JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Original Article

Biological activities of essential oil extracted from leaves of *Atalantia sessiflora* Guillauminin Vietnam

Nhan Trong Le[1#], Matthew Gavino Donadu[2,3#], Duc Viet Ho[1], Tuan Quoc Doan[1], Anh Tuan Le[4], Ain Raal[5], Donatella Usai[2], Giuseppina Sanna[6], Mauro Marchetti[7], Marianna Usai[3], Nicia Diaz[2], Paola Rappelli[2,8], Stefania Zanetti[2], Piero Cappuccinelli[2], Hoai Thi Nguyen[1]

[1] *Faculty of Pharmacy, Hue University of Medicine and Pharmacy, Hue University, Hue city, Viet Nam*
[2] *Department of Biomedical Sciences, University of Sassari, Sassari, Italy*
[3] *Department of Chemistry and Pharmacy, University of Sassari, Sassari, Italy*
[4] *Mientrung Institute for Scientific Research, VAST, Hue city, Viet Nam*
[5] *Institute of Pharmacy, Faculty of Medicine, University of Tartu, Tartu, Estonia*
[6] *Department of Biomedical Sciences, University of Cagliari, Monserrato, CA, Italy*
[7] *CNR, Institute of Biomolecular Chemistry, Li Punti, Sassari, Italy*
[8] *Mediterranean Center for Disease Control (MCDC), University of Sassari, Sassari, Italy*

# Authors equally contributed to this paper.

Abstract

Introduction: The present study aimed to determine the chemical compositions and bioactivities of the essential oil of *Atalantia sessiflora* Guillaumin (*A. sessiflora*), including antibacterial, antimycotic, antitrichomonas, anti-inflammatory and antiviral effects.

Methodology: The essential oil from leaves of *A. sessiflora* was extracted by hydrodistillation using a Clevenger apparatus. Chemical compositions of oil were identified by GC/MS. Antimicrobial and antitrichomonas activity were determined by the microdilution method; anti-inflammatory and antiviral were determined by the MTT method.

Results: The average yield of oil was 0.46 ± 0.01% (v/w, dry leaves). A number of 45 constituents were identified by GC/MS. The essential oil comprised four main components. The oil showed antimicrobial activities against Gram-positive strains as *Staphylococcus*; Gram-negative bacteria such as *Klebsiella pneumoniae* and *Escherichia coli*; and finally four *Candida* species. *Enterococcus faecalis* and *Pseudomonas aeruginosa* were least susceptible to the oil of *A. sessiflora*, as seen in their MIC and MLC values over 16% (v/v). Activity against *Trichomonas vaginalis* was also undertaken, showing IC_{50}, IC_{90} and MLC values of 0.016, 0.03 and 0.06% (v/v) respectively, after 48 hours of incubation. The oil of *A. sessiflora* displayed activity against the nitric oxide generation with the IC_{50} of 95.94 ± 6.18 µg/mL. The oil was completely ineffective against tested viruses, ssRNA+, ssRNA-, dsRNA, and dsDNA viruses.

Conclusions: This is the first yet comprehensive scientific report about the chemical compositions and pharmacological properties of the essential oil of *A. sessiflora*. Further studies should be done to evaluate the safety and toxicity of *A. sessiflora* oil.

Key words: *Atalantia sessiflora*; essential oil; bioactivity; bacteria, viruses; *T. vaginalis*.

J Infect Dev Ctries 2020; 14(9):1054-1064. doi:10.3855/jidc.12469

(Received 26 January 2020 – Accepted 26 August 2020)

Introduction

Atalantia Corrêa genus belong to the family Rutaceae which is composed of 17 species. The plants of this genus are found in tropical and subtropical regions of Southeast Asia and China [1,2].*A. sessiflora* is one of the 8 species of the genus *Atalantia* found in Vietnam. *A. sessiflora*is a shrub with green branches, spines or rarely unarmed [3]. Several species in the genus *Atalantia* for which the chemical compositions and bioactivities of their essential oils extractions have been well documented lately [4–6], yet none of such included *A. sessiflora*. Therefore, we aimed to identify the chemical composition and biological activities present in the essential oil of *A. sessiflora* which was collected in Quang Tri Province, Vietnam. To the best of our knowledge, this is the first scientific report regarding the chemical and biological analysis of essential oil in *A. sessiflora*.

Methodology

General experimental procedures

Hewlett-Packard Model 5890A Gas chromatography (Agilent, CA, USA) was used to determine retention times and mass spectrum, with a

flame-ionization detector and fitted with a 60 m × 0.25 mm, thickness 0.25 μm AT-5 fused SiO_2 capillary column (Alltech, Milan, Italy). MS analyses were carried out with an *Agilent Technologies model 7820A* connected with a MS detector *5977E MSD* (Agilent, Santa Clara, USA). All reference standards, homologous series of *n*-alkanes (C_9-C_{22}), organic solvents used for GC analysis, chemicals used in Diamond's Trypticase Yeast extract Maltose (TYM) medium, lipopolysaccharide (LPS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), sodium nitrite, sulfanilamide, N-1-napthylethylenediamine dihydrochloride, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), dimethyl sulphoxide (DMSO), sterile distilled water, penicillin G, streptomycin, Luria Bertani (LB) broth and Roswell Park Memorial Institute 1640 (RPMI-1640) medium were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM), and fetal bovine serum (FBS) were purchased from the Grand Island Biological Company (GIBCO, Invitrogen). Mueller Hinton Agar (MHA), Sabouraud Dextrose Agar (SDA) were manufactured from Microbiol, Cagliari, Italy. All microbial strains and *T. vaginalis* were isolated from different clinical samples at the Department of Biomedical Sciences, Section of Experimental and Clinical Microbiology, University of Sassari, Sassari, Italy. Cell lines and viruses used to screen antiviral activity were purchased from American Type Culture Collection (ATCC). RAW 264.7 cell lines were cultured at the University of Perugia, Perugia, Italy. Cell culture flasks and 96-well plates were provided from Corning Inc. (Corning, New York, USA). The ELISA Plate Reader (Bio-Rad, Pleasanton, California, USA) was used to measure the absorbance of cells in the MTT cell viability assay.

