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Antimicrobial, Antiquorum-sensing and *Ex-vivo* Antispasmodic Activity of *Adhatoda vasica*

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Authors' contributions

This work was carried out in collaboration among all authors. All authors contributed to the design of the protocol, review of literature, analyses and biological assays, interpretation of data, drafting the manuscript, reading and approving the final manuscript version to be published.

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ABSTRACT

Aims: To investigate the antimicrobial, antiquorum-sensing and *Ex-vivo* antispasmodic activity of the stem extract, fractions and isolated compounds of *Adhatoda vasica*.
Study Design: Preparation of the total extract, fractions of *A. vasica* and isolation of its phytoconstituents for investigation of antimicrobial, antiquorum-sensing and *Ex-vivo* antispasmodic activity.
Place and Duration of Study: Faculty of Pharmacy, Mansoura University, Egypt and College of Pharmacy, Prince Sattam Bin Abdulaziz University, KSA, between November 2014 and May 2019.
Methodology: Different phytoconstituents in obtained liquid-liquid fractionations were isolated by repeated column chromatography. The preliminary antimicrobial activity was measured via agar disc-diffusion method. The minimum inhibitory concentration (MIC) was further determined using broth microdilution method in 96-well plates. Antiquorum-sensing activity was tested against *Chromobacterium violaceum* in LB agar medium. While, antispasmodic activity was performed using Ach-induced contraction on rat ileum.

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Results: Seven compounds have been isolated from the stems extract of *Adhatoda vasica* viz., *β*-sitosterol, daucosterol palmitate, monopalmitin, vanillin, vanillic acid, vasicinolone and vasicinone. The petroleum ether fraction, daucosterol palmitate, monopalmitin, vanillin and vanillic acid showed strong antibacterial activity towards *E. coli* and *S. aureus*. Whereas daucosterol palmitate and vanillic acid showed pronounced antifungal activity against *C. albicans*. Antiquorum-sensing assay showed that *β*-sitosterol, vanillin and vanillic acid were the most active compounds. While, petroleum ether and methylene chloride fractions, vasicinolone and daucosterol palmitate showed moderate antiquorum-sensing activity. For antispasmodic activity, petroleum ether fraction showed a remarkable inhibition of Ach-induced contraction at 200 and 250 µg/mL (89.5 and 95.2%, respectively). Also, methylene chloride fraction showed remarkable inhibition (97.6%) at 150 µg/mL. Vasicinone and vasicinolone showed significant inhibition at 150 µg/mL (89.9% and 84.8%, respectively).
Conclusion: The isolated compounds and extracts from the stem of the studied plant showed remarkable activities. This work provides further evidences for the traditional medicinal uses of *A. vasica* in treatment of various ailments.

Keywords: Adhatoda vasica; vasicinone; vasicinolone; antimicrobial activity; antiquorum-sensing activity; antispasmodic activity.

1. INTRODUCTION

Adhatoda vasica Nees. (Syn. *Adhatoda zeylanica* Medic. and *Justicia adhatoda* L.) is a perennial plant belonging to family Acanthaceae, usually known as Vasaka [1]. It is distinguished with a disagreeable smell and bitter taste [2]. The titled plant has been reported for antibacterial, wound healing, hypoglycemic, abortifacient, antitussive, anti-inflammatory, and hepatoprotective activities [3]. This plant was particularly used as an herbal medicine for treating respiratory complaint including cold, cough, chronic bronchitis, asthma and as antispasmodic [4]. Previous phytochemical reports on *A. vasica* revealed that it mainly contains pyrroquinazoline alkaloids, flavonoids [3], triterpenes [5] steroids, tannins, saponins and glycosides [6]. Previous reports indicated the presence of variation in the alkaloid content based on the plant organ. For example, vasicine was the major alkaloid in the leaves along with several alkaloids such as vasicinone, vasicinol, adhatodine, adhatonine, adhavasinone, anisotine and peganine. However, the root was found to contain vasicinol, vasicinolone, vasicinone and adhatonine [1].

The aggravating worldwide hazard of the development of antimicrobial resistance is leading to an endless request for the discovery of new antimicrobial drugs. Bacterial cell to cell communication named as "quorum-sensing" represents a signaling system that is important for the regulation of several cellular processes such as virulence factors expression, biofilm formation, and competence [7]. Consequently, affecting the bacterial pathogenic potential through quorum-sensing inhibition is one of the new approaches that are used to fight the microbial resistance [8]. Natural products represent a valued viable source of antimicrobial drugs and resistance modifying agents [9,10]. It is important to recognize the pure compound(s) responsible for the antimicrobial activity since the use of total plant extracts as antimicrobial drugs is not the best choice because of their complex chemical nature and difficulty of standardization [9].

