of choice for the treatment of the disease in all its phases is doxycycline for 28 days (Tilley et al. 2003). However, the drugs of the tetracycline group may have some undesired effects when administered for prolonged periods of time. The most notable toxic effects of tetracyclines are observed in the bone and teeth. It causes a delay in bone formation and yellowish discoloration of teeth (Damaso 1990). Tetracycline bone toxicity results from neuromuscular blockade which is probably produced by the chelation of calcium ions (Goodman and Gilman 1990) and direct action on the mechanism of bone

absorption and resorption by osteoclasts (Donahue et al. 1992) interfering with the deposition of calcium in bone tissue (Van Linthoudt et al. 1991).

In addition, photosensitivity manifested by hyperpigmentation, erythema, and ultimately skin ulceration is frequently associated with tetracycline therapy (Cunha 2001). The most frequent clinical signs observed in patients treated with tetracyclines are digestive and include nausea, vomiting, diarrhea, abdominal pain, gastritis, and enterocolitis (Damaso 1990; Gugliemo and Jacobs 2001; Saucedo 2003; Torres 2000; Workowki 2000; Zimmerman 2000). This drug is able to cross the placental barrier and is therefore contraindicated during pregnancy. Tetracycline should not be given to puppies (Gugliemo and Jacobs 2001).

According to González-Lamothe et al. (2009), the products of secondary metabolism accumulated by plants can act as "potentiators of antibacterial activity", favoring the activity of antibiotics whose action is limited by multidrug resistance mechanisms developed by microorganisms; or as “virulence attenuators”, adapting the host's immune system response to infection.

Ageratum conyzoides L. is present in the vast majority of the Brazilian states. Common names for this plant are "mentrasto" and "catinga-de-bode". It occurs mainly in anthropic areas and in plantations (Kissmann and Groth 1999). This ethnomedicinal plant has anti-inflammatory, analgesic, antidiarrheal, and antimicrobial properties (Cimanga et al. 2014; Prajapati et al. 2014). Its anti-Leishmania activity was recently reported in the literature (Teixeira et al. 2014). Compounds found in Ageratum conyzoides L. (terpenes, monoterpenes, sesquiterpenes, quinolones, phenolic compounds and flavonoids) have antimicrobial activity well described in literature, methanolic extracts have already shown to be effective against Escherichia coli, Klebsiella oxytoca, Proteus mirabilis, Salmonella thyphimurium, Stahylococcus aureus and Shigella flexneri (Samy et al. 2013). Studies have also confirmed the efficacy of dichloromethane extracts against bacteria Staphylococcus aureus Escherichia coli (Kanyanga et al. 2014). In addition to the capacity to inhibit the replication of intracellular microorganisms (Singh et al. 2016). The present study aimed to analyze the anti-Ehrlichia activity of the dichloromethane extract (DCM) of Ageratum conyzoides L. in Ehrlichia-infected DH82 cells.

Materials And Methods

Plant material

The plant species *Ageratum conyzoides* L. was cultivated and collected at the Berta Langes de Morretes Medicinal Herb Garden of the Federal University of Maranhão (UFMA), municipality of São Luís, State of Maranhão (MA), northeast Brazil (2°33'13.5"S and 44°18'20.8"W) in July 2017 (rainy season), according to the method published in the Brazilian Pharmacopeia (ANVISA 2010). The plant was herborized and identified, and a sample (voucher specimen - MAR 9099) is deposited at the Herbarium of Maranhão (MAR), located in Federal University of Maranhão, Brazil.

The access was registered under the ID number ADBBA07 in the National System of Management of Genetic Heritage and Associated Traditional Knowlege. The collect did not involve endangered or protected species.