Plant material

*A. sessiflora*were collected from Dakrong District, Quang Tri Province, Vietnam in May 2019 (16°38′16.9″N 106°48′06.5″E) and were identified by Dr. Chinh Tien Nguyen, Vietnam National Museum of Nature. A voucher specimen (AS-03) was deposited at the Faculty of Pharmacy, Hue University of Medicine and Pharmacy, Vietnam.

Extraction of the essential oil

The leaves of *A. sessiflora*(5 Kg) were shredded and the essential oil was extracted by hydrodistillation using a Clevenger apparatus for 3.5 hours at normal pressure according to Vietnamese Pharmacopoeia [7]. The oil was then collected, dried by Na_2SO_4, and stored in sealed vials at 4°C. The experiments were performed in triplicate.

Analysis of the essential oil

Three replicates of each sample were analyzed using a *Hewlett-Packard Model 5890A*GC equipped with a flame-ionization detector and fitted with a 60m × 0.25mm, thickness 0.25 μm*AT-5* fused SiO_2 capillary column. Injection port and detector temperature were at 280°C. The column temperature was operated from 50 to 135°C at 5°C/minute (1 minute), 5°C/minute to 225°C (5 minutes), 5°C/minute to 260°C held for 10 minutes. The samples (0.1 μL each) generally analyzed without dilution (using 2,6-dimethylphenol as an internal standard), were injected using a split/splitless automatic injector *HP 7673* and He as carrier gas. The quantification of each compound was displayed as absolute weight percentage using internal standard and response factors. The detector response factors (RFs) were determined for key components relative to 2,6-dimethylphenol and assigned to other components based on similarity of functional groups and/or structures.

MS analyses were carried out with an *Agilent Technologies model 7820A* connected with a MS detector *5977E MSD* using the same conditions and column described above. The column was connected to the ion source of the mass spectrometer. Mass units were monitored from 10 to 900 at 70 eV.

The retention indices (RI) of constituents were determined by co-injection with reference to a homologous series of *n*-alkanes (C_9-C_{22}) under the same conditions to calculate the retention indexes with the generalized equation by Van del Dool and Kartz [8].

The identification of compounds was based on several methods [8]. Data were processed for ANOVA by means of the software MSTAT-C and mean separation was performed by application of the LSD test at $p \leq 0.05$ level of significance.

Antimicrobial activity

Microbial strains and growth conditions

In the present work, a collection of 12 strains were selected, including 5 Gram-negative strains namely *Escherichia coli* ATCC 35218, *Escherichia coli* clinical, *Pseudomonas aeruginosa* ATCC 27853*, Pseudomonas aeruginosa* clinical and*Klebsiella pneumoniae* clinical; 3 Gram-positive bacteria such as *Staphylococcus aureus* ATCC 43300*, Staphylococcus aureus* clinical and *Enterococcus faecalis* clinical; 4 *Candida* spp. strains: *Candida albicans* 556 RM,

Candida glabrata clinical, *Candida tropicalis* 1011 RM and *Candida parapsilosis* RM.

All the strains used in this study, except the reference strains, were isolated from different clinical samples at the Department of Biomedical Sciences, Section of Experimental and Clinical Microbiology, University of Sassari, Sassari, Italy. All the cultures were maintained on the appropriate media at 4 °C. The cells were cultivated at 37°C on Agar plates (Microbiol, Cagliari, Italy) for 18 hours prior to performing the experiments.

Determination of Minimum Inhibitory Concentrations (MIC) and Minimum Lethal Concentration (MLC)

In order to establish the MIC and MLC of bacteria and *Candida* species, the broth microdilution method was used as suggested by the Clinical and Laboratory Standard Institute [9]. The inoculum was prepared by diluting colonies in salt solution at a concentration of 0.5 McFarland, and the concentration was confirmed at a wavelength of 530 nm by a spectrophotometric. The sensitivity test was implemented in LB broth and RPMI-1640 medium using 96-well plates. Oil concentrations were prepared by serial dilutions from 16% (v/v) to 0.0005% (v/v) and added with 0.5% Tween 80. After shaking, 100 µL of each oil dilution and 100 µL of bacterial/ yeast suspension at a concentration of 10^6 CFU/mL were added to each well then incubated at 37°C for 24 to 48 hours. MIC values were determined as the lowest concentration of the essential oil that inhibit the visible growth of the strains after overnight incubation. In order to determine the MLC value, 10 µL were seeded on Mueller Hinton agar and Sabouraud Dextrose agar and the plates were incubated for 24 to 48 hours at 37°C. Minimal Lethal Concentration (MLC) was considered as the lowest concentration that reduces the viability of the initial microbial inoculum by ≥ 99.9%. Each experiment was performed in duplicate and repeated three times.

Antitrichomonas activity

Culture of *Trichomonas vaginalis*

T. vaginalis reference strain G3 was cultured axenically in vitro by daily passages in Diamond's Trypticase Yeast extract Maltose (TYM) medium (Sigma Chemical Co., St. Louis, MO, USA) plus 10% fetal bovine serum (FBS) at 37 °C in a 5% CO_2 atmosphere. Exponentially growing *T. vaginalis* cells were harvested and viability was assessed by microscopy. Trichomonas cells (viability > 95%) were centrifuged at 1500 rpm for 10 minutes and resuspended in Diamond's TYM medium at 2. × 10^5 cells/mL [10].