Meanwhile, *A. vasica* has been traditionally used as an antispasmodic however; there is no available scientific evidence for this use. Also, the antispasmodic effect is generalized to the herbal formulation and there is no studies addressed the antispasmodic potential of the isolated compounds from *A. vasica*.

Therefore, the aim of the current study is to isolate and identify the phytoconstituents of *A. vasica* and to evaluate the antimicrobial, anti-quorum-sensing, and anti-spasmodic activities of the different extracts and isolated compounds from the titled plant.

2. MATERIALS AND METHODS

2.1 Extraction and Isolation of the Phytochemicals

2.1.1 General experimental methods

^{1}H- and ^{13}C-NMR spectra were obtained using $CDCl_3$ or $(CD_3)_2SO$ solvents and TMS as an internal standard at 400 MHz for ^{1}H-NMR and 100 MHz for ^{13}C-NMR on BRUKER Avance III spectrometer (Bruker AG, Switzerland) or Jeol

500 MHz ^{TM}spectrometer (500 MHz for ^{1}H-NMR and 125 MHz for ^{13}C-NMR). Chemical shifts (δ) are reported in ppm relative to the solvent signal and coupling constants are given in Hz. Column chromatography was carried out on silica gel G 60-230 mesh (Merck, Germany). Thin layer chromatography (TLC) was performed on precoated silica gel 60 GF_{254} (20 x 20 cm, 0.2 mm thick) on aluminum sheets (Merck, Germany). Organic solvents were of reagent grade (ElNasr Co., Cairo, Egypt).

2.1.2 Plant material

The aerial parts of *Adhatoda vasica* Nees. were collected from Mansoura University's gardens, Dakhlia, Egypt in December 2013. The identity of plant was confirmed by Prof. Ibrahim Mashaly (Ecology and Botany Department, Faculty of Science, Mansoura University). A voucher sample was deposited at the herbarium in Department of Pharmacognosy, Faculty of Pharmacy, Mansoura University (014-Mansoura-6).The leaves were separated from the stems which were shade-dried, powdered and kept for further investigation.

2.1.3 Preparation of the crude extract

The air dried powdered stem (4.5 kg) was extracted by maceration with cold distilled methanol (4 x 8 L). The combined methanol extract was concentrated to a syrupy consistency under reduced pressure and then allowed to dry in a desiccator over anhyd. $CaCl_2$ to a constant weight (328 g). The dried methanol extract was suspended in distilled water and then partitioned successively, till exhaustion, with *n*-hexane, methylene chloride and ethyl acetate. The different extracts were evaporated to dryness under reduced pressure and kept for further investigation.

2.1.4 Chromatographic isolation of components in the *n*-hexane fraction

The *n*-hexane fraction (70.00 g) was chromatographed on silica gel packed column and eluted with pet. ether-ethyl acetate (gradient), 250 mL-fractions were collected. Residue of fraction 22 (30 mg), eluted with pet. ether-ethyl acetate (20%), afforded compound APE1. Repeated rechromatography of fraction 34-39 (4.023 g), on silica gel column, eluted with methylene chloride-methanol (gradient) and 50 mL-fractions were collected, crystallization of fractions eluted with methylene chloride–methanol (1%) afforded compound APE3. Subfraction 68-78 (400 mg) eluted with methylene chloride-methanol (2%) was further chromatographed on a Sephadex LH20 MPLC column using methylene chloride-methanol (gradient) and 20 mL-fractions were collected. Fractions 2-6 (141.6 mg) eluted with methylene chloride (100%) were further chromatographed on a silica gel column, eluted with methylene chloride-methanol (gradient) and 20 mL-fractions were collected. Fractions 27-33 (50 mg) eluted with methylene chloride-methanol (2%) were further chromatographed on a reversed phase RPC_{18} column using water–methanol (gradient) and 10 mL-fractions were collected to yield APE2 from fraction 30-48 (30 mg) eluted with methanol (100%).

2.1.5 Chromatographic isolation of components in the methylene chloride fraction

The methylene chloride fraction (10.00 g) was chromatographed on silica gel column and eluted with pet. ether-ethyl acetate (gradient) and 100 mL-fractions were collected. Fraction 49-72 (155.8 mg) eluted with pet. ether/ethyl acetate mixtures (14-25% v/v) was further chromatographed on a silica gel column using *n*-hexane-methylene chloride (gradient) and 10 mL-fractions were collected, crystallization of residue of fraction 13-17 (7.5 mg), eluted with *n*-hexane-methylene chloride (30-40% v/v), afforded compound AMC2.