Method to obtain the extract and the extract of the plant

A total of 1.7 kg of the aerial parts of *Ageratum conyzoides* L. was collected in the early hours of the morning. Samples were dried at room temperature (25 °C) for a 7-day period. An infrared moisture analyzer (GEHAKA IV 2500) was used to measure the plant moisture and calculate the yield. Shortly after that, the dry aerial parts of *A. conyzoides* were crushed in a mechanical turbolizer. The exhaustive percolation in H_2O- CH_2Cl_2 (2:8) was the extraction method used in this study. Percolation was carried out at room temperature (25 °C) and protected from light. The extractor liquid was completed every 24h Corresponding to a total of 72h until the plant material exhaustion was finished. These compounds were evaporated to dryness under vacuum at approximately 40 °C. Shortly after that, the specimen was ultra-refrigerated at -80 °C to be lyophilized and to obtain the dry extract.

Analysis of the Extract of *Ageratum conyzoides* L. by High Performance Liquid Chromatography (HPLC) with ultraviolet detection

After cleaning up the extract, the sample was analyzed by HPLC using a Shimadzu® chromatograph (Shimadzu Corp., Kyoto, Japan) consisting of a solvent injection module with a binary pump and UV detector -Vis (SPA-10A). The column used was a Luna 5 µm C18 100 A (150 µm x 4.6 µm). The elution solvents used were elution solvent A (water + 0.01% formic acid) and elution solvent B (methanol + 0.01% formic acid). Samples were eluted according to the following exploratory gradients: 5% to 100% B in 60 min and 100% to 100% in 60 to 70 min. The flow rate was 1 mL/min, and the column temperature was 20 °C. The injection volume of the sample was 20 µL. Data were collected and processed using the LC Solution software (Shimadzu). In this study, baseline separation for the major components of the sample was obtained in a 70-min chromatographic run and evaluated at a 254 nm wavelength.

Characterization of the components of *Ageratum conyzoides*L. by liquid chromatography-mass spectrometry (LC-MS) and electrospray ionization mass spectrometry (FIA-ESI-IT/MS)

Characterization of the compounds extracted from *Ageratum conyzoides* L. was carried out at the Institute of Biosciences, São Paulo State University (Unesp), São Vicente, SP, southeast Brazil. By infusing the samples directly into a mass spectrometer with an ion-trap linear analyzer (Thermo Scientific LTQ XL) equipped with an electrospray (ESI) in negative mode (Thermo, San Jose, CA, USA). A stainless-steel capillary tube at 280 °C, a spray voltage of 5.00 kV, a capillary voltage of -90 V, and a -100 V tube lenses at a flow of 5 µL/min were used in this procedure. Samples were infused into the mass spectrometer from the HPLC system in which samples were analyzed online by ESI-MS in negative mode and with an associated UV detector. The mass spectra data were obtained in the same Fleet LCQ mass spectrometer from Thermo Scientific® with direct insertion of the sample device via continuous flow injection analysis (FIA). Samples were ionized with an ESI source. Fragmentations were obtained in multiple stages (MS^n)

in an ion trap (IT)-type interface. The negative mode was used for the generation and analysis of all spectra. The experimental conditions were the following: capillary voltage of −35 V, spray voltage −5000 V, capillary temperature 350 °C, carrier gas N_2 and flow 60 (arbitrary units). The track acquisition was in a mass range of *m/z* 100–2000 with two or more sweep events performed simultaneously in the spectrum. Compounds were identified by comparing between data from literature and fragmentation patterns.

Culture of *Ehrlichia canis*-DH82 cells

The *Ehrlichia canis* strain was obtained from the 35th passage of *E. canis* of the Cuiabá #1 isolate which belongs to the collection (library) of Rickettsiae and *Ehrlichia* from the Laboratory of Virology and Rickettsioses of the School of Veterinary Medicine of the Federal University of Mato Grosso (FMVZ/UFMT), Cuiabá, MT, central-west Brazil. This rickettsial strain multiplied in DH82 cell monolayers (ATCC number: CRL-10389) and was maintained at 37 °C and 5% CO_2.