Determination of anti-*T.vaginalis* activity

A 1% stock solution of essential oil from leaves of *A. Sessiflora* was prepared in Diamond's medium plus 4% of DMSO. Twofold serial dilutions of the stock solution in 100 µl of Diamond's TYM medium, ranging from 0.5% to 0.002% (v/v), were distributed in 96-well plates; 100 µl of the trichomonas suspension prepared as described above were then added to each well. The same dilutions of Diamond's TYM medium plus DMSO were used as a control. The culture plate was kept at 37 °C in a CO_2 incubator and checked after 1, 4, 24, and 48 hours. The percentage of viable *T. vaginalis* cells was observed by microscope. The MLC was defined as the lowest essential oil concentration at which no viable protozoa were observed. The 50% inhibitory concentration (IC_{50}) and ≥ 90% inhibitory concentration (IC_{90}) values were considered as the essential oil concentration at which 50% and ≥ 90% of *T. vaginalis* cells were killed, respectively. Each assay has been repeated independently at least two times [10].

Anti-inflammatory activity

Cell culture

RAW 264.7 cell lines were cultured in DMEM supplemented with 2 mM L-Glutamine, 10 mM HEPES, 1 mM sodium pyruvate and 10% FBS. Cells were maintained at 37ºC with 5% CO_2 in a humidified chamber. The cells were sub-cultured every 3-5 days with the ratio of (1:3) and incubated at 37°C under humidified 5% carbon dioxide atmosphere [11,12].

Inhibition of nitric oxide (NO) production

The inhibition of NO production was evaluated on different concentrations of essential oil of *A. sessiflora* (100, 20, 4, and 0.8 µg/mL). The RAW 264.7 cells were seeded at a density of 2×10^5 cells/well in 96-well plates and incubated for 24 hours at 37°C and 5% CO_2. Then, the media of each well were aspirated and fresh FBS-free DMEM media were replaced for 3 hours. Tested samples were added carefully into each well of 96-well plates and the cultivation was continued under the same conditions. After 2 hours treatment, cells were stimulated with 1 µg/mL of LPS for 24 hours. The presence of nitrite was determined in cell culture media using commercial NO detection kit Griess Reagent System (Promega Cooperation, WI, USA). Protocols supplied with assay kit were used for the application of assay procedure. Briefly, 100 µL of cell culture medium with an equal volume of 100 µL Griess reagent: 50 µL

of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid, and 50 µL 0.1% (w/v) N-1-naphthylethylenediamine dihydrochloride in a 96-well plate was incubated at room temperature for 10 minutes. Then the absorbance was measured at 540 nm in a microplate reader (Bio-Rad, California, USA). The amount of nitrite in the media was calculated from sodium nitrite ($NaNO_2$) standard curve. FBS-free DMEM media was used as blank sample while L-N^G-monomethyl arginine citrate (L-NMMA) was used as positive control and macrophages stimulated with LPS at 1 µg/mL and untreated was used as negative control. The ability to inhibit the nitric oxide (NO) was measured at doses of 100, 20, 4, and 0.8 µg/mL, and estimated as a IC_{50} which was calculated by the program Table Curve Version 4.0 [11,12].

MTT cell viability assay

RAW 264.7 cells were seeded in 96-well plates in the presence of various concentrations of essential oil of *A. sessiflora*. After 24 hours of incubation, MTT was added to a final concentration of 0.5 mg/mL, and the cells were incubated for 4 hours at 37°C and 5% CO_2. Finally, the supernatant was removed and the formazan crystals were dissolved in dimethyl sulfoxide (DMSO). The absorbance was measured at 540 nm. The percentage of dead cells was determined relative to the control group [11,12].

Antiviral activity

Cells and viruses

Cell lines were purchased from American Type Culture Collection (ATCC). Cell lines supporting the multiplication of RNA and DNA viruses were the following: CD4+ human T-cells containing an integrated HTLV-1 genome (MT-4); Madin Darby Bovine Kidney (MDBK) (ATCC CCL 22 (NBL-1) Bos taurus); Baby Hamster Kidney (BHK-21) (ATCC CCL 10 (C-13) *Mesocricetus auratus*); Monkey kidney (Vero-76) (ATCC CRL 1587 *Cercopithecus aethiops*).

Viruses were purchased from American Type Culture Collection (ATCC), with the exception of Human Immunodeficiency Virus type-1 (HIV-1) and Yellow Fever Virus (YFV). Viruses representative of positive-sense, single-stranded RNAs ($ssRNA^+$) were: i) *Retroviridae*: the III_B laboratory strain of HIV-1, obtained from the supernatant of the persistently infected H9/III_B cells (NIH 1983); ii) *Flaviviridae*: yellow fever virus (YFV) (strain 17-D vaccine (Stamaril Pasteur J07B01)) and bovine viral diarrhoea virus (BVDV) (strain NADL (ATCC VR-534)); iii) *Picornaviridae*: coxsackie type B4 (CV-B4) (strain J.V.B. (ATCC VR-184)) and human enterovirus C (poliovirus type-1 (Sb-1) (Sabin strain Chat (ATCC VR-1562)). Viruses representative of negative-sense, single-stranded RNAs ($ssRNA^-$) were: iv) *Rhabdoviridae*: vesicular stomatitis virus (VSV) (lab strain Indiana (ATCC VR 1540)); v) *Pneumoviridae*: human respiratory syncytial virus (hRSV) (strain A2 (ATCC VR-1540)). The virus representative of dsRNA was: vi) Reoviridae: reovirus type-1 (Reo-1) (simian virus 12, strain 3651 (ATCC VR- 214)). DNA virus representatives were: vii) *Poxviridae*: vaccinia virus (VV) (vaccine strain Elstree-Lister (ATCC VR-1549)); viii) *Herpesviridae*: human herpes 1 (HSV-1) (strain KOS (ATCC VR-1493)).