2.1.6 Chromatographic isolation of the ethyl acetate fraction substances

The ethyl acetate fraction (17.00 g) was chromatographed on silica gel column. Elution was started with methylene chloride-methanol (gradient) and 100 mL-fractions were collected. Crystallization of the residue of fraction 14 eluted with methylene chloride/methanol (15%) afforded compound EA1 (66 mg). Fractions 12-13, (100 mg), eluted with methylene chloride/methanol (15%) was further chromatographed on a Sephadex LH20 MPLC column, using methylene chloride-methanol (gradient) and 10 mL-fractions were collected, crystallization of residue of 30-33 (9.3 mg), eluted with methylene chloride-methanol (2%) afforded compound EA2.

2.1.7 Chromatographic isolation of the aqueous fraction

The aqueous extract left after partitioning with ethyl acetate (500 mL), was passed over a chromatographic column packed with Diaion HP20 resin. The column was washed

several times with distilled H_2O then eluted with metanol. This metanol fraction was concentrated using rotavap and left to dry to yeild 15.078 g. Column chromatography of this residue over a silica gel column, eluted with methylene chloride-methanol (gradient) and 250 mL fractions were collected. Fraction 26-31 (675.7 mg), eluted with methanol (15% v/v) was chromatographed over a silica gel column, methylene chloride-methanol (gradient) and 10 mL-fractions collected. Residue of fractions 22-24 (35 mg), eluted with methylene chloride-methanol (98:2 v/v) afforded compound AA1.

2.2 Antimicrobial Activity

2.2.1 Microorganisms

The antimicrobial activity was measured against a panel of sensitive microorganisms provided form the laboratory of Microbiology and Immunology Department at Faculty of Pharmacy, Mansoura University, Egypt, including the Gram-positive bacteria (*Staphylococcus aureus*), the Gram-negative bacteria (*Escherichia coli*, *Klebsiella pneumonia*, and *Pseudomonas aeuroginosa*), and the yeast-like pathogenic fungus *Candida albicans*.

2.2.2 Antimicrobial assay

The primary screening was carried out via agar disc-diffusion method using Mueller Hinton agar medium (for the antibacterial assay) and YPD agar medium (for the antifungal assay). Briefly, the overnight cultures of the different strains were diluted to OD_{600nm} of 0.1 (equivalent to 8 $x10^7$ cells/mL), except for *C. albicans* that was diluted to OD_{600nm} of 0.5 (equivalent to 8 $x10^7$ cells/mL). The diluted cultures were spread on Mueller Hinton agar plates using sterile swab followed by the application of sterile cellulose disc papers (6 mm each). The prepared fractions and isolated compounds from *A. vasica* were dissolved in DMSO as a stock solution with a concentration of 10 mg/300 µL. From each derivative, 100 µg were loaded from the stock solution (3 µL) into the disc papers. The standard antibacterial compounds; ampicillin (100 µg/disc) and gentamicin (100 µg/disc) were applied in the antibacterial assay, while fluconazole (40 µg/disc) was applied in the antifungal assay. DMSO alone was used as a negative control. The plates were incubated at 37ºC for 24 h. Diameter of the growth inhibition zone was determined to the nearest millimeter using a caliber.

2.2.3 Determination of the minimum inhibitory concentration

The minimum inhibitory concentration (MIC) for the most active compounds and/or extracts was further determined using broth microdilution method in 96-well plates as reported previously [11]. Briefly, the overnight cultures of the bacterial strains were diluted to OD_{600nm} of 0.01 (equivalent to 8 $x10^6$ cells/mL), while that of *C. albicans* was diluted to OD_{600nm} of 0.5 (equivalent to 8 $x10^7$ cells/mL). The selected compounds and/or extracts, as well as standard antibiotics were diluted by two-fold serial dilution. The dilutions of the compounds in DMSO (5000, 2500, 1250, 625, 312.5, 156.25, 78.125, 39.062, and 19.531 µg/mL) were applied to the diluted cultures in the microtiter plates as 5 % of the final volume in each well. The plates were incubated at 37ºC for 24 h. The MIC values were determined as the lowest concentration that completely inhibits the visible growth of the microorganism after overnight incubation.

2.3 Antiquorum-sensing Activity

In addition to the antimicrobial screening of the compounds and extracts, their anti-pathogenic potential was checked by examining their antiquorum-sensing activity against *Chromobacterium violaceum* in LB agar medium as described previously (El-Gohary and Shaaban [12].