The access was registered under the number A9463BB in the National System of Management of Genetic Heritage and Associated Traditional Knowledge according to art. 41 of Decree Nº. 8772/2016 of the Ministry of the Environment in Brazil.

DH82 cells (Canine Histiocyte: ATCC in CRL-10389) grew in Dulbecco's Modified Eagle's (DMEM) medium (Sigma Chemical Co., St. Louis, MO, USA), plus 5% fetal serum of calf (HyClone Laboratories, Logan, Utah, USA) and culture bottle of 25 cm^2 at 37 °C and 5% CO_2 as recommended by Aguiar et al. (2007). The rate of *E. canis* infection was determined by screening Diff-Quik stained cell monolayer smears (Laborclin, Pinhais, PR, Brazil) under the light microscope.

When an infection rate of 70% was detected, the cells were resuspended in the same medium and the cell suspension was centrifuged at 4,000 g for 5 min. The experiments were run in 24-well culture plates at 37 °C and 5% CO_2. The infection rate was standardized at 3,000 cells per well and 70% of infected cells (Aguiar et al. 2007).

Anti-*Ehrlichia*assay

The assays were performed in the IC50 determination of the treatments studied against *E. canis* was determined from the test concentrations of 25 µg.mL^{-1}, 50 µg.mL^{-1}, 100 µg.mL^{-1}, 200 µg.mL^{-1}, 300 µg.mL^{-1}, 400 µg.mL^{-1} and 500 µg.mL^{-1} in cell monolayers DH82 infected with *E. canis* at a 70% infection rate, cell quantities were standardized at 3,000 cells / well, in 24-well plates, assays were performed in triplicate, where treatment control used spun doxicycline1µg.mL^{-1}, according to the package insert, and as a control of bacterial culture, wells treated only with distilled water. The protocol used to determine the antimicrobial effect of the test treatments was an adaptation by Rolain et al. (1998) and Rolain et al. (2002).

Cell viability analyses were performed using the trypan blue assay (Trypan blue exclusion test of cell viability) (Sigma-Aldrich, St. Louis, MO) according to the protocol and guidelines provided by Barile (Barile

1994).

Statistical analysis

The experimental design used in all biological assays of this study was completely randomized. The mean of each treatment was compared to its respective control. Data were initially transformed to Log(X), normalized, and then nonlinear regression was calculated to obtain IC_{50} (50% inhibition concentration) using the GraphPad Prism 7.0 software (Graph-Pad Inc., San Diego, CA, USA).

Results

This is the first study, the anti-*Ehrlichia* potential of *A. conyzoides* was calculated after 18h and 36h of treatment with the extract botanic (Fig 1). In the triplicate assays, the IC_{50} of the proposed treatment was 200 $\mu g.mL^{-1}$.

Were assessed the viability of the DH82 cells against different concentrations of the tested treatment. Were noted that they were not toxic in the highest concentration, i.e. 500 $\mu g.mL^{-1}$ (Fig 2) when compared with the control group which was formed by DH82 cells treated with ultrapure distilled water forlornly a 24-hour period.

In Fig. 3 shows the chromatogram of the extract dichloromethane of *A. conyzoides* with the UV absorption profiles of the 5 chromatographic peaks as evidenced by overlapping peaks high resolution in reverse phase (Table 1).

Lignans was the major class of the extract dichloromethane of *A. conyzoides* collected in São Luís, MA, northeast Brazil. This class of compounds was first identified in this specimen.

Discussion

To the best of the knowledge, this is the first study to report the anti-Ehrlichia activity of the botanical extract of Ageratum conyzoides L. Recently a scientific paper on the efficacy of A. conyzoides against bacteria Ehrlichia canis has been published, but the product used by the researchers was the essential oil (Rosario et al. 2019).