Cell cultures were checked periodically for the absence of mycoplasma contamination with MycoTect Kit (Gibco, Waltham, Massachusetts, USA). Viruses were maintained in our laboratory and propagated in appropriate cell lines. The viruses were stored in small aliquots at -80 °C until use.

Cytotoxicity assays

Exponentially growing MT-4 cells were seeded at an initial density of 4×10^5 cells/mL in 96-well plates in RPMI-1640 medium, supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin G and 100 µg/mL streptomycin. BHK cells were seeded in 96-well plates at an initial density of 6×10^5 cells/mL, in Minimum Essential Medium with Earle's salts (MEM-E), L-glutamine, 1mM sodium pyruvate and 25 mg/L kanamycin, supplemented with 10% fetal bovine serum (FBS). MDBK cells were seeded in 96-well plates at an initial density of 1×10^6 cells/mL, in Minimum Essential Medium with Earle's salts (MEM-E), L-glutamine, 1mM sodium pyruvate and 25mg/L kanamycin, supplemented with 10% horse serum (MDBK). Vero-76 cells were seeded in 96-well plates at an initial density of 5×10^5 cells/mL, in Dulbecco's Modified Eagle Medium (D-MEM) with L-glutamine and 25 mg/L kanamycin, supplemented with 10% FBS. Cell cultures were then incubated at 37 °C in a humidified, 5% CO_2 atmosphere, in the absence or presence of serial dilutions of test compounds. The test medium used for the cytotoxic assay as well as for antiviral assay contained 1% of the appropriate serum. Cell viability was determined at 37 °C by the 3-(4,5-dimethylthiazol-1-yl)-2,5-diphenyltetrazolium bromide (MTT) method after 72 hrs for BHK, MDBK and Vero-76 or 96 hrs for MT-4 [13]. The cytotoxicity of test compounds was evaluated in parallel with their antiviral activity through the viability of mock-infected, treated cells, as monitored by the MTT method.

Antiviral assays

Essential oil's activity against HIV-1 was based on inhibition of virus-induced cytopathogenicity in exponentially growing MT-4 cell acutely infected with a multiplicity of infection (m.o.i.) of 0.01. Compound's activity against YFV, BVDV and Reo-1 was based on inhibition of virus-induced cytopathogenicity in BHK-21 cells acutely infected at an m.o.i. of 0.01. After a 3, or 4 -day incubation at 37 °C, cell viability was determined by the MTT method [13]. Compound's activity against CV-B4, Sb-1, VV, VSV, hRSV A2 and HSV-1 was determined by plaque reduction assays in infected cell monolayers as described previously [14]. Briefly, monolayer of Vero-76 cells was grown overnight on 24-well plate. The cells were then infected for 2 hrs with 250 µL of proper virus dilutions to give 50-100 PFU/well. Following removal of unadsorbed virus, 500 µL of medium (D-MEM with L-glutamine and 4500 mg/L D-glucose, supplemented with 1% inactivated FBS) containing 0.75% methyl-cellulose, with serial dilutions of test products, were added. The overlayed medium was also added to not treat wells as non-infection controls. Cultures were incubated at 37°C for 2 (Sb-1 and VSV), 3 (CV-B4, hRSV A2, VV, and HSV-1) and then fixed with PBS containing 50% ethanol and 0.8% crystal violet, washed and air-dried. The number of plaques in the control (no inhibitor) and experimental wells were then counted.

Linear regression analysis

The extent of cell growth/viability and viral multiplication, at each drug concentration tested, were expressed as percentage of untreated controls. Concentrations resulting in 50% inhibition (CC_{50} or EC_{50}) were determined by linear regression analysis.

Results

Extraction yield and chemical composition of essential oil

In *A. sessiflora* leaves, the average yield of extracted essential oil was of 0.46 ± 0.01%, calculated on a dry weigh of three samples. The obtained essential oil was a pale, yellow liquid with odor and lighter than water. The GC/MS analysis indicated that the leaves 'essential oil contained 45 constituents representing 98.71% of the total oil content (Supplementary Table 1). The main classes of compounds in this oil were oxygenated monoterpenes (35.82%), sesquiterpene hydrocarbons (35.68%), oxygenated sesquiterpenes (13.92%) and monoterpene hydrocarbons (12.36%). The constituents accounted for higher amounts in the leaves oil of *A. sessiflora* were linalool (16.21%), *E*-β-caryophyllene (11.01%), ledene (8.59%), α-humulene (8.02%) and L-α-terpineol (6.99%). The other components found at lower concentration were linalyl acetate (4.89%), (+)-limonene (4.22%), (+)-spathulenol (4.09%), geranyl acetate (3.87%), (-)-globulol (3.28%) and β-myrcene (2.94 %). In addition, the oil also contained two aldehydes with open chais, *n*-decanal (0.34%) and *n*-dodecanal (0.35%) having nonterpenic structure, and *CIS*-methylisoeugenol (0.26%), also being a nonterpenic derivative.