Cultures were prepared by growing the bacterium in LB broth and incubated for 16–18 h in an orbital incubator running at 28ºC and 150 rpm. Cultures were then adjusted to 0.5 McFarland standard (equivalent to 1 $x10^6$ cells/mL). *Ch. violaceum* (50 µL) was inoculated into LB agar (50 mL), poured into plates, and solidified. Wells were made in LB agar medium using cork borer. The compounds and extracts were dissolved in DMSO as a stock solution with a concentration of 5 mg/mL. From each compound, 50 µL were applied from the stock solution into the wells. The positive control (catechin) and negative control (DMSO) were added at the same concentration and volume to each plate. Plates were incubated at 30°C for 48 h to check the inhibition of pigment production around the wells. Bacterial growth inhibition would result in a clear halo around the disk, while a positive quorum sensing inhibition is exhibited by a turbid halo harboring pigmentless bacterial cells of *Ch. violaceum* monitor strain. Bacterial growth inhibition by the tested compounds was

measured as radius (r1) in mm, while both growth and pigment inhibition were measured as radius (r2) in mm. The pigment inhibition (QS inhibition) was determined by subtracting bacterial growth inhibition (r1) from the total radius (r2); thus, QS inhibition = (r2 - r1) in mm.

2.4 Ex-vivo Antispasmodic Activity Using Rat Ileum Method

2.4.1 Materials and instruments

For antispasmodic assay, acetyl choline chloride and atropine sulfate (LOBA Chemie, India) were used for induction of contraction and as an inhibitor, respectively. For preparation of Tyrode solution, NaCl, $NaHCO_3$, $CaCl_2$ dihydrate, $MgCl_2$ hexhydrate, (LOBA Chemie, India), KCl (Sigma-Aldrich, Germany), NaH_2PO_4 dihydrate, and D(+)-glucose anhydrous (Scharlab S.L., Spain), were used. Inhibitions of induced contractions were tested on an EmkaBATH2 instrument (emka technologies, France).

2.4.2 Methods

The antispasmodic activity of the different extract fractions and the isolated compounds of *A. vasica* was performed using Ach-induced contraction on rat ileum as described before with simple modifications [13].

2.4.2.1 Preparation of test solutions

For Ach or atropine, stock solution of 10 mg/mL was prepared in dist. H_2O, followed by tenfold dilution to obtain a solution of 1000 µg/mL. A final concentration 50 µg/mL was obtained considering the volume of EmkaBath organ chamber (20 mL). For extracts or compounds, a stock solution of 5 mg/mL was prepared in DMSO. The final concentrations for extracts or compounds were as follows: 0.1 mL (25 µg/mL), 0.2 mL (50 µg/mL), 0.3 mL (75 µg/mL), 0.4 mL (100 µg/mL), 0.8 mL (200 µg/mL), and 1 mL (250 µg/mL).

2.4.2.2 Preparation of Tyrode solution

Tyrode solution (5 L) was prepared by simple dissolution of the following: NaCl (8.00 g/L), KCl (0.2 g/L), $CaCl_2$ (0.2 g/L), $MgCl_2$ (0.1 g/L), NaH_2PO_4 (0.05 g/L), $NaHCO_3$ (1.00 g/L) and glucose (1.00 g/L) in dist.H_2O. The pH was adjusted to 6.5 (https://medical-dictionary.thefreedictionary.com/Tyrode+solution, March 2019).

2.4.2.3 Screening of the smooth muscle relaxant effect of the fractions and isolated compounds from A. vasica

EmkaBath2 instrument was used to record the effect of the different plant extracts and major alkaloids of *Adhatoda vasica* on acetylcholine (ACh) induced contraction using isolated rat's intestinal smooth muscle (ileum) by the following procedure:

2.4.2.4 Calibration of emkaBath2 apparatus

The organ bath (EmkaBath2) was calibrated with weights 1 and 10 grams mass units then, maintained at a temperature equals 37ºC and bubbled with carbogen gas (95% oxygen + 5 % carbon dioxide).

2.4.2.5 Isolation of rat-ileum

After making the organ bath ready, chloroform-anesthetized rat (adult male Wistar rat; 150-200 g), was sacrificed by cervical dislocation to death, and lay it on its back on the dissecting board. The abdomen was opened, the caecum was exposed and the ileum was identified. The ileum was cut into pieces of 2-3 cm-length, and the tissue was tied from both ends using a string to be fixed between upper and lower tissue holder.

2.4.2.6 Recording Ach-induced contractions

The ileum preparation was mounted in the organ bath containing Tyrode solution (just enough to submerge the tissue) in an upright position. The tissue tension was adjusted by using the micro positioner. The tissue was relaxed for 15-20 min, during this time; it was washed with fresh Tyrode solution for at least four times for equilibration. Once the tissue is stabilized, the experiment was started by recording a baseline for 30 seconds. Graded doses of acetylcholine (viz., 0.1, 0.2, 0.4, 0.8 and 1.0 mL) were added separately to obtain contractile responses for 30 sec, followed by at least 2-3 washings in each case. The response was recorded till maximum response (ceiling dose). For testing the antispasmodic potential of the plant extracts or pure compounds, doses of 0.1, 0.2, 0.3, 0.4, 0.8 and 1.0 mL were added to the tissue bath and left for 3 minutes. Then a ceiling dose of Ach was added to the tissue bath and the response was observed and recorded. These steps were repeated for each test sample. Contractions were measured as the reduction in area under the peak (A) given by the tissue after addition of the test sample (A *test*), followed by ceiling dose of Ach that produced maximum