The DCM extract of this especimen showed remarkable anti-Ehrlichia activity at the concentration of 200 $\mu g.mL^{-1}$ (Fig. 1). The botanical extract of A. conyzoides was active against intracellular microorganisms, such as Trypanosoma brucei rhodesiense, Leishmania donovani (Nour et al. 2010), and against Plasmodium falciparum (Owuor et al. 2012) as previously described in the literature by other authors. In addition, the efficacy of A. conyzoides extract against Leishmania infantum (Joshi et al. 2016) and Leishmania amazonensis (Teixeira et al. 2014) has been demonstrated by a number of researchers.

The chemical characterization of the dichomethane extract of A. conyzoides obtained in this study shows that almost all of it is composed of secondary metabolites of the lignan class (Fig. 3). According to the literature, lignans has remarkable biological activities including their anti-Leishmania action (Royo et al. 2003) and trypanocidal action which results in mitochondrial dysfunction and oxidative damage which can trigger destructive effects on the biological molecules of these microorganisms leading to death (Bernardes et al. 2006; Izumi et al. 2011; Luize et al. 2006; Pelizzaro-Rocha et al. 2011).

The semi-synthetic derivatives of the lignans including (-) - quinoline, (-) - O-benzyl cubebin, and (-) - (N, N-dimethylaminoethyl) cubebine showed also antiprotozoal activity against amastigote stages of T. cruzi with IC50 = 0.7; 5.7 and 4.7 µm respectively, after 24 h of incubation (Souza et al. 2005). A number of studies have shown that lignan (-) - hinoquinine is effective in reducing chromosomal damage induced by doxorubicin as it has an antioxidant effect on the mitochondria of the parasite (Saraiva et al. 2007; Izumi et al. 2011). Syringaresinol likely compound found in the botanical extract (Table 1) has antitrypanosomal and anti-Leishmania activity (Costa et al. 2018) in addition to its antibacterial and antitrypanosomal activity (Alamzeb et al. 2013).

To date, the mechanism of action of plant lignans against E. canis is unknown. Were suggest that these substances (polyphenols) inhibit the formation of microtubules, preventing cell division (Wink 2015) since the microtubules are responsible for the organization of the mitotic spindle. Ehrlichia replicates by binary fission and subsequently form elemental corpuscles which are seen as pleomorphic inclusions (initial corpuscles) inside leukocytes (Almosny et al. 2002).

The dichloromethane extract of A. conyzoides collected in São Luís, MA, northeast Brazil, has a promising lignan composition with remarkable anti-Ehrlichia activity, and may be a potential alternative treatment for one of the most important diseases of companion animals.

Declarations

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Authors' contributions

CJRMR and FAM designed the study. CJRMR, CQR, DMA, CAAL, DPBS, JACL, DFC and FAM carried out experiments and analyzed the data. CJRMR wrote the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data generated or analyzed during this study are included in this published article in the main manuscript and additional supporting file.

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Not applicable.

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Not applicable.

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Table

Table 1: Identification of compounds in *Ageratum conyzoides* dichloromethane extract by LC-ESI-IT/MS.

Peaks	$[M-H]^-$	Ms^n	Proposed compound
1	175	159; 145; 132; 115	Hydroxy methyl coumarin
2	417	402;387;372;356;328;300	Syringaresinol
3	403	390; 345	Derived from syringaresinol 1
4	431	415; 400;369	Derived from syringaresinol 2
5	387	372; 356; 341; 313; 285	Derived from syringaresinol 3