Antimicrobial activities

The antimicrobial activities of essential oil of *A. sessiflora* were displayed in Table 1. The results exhibited potential antibacterial activities of the essential oil against i) Gram-positive bacterium: *S. aureus* (two strains) with MIC and MLC from 2 to 4% (v/v); ii) Gram-negative bacteria such as *K.*

Table 1. Antibacterial activity (MIC and MLC) of essential oil from the leaves of *A. sessiflora.*

Strains	[a]MIC (% v/v)	[b]MLC (% v/v)
Gram-positive bacteria		
S. aureus ATCC 43300	2	4
S. aureus clinical strain	4	4
E. faecalis clinical strain	>16	>16
Gram-negative bacteria		
E. coli ATCC 35218	4	4
E. coli clinical strain	4	4
P. aeruginosa ATCC 27853	>16	>16
P. aeruginosa clinical strain	16	>16
K. pneumonia clinical strain	0.25	0.25
Yeast		
*C. albicans*556 RM	0.5	1
C. glabrata clinical	2	2
*C. tropicalis*1011 RM	1	1
C. parapsilosis RM	1	1

[a]MIC: Minimum Inhibitory Concentrations; [b]MLC: Minimum Lethal Concentrations.

pneumoniae, with MIC and MLC were both 0.25% (v/v) and *E. coli (*two strains*),* with MIC and MLC were both 4% (v/v); iii) *Candida species* showed MIC and MLC as follows: *C. albicans,* with MIC and MLC were 0.5 and 1% (v/v), *C. glabrata,* with MIC and MLC were both 2% (v/v), *C. tropicalis* and *C. parapsilosis,* with MIC and MLC were both 1% (v/v).*E. faecalis* and *P. aeruginosa*were the least susceptible to the essential oil of *A. sessiflora*, their MIC and MLC values over16 % (v/v). To the best of our knowledge, we are unaware of any previous studies investigating the antimicrobial activity of essential oil from *A. sessiflora*.

Anti- Trichomonas vaginalis activity

As shown in Table 2, the essential oil obtained from leaves of *A. sessiflora* has a remarkable activity against *T. vaginalis.* Results obtained indicate a prompt effect, as demonstrated by the IC_{50} after one hour of incubation (0.06%). The anti-trichomonas activity of *A. sessiflora* essential oil is time-dependent, reaching IC_{50}, IC_{90} and MLC values of 0.016, 0.03, and 0.06% (v/v) respectively, after 48 hours of incubation.

Anti-inflammatory activity

The essential oil from the leaves of *A. sessiflora*have shown the ability to inhibit macrophage cells producing NO with IC_{50} of 95.94 ± 6.18 µg/mL, while the IC_{50} of positive control was of 7.61 ± 0.59 µg/mL (Table 3). The anti-inflammatory effect of the oil may be attributed to the presence of high concentrations of linalool, linalyl acetate, *E*-β-caryophyllene and α-humulene, compounds with well documented anti-inflammatory potentials [15–18]. This is the first report about anti-inflammatory activity of essential oil from *A. sessiflora*.

Antiviral activity

Here, we explored the antiviral properties of leaves essential oil of *A. sessiflora* against RNA and DNA viruses belonging to different families, including several important human pathogens (Table 4). In order to be able to establish whether tested *A. sessiflora* oil were endowed with selective antiviral activity, their cytotoxicity was evaluated in parallel assays with uninfected cell lines. *In vitro* cytotoxicity was measured based on cell proliferation and viability. The CC_{50} (drug concentration inhibiting cell growth by 50% referred to untreated control) was > 100µg/mL and no cell toxic effect was observed (Table 4). However, results obtained from our screening pointed out *A. sessiflora*essential oil was completely ineffective against the tested viruses with ED_{50} values over 100µg mL^{-1}.

Discussion

Essential oils contain 20 to 60 compounds and nearly 85% of the essential oils are major components [19,20]. Each compound displays its own perfume and bioactivities on the human body [21]. The place of origin, climatic conditions, plant species and seasons are key factors affecting the chemical compositions of essential oils [22,23]. Some constituents are resulted from the decomposing process of the precursor components during distillation [24]. Furthermore, numerous components are generated from the hydrolysis of several compounds found in the plants [24]. In this study, the qualities and contents of the

Table 2. *In vitro* anti-*T. vaginalis* activity of essential oil from the leaves of *A. sessiflora.*

Time	IC_{50} (% v/v)	IC_{90} (% v/v)	MLC (% v/v)
1 hour	0.06	0.12	0.25
4 hour	0.06	0.12	0.25
24 hour	0.03	0.06	0.12
48 hour	0.016	0.03	0.06

IC_{50}: The concentration that causes 50% *T. vaginalis* growth inhibition; IC_{90}: The concentration that causes ≥ 90% *T. vaginalis* growth inhibition; MLC: The concentration that causes the death of 100% protozoan cells.

Table 3. Biological activity of the essential oil of *A. sessiflora* on the NO inhibitory activity.