induced response for each tissue (A *control*) and the results were expressed as percentage of inhibition according to the following equation:

$$\%\text{Inhibition} = \frac{A\ control - A\ test}{A\ control} x100$$

2.4.2.7 Statistical analysis

Statistical calculations were carried out with Microsoft Excel®. The results are expressed as the mean ± standard deviation (SD) calculated from three independent experiments. The data was further analyzed with student's *t*-test for calculating the significance differences in comparison with control data. The results are considered as statistically significant at $p \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1 Phytochemical Investigation of *Adhatoda vasica*

To explore the chemical composition and bioactive compounds of *Adhatoda vasica*, the different fractions viz., petroleum ether (PE), methylene chloride (MC), ethyl acetate (EA) and the aqueous (Aq) fractions, were subjected to an intensive phytochemical study. Chromatographic separation and purification of phytochemicals from the different extract fractions afforded seven known compounds (Fig. 1). The PE fraction afforded three compounds; APE1, APE2 and APE3, while the MC fraction afforded compound AMC1, the EA fraction afforded EA1 and EA2, and finally, the Aq fraction afforded AA1.

Compound APE1 was identified as *β*-sitosterol using co-chromatography against an authentic *β*-sitosterol. Compound APE2 and APE3 were identified by comparing IR, ^{1}H-NMR, APT experiment, and mass spectra against published data [14,15] as *β*-sitosterol-3-*O*-*β*-D-glucopyranoside-6`-palmitate (daucosterol-6`-palmitate) and monopalmitin, respectively [14, 15]. Monopalmitin is isolated for the first time from *A. vasica*. Similarly, IR, ^{1}H-NMR and APT spectral data confirmed the identity of AMC1 as vanillin [16], which is reported for the first time from *A. vasica*. The identity of compounds EA1 and EA2 were confirmed using IR, ^{1}H-NMR and APT spectral data and by comparison with reported data for vasicinolone and vanillic acid, respectively [17]. EA1 was reported before from *A. vasica* while vanillic acid is reported here for the first time from this plant. Compound AA1 was confirmed as vasicinone, using IR, ^{1}H-NMR and APT spectral data [18], which was reported before from *A. vasica.*

β-sitosterol-3-O-β-D-glucopyranoside-6`-palmitate (Daucosterol-6`-palmitate) ***APE2:*** ^{13}C-NMR ($CDCl_3$ at 100 MHz); δ_c (ppm) 37.32 (CH_2, C-1), 29.50 (CH_2, C-2), 79.56 (CH, C-3), 38.97 (CH_2, C-4), 140.39 (qC, C-5), 122.06 (CH, C-6), 31.88 (CH_2, C-7), 31.94 (CH, C-8), 50.29 (CH, C-9), 36.76 (qC, C-10), 21.09 (CH_2, C-11), 39.83 (qC, C-12), 42.38 (qC, C-13), 56.82 (CH, C-14), 24.28 (CH_2, C-15), 28.18 (CH_2, C-16), 56.21 (CH, C-17), 11.83 (CH_3, C-18), 19.30 (CH_3, C-19), 36.13 (CH, C-20), 18.77 (CH_3, C-21), 34.24 (CH_2, C-22), 26.36 (CH_2, C-23), 45.96 (CH, C-24), 29.33 (CH_3, C-25), 19.06 (CH_3, C-26), 19.73 (CH_3, C-27), 23.16 (CH_2, C-28), 11.95 (CH_3, C-29), 101.24 (CH, C-1`), 73.68 (CH, C-2`), 76.23 (CH, C-3`), 70.37 (CH, C-4`), 73.98 (CH, C-5`), 63.29 ($CH_2$, C-6`), 174.31 (qC, C-1``), 34.05 ($CH_2$, C-2``), 24.95 (CH_2, C-3``), 29.18 ($CH_2$, C-4``), 29.62 (CH_2, C-5``), 29.70 ($CH_2$, C-6``), 29.66 (CH_2, C-7``-12``), 29.28 (CH_2, C-13``), 31.96 ($CH_2$, C-14``), 22.61 (CH_2, C-15``), 13.98 ($CH_3$, C-16``).