Figures

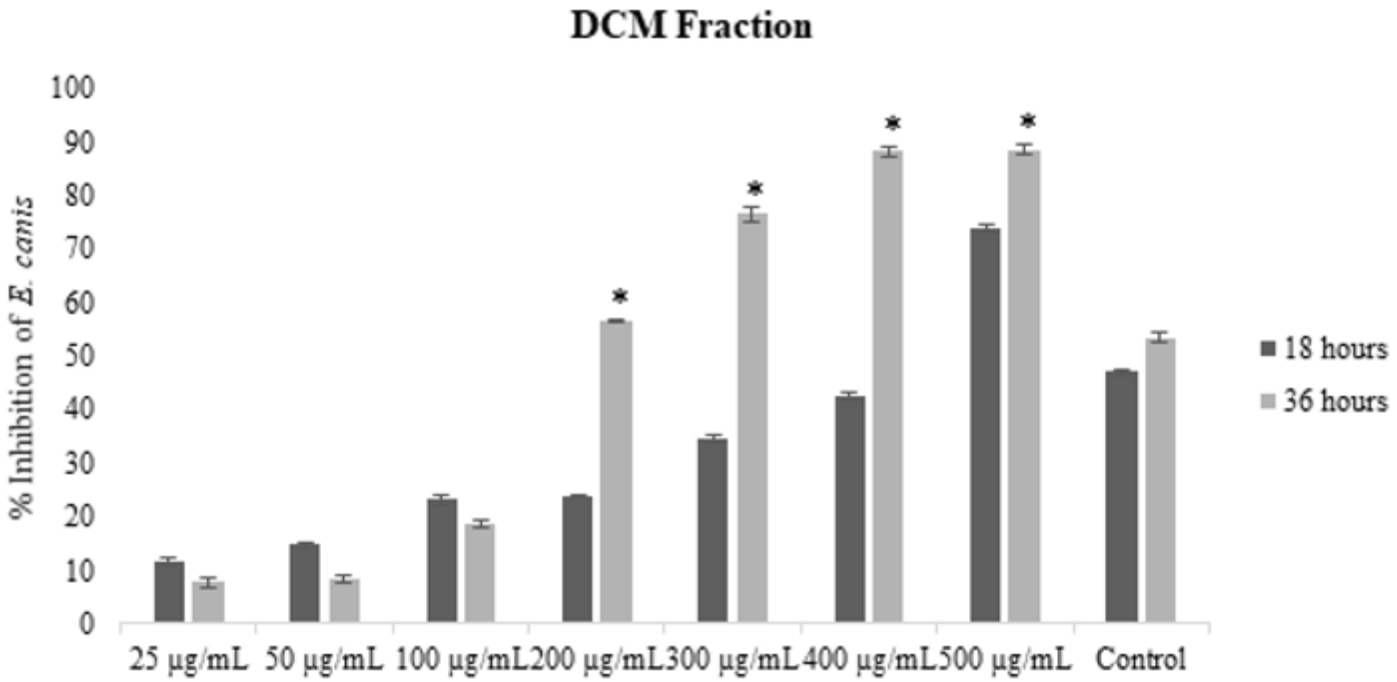

Figure 1

Percentage of inhibition of Ehrlichia canis in DH82 cells after 18h and 36h of treatment with the A. conyzoides DCM extract and with doxycycline (control group) at different concentrations. Each row represents mean and standard deviation of three independent assays in which *$p<0.05$ demonstrated that there was no statistically significant difference in relation to the control group according to the Tukey-test with a 95% confidence index.