Concentration	Essential oil of *A. sessiflora*		L-NMMA	
(µg/mL)	% Inhibition NO	% Survival cells	% Inhibition NO	% Survival cells
100	51.22	84.67	85.38	85.34
20	23.93	91.77	72.98	90.93
4	14.63	> 100	37.41	> 100
0.8	3.62	> 100	15.02	> 100
IC_{50}	**95.94±6.18**		**7.61±0.59**	

essential oil from the leaves of *A. sessiliflora* from Quang Tri Province was significantly different from those in Thanh Hoa [25]. This might be attributed to the distribution of species in different geographic areas, analysis and identification methods of the constituents in the essential oil [17,18]. The leaves' essential oil of *A. sessiliflora* in Thanh Hoa was consisted of monoterpene hydrocarbons (40.8%) and oxygenated sesquiterpenes (27.7%), with major constituents were β-pinen (17.2%), limonen (9.7%), α-humulen (7.7%) and β-caryophyllen (7.6%). Generally, the content of essential oil from *A. sessiliflora* has previously been described just in one thesis [25], no more publications available about the composition of the essential oil. In comparison with previous studies in the chemical compositions of the essential oils from *Atalantia* species, Kathirvelu *et al*. [4] indicated that essential oil of *Atalantia monophylla* contains high content of sabinene, eugenol, 1,2-dimethoxy-4-(2-methoxyethenyl)benzene, and β-asarone. This oil has also shown larvicidal and repellent activity against *Anopheles stephensi*, *Aedes aegypti*, and *Culex quinquefasciatus*. The main constituents of essential oil of *Atalantia roxburghiana* were p-cymene, γ-terpinene, α-pinene, and β-pinene [5]. Arun *et al*. [6] reported that T-cadinol, caryophyllene, and caryophyllene oxide are the main constituents in essential oil from *Atalantia racemosa*, while caryophyllene, decanal, and D-limnonene found were found with large amounts in essential oil of *Atalantia wightii*.

In plants, essential oils play several important roles e.g. in pollination, or in a defense mechanism as a repellent or irritant. Studies have shown that essential oils include a large content of antioxidants donating hydrogen in oxidative reactions, especially in the presence of light. Essential oils were also recognized to exhibit antifungal and antibacterial properties hence preventing plants from possible pathogenic triggers [26-28]. Upon biotic/abiotic stress conditions, essential oils can release their chemical substituents through a range of molecular interactions [29] and each of them exhibits different mechanisms of actions on microorganism [30]. These mechanisms synergize and amplify one another to form many effective antimicrobial properties for essential oils [31]. As such, essential oils whose chemical compositions can inhibit or kill bacteria and fungi in different pathways [32]. For example, essential oils can inhibit bacteria in various approaches including triggering the degradation of bacterial cell wall, leakage of cell contents, membrane protein damage, disruption of cytoplasmic membrane, depletion of the proton motive force or coagulation of cytoplasm [30]. Additionally, the hydrophobicity of essential oils enables the permeation of essential oils into cell membrane, and thus facilitating the spillage of ions and molecules out of the cells and cell apoptosis occurs [33]. In our study, the antimicrobial activities of the essential oil could potentially stem from their main components such as linalool and *E*-β-caryophyllene as their antimicrobial potentials were well-reported

Table 4. Cytotoxicity and antiviral activity of essential oil from *A. sessiflora* against representatives of $ssRNA^+$ (HIV-1, YFV, BVDV, Sb-1, CV- B4), $ssRNA^-$ (RSV, VSV), dsRNA (Reo-1), and dsDNA (HSV-1, VV) viruses.

Cell lines and virus	MT4	$HIV\text{-}1_{IIIB}$	BHK	YFV	Reo-1	MDBK	BVDV	Vero-76	RSV	VSV	HSV-1	VV	Sb-1	CV-B4
	CC_{50}[a]	EC_{50}[b]	CC_{50}[c]	EC_{50}[d]	EC_{50}[d]	CC_{50}[e]	EC_{50}[f]	CC_{50}[g]	EC_{50}[h]					
Oil	> 100	> 100	> 100	>100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
*Reference Compds														
RC1	40	0.003 ± 0.0003	-	-	-	-	-	-	-	-	-	-	-	-
RC2	-	-	80	1.4 ± 0.2	-	>100	1.7± 0.3	-	-	-	-	-	-	-
RC3	-	-	-	-	-	-	-	> 100	-	-	-	-	2	2 ± 0.5
RC4	-	-	-	-	-	-	-	≥ 14	2 ± 0.2	-	-	-	-	-
RC5	-	-	-	-	-	-	-	> 100	-	-	3.0 ± 0.1	-	-	-
RC6	-	-	-	-	-	-	-	19	-	-	-	1.7 ± 0.1	-	-
RC7	-	-	> 100	-	17	-	-	-	-	-	-	-	-	-

Data represent mean values ± SD for three independent determinations. For values where SD is not shown, variation among duplicate samples was less than 15%. Oil: essential oil from the leaves of *A. sessiflora;* RC1: Efavirenz; RC2: 2′-C-methylguanosine; RC3: Pleconaril; RC4: 6-aza-uridine; RC5: Acycloguanosine; RC6: Mycophenolic acid; RC7: 2′-C-methylcytidine. [a] Compound concentration (μg ml^{-1}) required to reduce the proliferation of mock-infected MT-4 cells by 50%, as determined by the MTT method. [b] Compound concentration (μg ml^{-1}) required to achieve 50% protection of MT-4 cells from HIV-1 induced cytopathogenicity, as determined by the MTT method. [c] Compound concentration (μg ml^{-1}) required to reduce the viability of mock-infected BHK cells by 50%, as determined by the MTT method. [d] Compound concentration (μg ml^{-1}) required to achieve 50% protection of BHK cells from YFV or Reo-1 induced cytopathogenicity, as determined by the MTT method. [e] Compound concentration (μg ml^{-1}) required to reduce the viability of mock-infected MDBK cells by 50%, as determined by the MTT method. [f] Compound concentration (μg ml^{-1}) required to achieve 50% protection of MDBK cells from BVDV induced cytopathogenicity, as determined by the MTT method. [g] Compound concentration (μg ml^{-1}) required to reduce the viability of mock-infected Vero-76 cells by 50%. as determined by the MTT method. [h] Compound concentration (μg ml^{-1}) required to reduce the plaque number of RSV, VSV, HSV-1, VV, Sb-1, and CV-B4 by 50% in Vero-76 monolayers. * Reference Compds:CC_{50} and EC_{50} are in μM.