Monopalmitin ***APE3****:* ^{13}C-NMR (100MHz, $CDCl_3$); δ_c (ppm) 173.6 (qC, C-1), 33.5 (CH_2, C-2), 24.3 (CH_2, C-3), 29.1-29.7 (CH_2, C4-13), 31.3 (CH_2, C-14), 22.01 (CH_2, C-15), 13.4 (CH_3, C-16), 64.6 (CH_2, C-1`), 69.7 (CH, C-2`), 62.8 (CH_2, C-3`).

Vanillin ***AMC1:*** ^{3}C-NMR (125 MHz, $CDCl_3$); δ_c (ppm) 129.0 (qC, C-1), 107.9 (CH, C-2), 146.2 (qC, C-3), 150.7 (qC, C-4), 113.4 (CH, C-5), 126.4 (CH, C-6), 55.2 (CH_3, OMe), 189.7 (CH, C-α).

Vanillic acid ***EA2:*** ^{13}C-NMR (125 MHz, $CDCl_3$); δ_c (ppm) 121.9 (qC, C-1), 112.2 (CH, C-2), 146.5 (qC, C-3), 150.4 (qC, C-4), 114.3 (CH, C-5), 124.4 (CH, C-6), 55.9 (OCH_3), 168.7 (COOH)

Vasicinone ***AA1*****:** ^{13}C-NMR (125 MHz, $CDCl_3$); δ_c (ppm) 43.6 (CH_2, C-1), 29.3 (CH_2, C-2), 71.7 (CH, C-3), 160.4 (qC, C-3a), 147.9 (qC, C-4a), 126.6 (CH, C-5), 134.5 (CH, C-6), 127.0 (CH, C-7), 126.3 (CH, C-8), 120.8 (qC, C-8a),160.6 (qC, C-9).

Vasicinolone ***EA1:*** ^{13}C-NMR (125 MHz, $(CD_3)_2SO$)); δ_c (ppm) 43.5 (CH_2, C-1), 30.0 (CH_2, C-2), 71.6 (CH, C-3), 158.0 (qC, C-3a), 142.7 (qC, C-4a), 129.1 (CH, C-5), 124.1 (CH, C-6), 156.4 (qC, C-7), 109.2 (CH, C-8), 122.1 (qC, C-8a), 160.1 (qC, C-9).