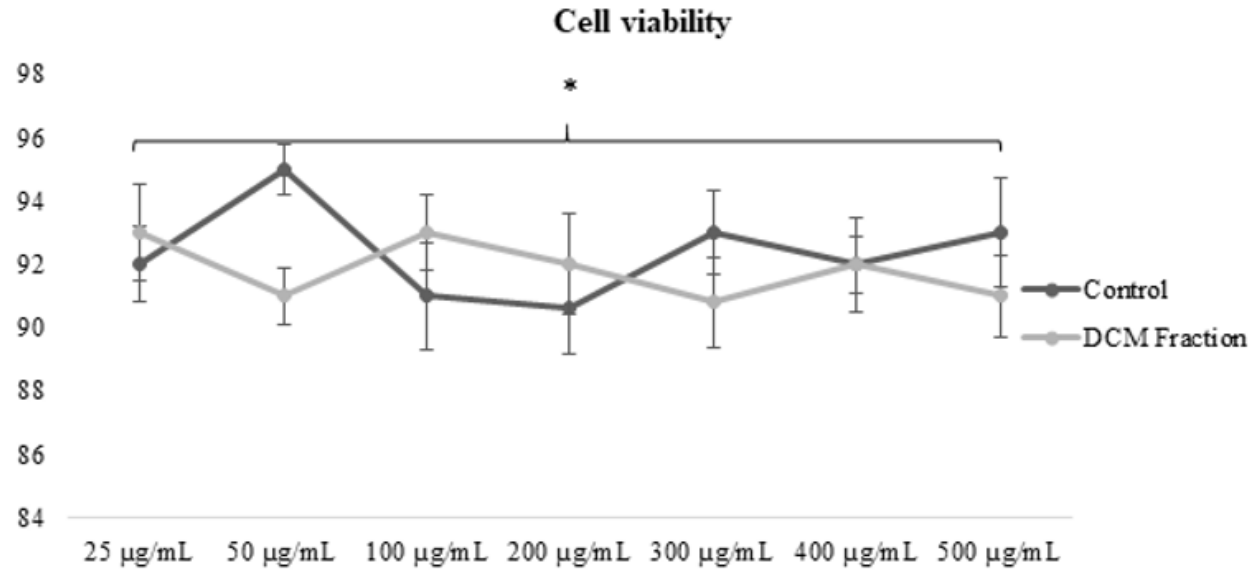

Figure 2

Each row represents mean and standard deviation of three independent assays. Where *p<0.05 demonstrated that there was no statistically significant difference in relation to the control group according to the unpaired Student's t-test with a 95% confidence index.

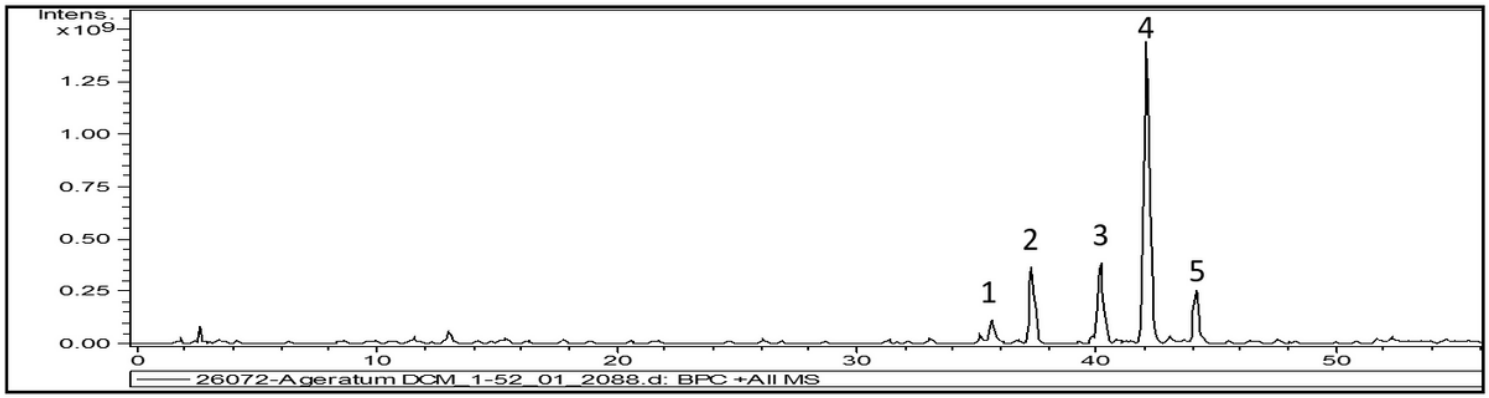

Figure 3

HPLC-UV chromatogram (254 nm) of Ageratum conyzoides dichloromethane extract.