[34,35]. Furthermore, the presence of many individual antibacterial components in the essential oil is more likely to produce a synergy, and that amplifying the antimicrobial activities of the essential oil as a whole [36,37].

Candida is referred to a relatively common yeast in the human mucosa, present in the digestive, and reproductive tract as well as oral cavity. Approximately 80% of the human population may be exposed to the *Candida* infection. *Candida* infections usually display a broad spectrum from superficially oral thrush and vaginitis to candidemia [38]. Fluconazole is a triazole antifungal and used to treat *Candida* infections. Fluconazole has fungistatic effect making it an ideal condition the emergence of the resistant strains [39]. Some clinical isolates of *Candida* species have been resistant against fluconazole and other triazole antifungal drugs. Therefore, the demand for newly effective antifungals is urgently required [40–42]. Natural products have long been known as key therapeutic agents providing many potent bioactivities while exhibiting high safety profiles. Accordingly, essential oils have gained growing attention as a powerful fungal inhibitor [43]. It was reported some essential oils yield antimicrobial activity against *Candida* species, including tea tree [44,45], thyme, clove [43], lemongrass, geranium, cinnamon, Japanese mint, ginger grass, and motiarosha essential oils [46]. Here, the current work showed that essential oil from leaves of *A. sessiflora* were effective against *C. albicans, C. glabrata, C. tropicalis,* and *C. parapsilosis* with MIC and MLC values from 0.5 to 2% (v/v).

T. vaginalis has been known as one of the most non-viral sexually transmitted infection worldwide, with an estimated 156 million cases of infections in 2016. The infection is ubiquitous, but it is mostly diffused in the depeloping countries [47,48]. *T. vaginalis* can cause a severe vaginitis, even if 50% of women shows no clear symptoms. Infection is associated to severe pregnancy complications, such as premature birth, premature rupture of membranes and low birth weight [49,50]. Trichomoniasis also contributes to an increasing risk of human immunodeficiency virus (HIV) infection and prostate cancer acquisition [51,52]. Metronidazole is recognized as the first-line drug option for *T. vaginalis* infections; nonetheless, the last few years have witnessed the new emergences of many resistant microbes very likely resulting in poor treatment efficacies [53]. To this end, plant-based products are of great interest for the treatment of *T. vaginalis* and other parasites attributed to relatively similar therapeutic proficiency and high safety profile. Up to now, a limited number of essential oils have been tested *in vitro* on *T.vaginalis*, including [44,54-58]. Among them, *Ocimum basilicum* shows one of the most efficient antitrichomonas activity, with 100% inhibition of of parasitic growth at a concentration of 30 mg/ml after 24 h of incubation [59].In the present work,the authors demonstrated that essential oil of *A. sessiflora* display a very good inhibitory effect on *T. vaginalis,* with IC_{50} value of 0.016% (v/v) after 48 hours, corresponding to 120 µg/ml. Impressively, *A. sessiflora* oil against *T. vaginalis* was quickly exhibited only 1 h following incubation as the values of IC_{50}, IC_{90} and MLC were in turn at 0.06, 0.12 and 0.25% (v/v).

Bacteria, fungi and parasites have consecutively been developing numerous resistant mechanisms against current antibiotics, hampering the success of anti-infectious therapies, and thus leaving severe consequences on patients' health [30,31–34]. In addition, the use of synthetic chemicals to control microorganisms is still limited due to their carcinogenic effects, acute toxicity and environmental hazards [32]. The therapeutic agents from herbal medicines have long emerged as a potential natural source for treating infectious diseases [11,36,37]. Herein, the antimicrobial, antitrichomonas, anti-inflammatory, and antiviral effects of essential oil from *A. sessiflora* were first studied, showing strong activity against *K. pneumoniae*, *Candida* species, and *T. vaginalis.*Therefore, the essential oil of *A. sessiflora*can be employed in the development of new anti-infectious agents thanks to its strong bactericidal effects.

Conclusions

The dried leaves essential oil of *A. sessiflora* after collecting from Quang Tri Province, Vietnam was composed of 45 constituents in which linalool (16.21%), *E*-β-caryophyllene (11.01%), ledene (8.59%), α-humulene (8.02%) were 4 main components. *A. sessiflora* essential oil displayed antimicrobial activities against one Gram-positive strain, *S. aureus,* two Gram-negative bacteria, *K. pneumoniae*, *E. coli*, and finally four *Candida* species. Meanwhile, *E. faecalis* and *P. aeruginosa* were the least susceptible to the essential oil of *A. sessiflora.*The oil also exhibited repellency against *T. vaginalis* and inhibited macrophage cells producing NO. It was ineffective against HIV-1, YFV, BVDV, Sb-1, CV- B4, RSV, VSV, Reo-1, HSV-1, VV viruses. Further studies should be done, such as investigating pharmacological effects of major components of *A. sessiflora* essential oil, testing on other cells or pathogens, studying on

resistant strains, elucidating the mechanisms of action of *A. sessiflora* oil, and evaluating the safety and toxicity of *A. sessiflora* oil in animal and human before considering the development new anti-infectious agents for using in clinical settings.

Acknowledgements

We feel thankful for the support of colleagues at the University of Sassari and University of Cagliari who enabled us to complete the present work. We would like to thank the Hue University for a partial financial funding and Mrs. Thao Thi Do (Institute of Biotechnology, VAST, Hanoi, Vietnam) for evaluating the anti-inflammatory activity.