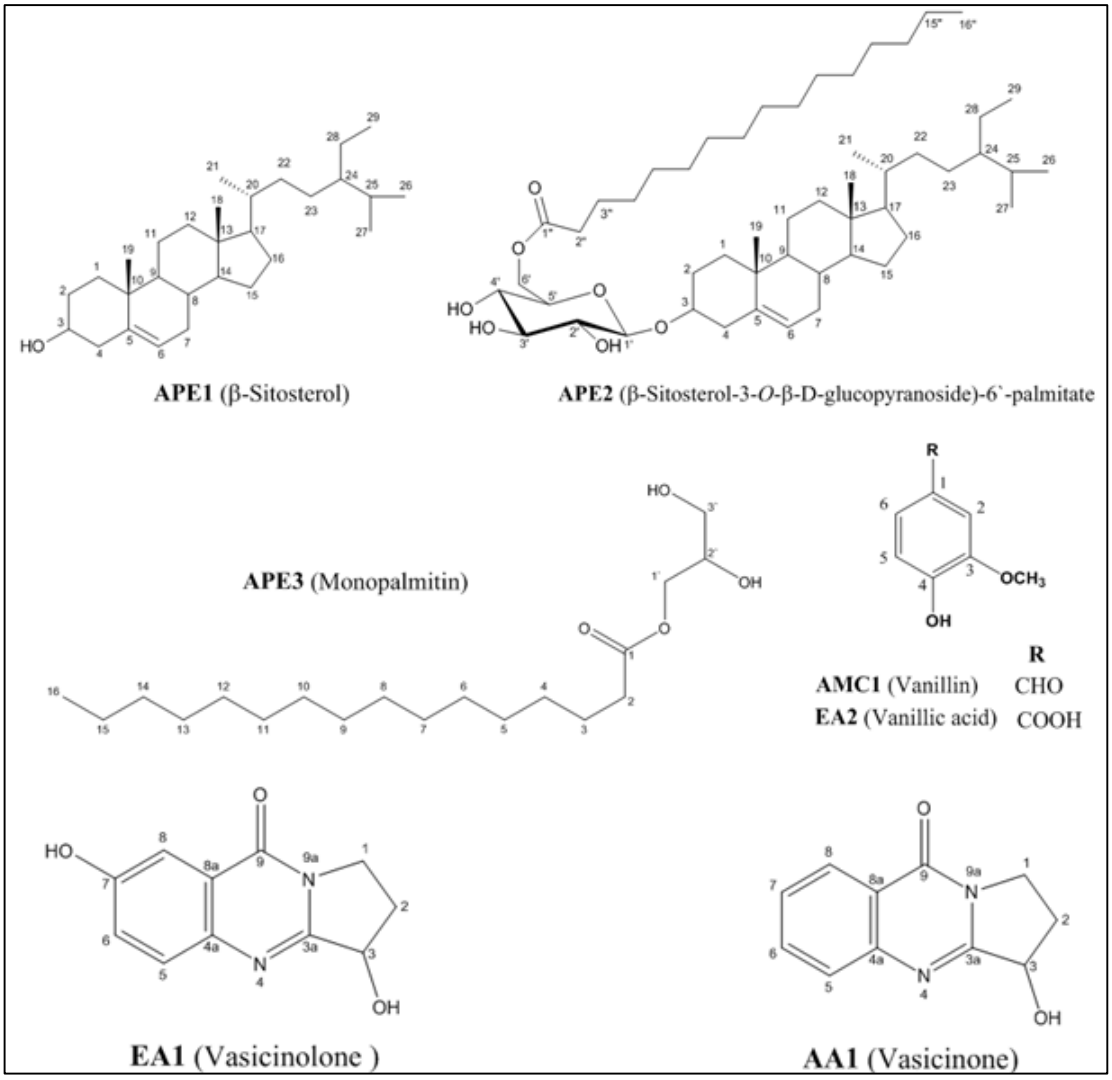

Fig. 1. Structures of the isolated compounds from the stem of *Adhatoda vasica*

3.2 Antimicrobial Activity

The primary *in-vitro* antimicrobial activity of the petroleum ether, methylene chloride, ethyl acetate as well as aqueous fractions of the stem extract of *A. vasica* were investigated according to procedure described previously [11]. In addition, the isolated compounds from this plant including; *β*-sitosterol (APE1), daucosterol palmitate (APE2), monopalmitin (APE3), vanillin (AMC1), vasicinolone (EA1), vanillic acid (EA2) and vasicinone (AA1) were also investigated. The antimicrobial activity was measured against a panel of sensitive microorganisms provided form the laboratory of Microbiology and Immunology Department at Faculty of Pharmacy, Mansoura University, Egypt, including the Gram-positive bacteria (*Staphylococcus aureus*), the Gram-negative bacteria (*Escherichia coli*, *Klebsiella pneumonia*, and *Pseudomonas aeuroginosa*), and the yeast-like pathogenic fungus *Candida albicans*. Ampicillin and gentamicin were used as standard antibacterial drugs (positive control), while DMSO alone was used as a negative control.

The obtained results (Table 1) indicated that compound (EA2) showed the highest activity among the tested compounds and *A. vasica* extracts. It showed moderate activity against *K. pneumoniae* and *P. aeruginosa*, while, strong activity against *E. coli* and *S. aureus*. Compound (APE1) showed moderate activity against *E. coli* and *K. pneumonia,* weak activity against

Table 1. Susceptibility testing by measuring Inhibition Zone Diameter (IZD, mm)[a, b], and MIC values (µg/ml)[c] of *A. vasica* isolated compounds and extracts

Organism	*E. coli*		*K. pneumoniae*		*P. aeruginosa*		*S. aureus*		*C. albicans*	
	IZD	**MIC**	**IZD**	**MIC**	**IZD**	**MIC**	**IZD**	**MIC**	**IZD**	**MIC**
APE1	13 ± 0.5	1250	12 ± 0.0	1250	8 ± 1.0	2500	17 ± 0.5	312.5	14 ± 1.0	1250
APE2	16 ± 1.0	312.5	9 ± 0.0	2500	-	ND	16 ± 0.5	625	9 ± 1.0	2500
APE3	16 ± 1.0	625	13 ± 0.0	1250	10 ± 0.5	1250	18 ± 2.0	312.5	16 ± 1.0	625
AMC1	17 ± 2.0	312.5	8 ± 0.0	5000	12 ± 1.0	1250	16 ± 0.5	625	15 ± 0.0	625
EA1	16 ± 0.0	312.5	10 ± 1.0	2500	10 ± 1.0	1250	10 ± 1.0	1250	15 ± 1.0	625
EA2	19 ± 1.0	156.25	9 ± 0.0	2500	9 ± 0.0	2500	17 ± 2.0	312.5	16 ± 1.0	312.5
AA1	13 ± 1.0	1250	7 ± 0.0	5000	9 ± 1.0	2500	9 ± 0.0	2500	13 ± 1.0	1250
Aq. fraction	9 ± 0.0	2500	-	ND	-	ND	8 ± 0.0	2500	8 ± 0.0	2500
Meth. chloride	14 ± 1.0	1250	11 ± 0.0	1250	10 ± 1.0	2500	12 ± 0.5	625	11 ± 1.0	1250
EtOAc	8 ± 0.0	2500	-	ND	-	ND	8 ± 0.0	2500	-	ND
Pet. ether	16 ± 2.0	625	13 ± 0.0	1250	12 ± 0.5	1250	17 ± 2.0	312.5	15 ± 0.0	625
Ampicillin	25 ± 0.0	39.062	18.8 ± 0.2	78.125	18.1 ± 0.1	78.125	21 ± 0.0	156.25	-	ND
Gentamicin	26 ± 0.0	19.531	25 ± 0.0	78.125	20.4 ± 0.2	39.062	31 ± 0.0	19.531	-	ND
Fluconazole	-	ND	-	ND	-	ND	-	ND	24.7 ± 0.0	78.125