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Corresponding authors

Professor Piero Cappuccinelli
Department of Biomedical Sciences, University of Sassari
Viale San Pietro 43/b, Sassari, Italy
Tel: +39 320 4299672
Fax: +39 079 228563
Email: pcappuc@gmail.com

Professor Hoai Thi Nguyen
Faculty of Pharmacy, Hue University of Medicine and Pharmacy, Hue University
No. 06 Ngo Quyen Street, Hue city, Viet Nam.
Tel: +84914019691
Fax: +84914019691
Email: hoai77@gmail.com

Conflict of interests: No conflict of interests is declared.

Annex – Supplementary Items

Supplementary Table 1. Chemical compositions of the essential oil from the leaves of *A. sessiliflora.*

No.	RT	[a]KI	Components	[b]% ± SD	[c]IM	[d]References
1	21.17	994	β-Myrcene	2.94 ± 0.03	Std	
2	22.18	1,003	α-Phellandrene	0.36 ± 0.01	Std	
3	23.45	1,028	(+)-Limonene	4.22 ± 0.04	Std	
4	23.56	1,032	β-Phellandrene	1.64 ± 0.01	Std	
5	23.67	1,035	*CIS*-β-Ocimene	0.61 ± 0.01	MS	
6	24.26	1,051	*TRANS*-β-Ocimene	1.70 ± 0.03	MS	
7	24.99	1,058	γ-Terpinene	0.30 ± 0.01	Std	
8	25.67	1,071	*CIS*-Linalooloxide	0.38 ± 0.02	MS	
9	26.47	1,089	Terpinolene	0.59 ± 0.02	Std	
10	27.18	1,094	Linalool	16.21 ± 0.11	Std	
11	32.17	1,188	L-α-Terpineol	6.99 ± 0.07	MS	
12	32.55	1,204	*n*-Decanal	0.34 ± 0.01	MS	
13	34.69	1,248	Linalyl acetate	4.89 ± 0.03	MS-RI	[1]
14	34.74	1,251	Geraniol	1.67 ± 0.01	Std	
15	39.16	1,362	Meryl acetate	1.81 ± 0.02	Std	
16	39.88	1,382	Geranyl acetate	3.87 ± 0.04	Std	
17	40.13	1,388	α-Cubebene	0.37 ± 0.01	Std	
18	40.46	1,389	β-Bourbonene	0.42 ± 0.01	MS	
19	40.55	1,390	β-Elemene	0.45 ± 0.01	Std	
20	41.02	1,409	*n*-Dodecanal	0.35 ± 0.01	Std	
21	41.55	1,418	α-Santalene	0.52 ± 0.01	MS	
22	41.74	1,421	*E*-β-Caryophyllene	11.01 ± 0.08	Std	
23	41.97	1,435	*TRANS*-α-Bergamotene	0.27 ± 0.01	MS	
24	42.34	1,440	(+)-Aromadendrene	0.38 ± 0.02	MS	
25	42.43	1,443	*TRANS*-β-Farnesene	0.56 ± 0.01	MS	
26	42.88	1,452	α-Humulene	8.02 ± 0.05	Std	
27	43.03	1,463	Alloaromadendrene	0.83 ± 0.02	MS	
28	43.65	1,484	Dermacrene D	0.22 ± 0.01	Std	
29	43.72	1,491	*CIS*-Methylisoeugenol	0.26 ± 0.01	MS	
30	43.98	1,493	Ledene (Viridiflorene)	8.59 ± 0.06	MS-RI	[2]
31	44.06	1,501	α-Muurolene	0.22 ± 0.01	Std	
32	44.22	1,525	β-Bisabolene	0.93 ± 0.02	MS-RI	[3]
33	44.63	1,523	(+)-δ-Cadinene	1.30 ± 0.04	MS	
34	44.72	1,560	β-Sesquiphellandrene	1.36 ± 0.05	MS	
35	45.07	1,561	γ-Cadina-1,4-diene	0.23 ± 0.01	MS	
36	45.65	1,566	*TRANS*-Nerolidol	1.81 ± 0.03	Std	
37	46.45	1,578	(+)-Spathulenol	4.09 ± 0.07	Std	
38	46.71	1,585	(-)-Globulol	3.28 ± 0.05	Std	
39	46.94	1,593	(+)-Viridiflorol	2.03 ± 0.06	Std	
40	47.21	1,609	Eudesmol<5epi-7epi-α>	0.68 ± 0.04	MS	
41	47.70	1,614	Cubenol	0.43 ± 0.03	Std	
42	47.86	1,619	(-)-Spathulenol	0.37 ± 0.02	Std	
43	48.02	1,639	α-epi-Cadinol	0.45 ± 0.02	MS	
44	48.08	1,640	α-epi-Muurolol	0.46 ± 0.02	MS	
45	48.41	1,654	α-Cadinol	0.30 ± 0.01	Std	
			Total	**98.71**		
			Monoterpene hydrocarbons	**12.36**		
			Oxygenated monoterpenes	**35.82**		
			Sesquiterpene hydrocarbons	**35.68**		
			Oxygenated sesquiterpenes	**13.90**		
			Others	**0.95**		

Data are the mean of three replicates ± SD; [a] Retention index (Kovalts) relative to *n*-alkanes (C_9–C_{22}) ; [b] Percentage of compounds; [c] Identification methods (IM): MS by comparison of the Mass spectrum with those of the computer mass libraries Adams, NIST 11 and by interpretation of the mass spectra fragmentations. RI by comparison of retention index with those reported in literature. Std: by comparison of the retention time and mass spectrum of available authentic standards. [d] Papers take as reference to compare the relative RI.