E. coli: Escherichia coli; K. pneumonia: Klebsiella pneumoniae; P. aeruginosa: Pseudomonas aeruginosa; S. aureus: Staphylococcus aureus and C. albicans: Candida albicans. a Results are calculated after subtraction of DMSO activity, and they are expressed as the mean of triplicates ± SD. b Not active [19]; weak activity (2-8 mm); moderate activity (9-15 mm); strong activity (> 15 mm). c The results are reproducible; ND: not determined

Table 2. Antiquorum-sensing activity of *A. vasica* isolated compounds and extracts

Compound	Quorum-sensing inhibition against *Ch. violaceum* [a]
APE1	5.2 ± 0.2
APE2	2.5 ± 0.5
APE3	-
AMC1	5.8±0.4
EA1	3.2 ± 0.1
EA2	6.5 ± 0.5
AA1	2 ± 0.2
Aq. fraction	-
Meth. chloride	2.6 ± 0.4
EtOAc	-
Pet. ether	3.5 ± 0.0
Catechin	4.8 ± 0.0

[a] *QS inhibition (radius of pigment inhibition in mm) = radius of growth and pigment inhibition (r2) - radius of bacterial growth inhibition (r1). Results are expressed as the mean of triplicates ± SD*

K. pneumonia, while strong activity against *S. aureus*. Compound (APE2) showed moderate activity against *K. pneumonia*, strong activity against *E. coli* and *S. aureus* but it had no activity against *P. aeruginosa.* In addition, compound (APE3) showed moderate activity against *K. pneumonia*e and *P. aeruginosa*, while, strong activity against *E. coli* and *S. aureus*. Moreover, compound (AMC1) exhibited moderate activity against *P. aeruginosa*, weak activity against *K. pneumonia* but is showed high activity against *E. coli* and *S. aureus.* Compound (EA1) showed moderate activity against *K. pneumonia*e and *P. aeruginosa*, *S. aureus* while, strong activity against *E. coli*. Compound (AA1) exhibited moderate activity against *P. aeruginosa*, *E. coli*, *S. aureus* and weak activity against *K. pneumonia*. The aqueous fraction *A. vasica* showed moderate antibacterial activity against *E. coli* and weak activity against *S. aureus* but it was not active against *K. pneumonia*e and *P. aeruginosa*. The methylene chloride fraction showed moderate activity against all tested bacterial strains. Moreover, the ethyl acetate fraction showed weak antibacterial activity against *E. coli*, *S. aureus* bacterial strain and no activity towards *K. pneumonia* and *P. aeruginosa*. In addition, the petroleum ether fraction showed strong activity against *E. coli*, *S. aureus* bacterial strain and moderate activity against *K. pneumonia* and *P. aeruginosa*. The activity of some pure compounds however their extract fraction showed inactivity could be a matter of concentration.

The antifungal activity of different compounds and extracts of *A. vasica* against the yeast-like pathogenic fungus *C. albicans* and fluconazole as an antifungal standard drug, and DMSO as a negative control proved that compounds APE2 and EA2 showed the highest antifungal activity among all tested compounds and extracts. Other compounds of the methylene chloride and the petroleum ether fractions showed moderate antifungal activity. However, the ethyl acetate fraction, showed no antifungal activity which could be a matter of concentration. The aqueous fraction showed weak antifungal activity. In all antimicrobial assays, the negative control, DMSO, did not show any obvious activity.

3.3 Antiquorum-sensing Activity

In addition to the antimicrobial testing, all compounds and extracts have been evaluated for their antiquorum-sensing activity against *Ch. violaceum* using catechin as a positive and DMSO as a negative control (Table 2). The antiquorum-sensing activity of the pure compounds and extract fractions was evaluated by testing their ability for preventing the release of a violet pigment (violacein), which is released in response to acyl homoserine lactones signals [20]. Measurement of the pigment inhibition radii in this assay indicated that compound (EA2) showed the highest antiquorum-sensing activity between the tested compounds and extracts. Also compounds (APE1) and (AMC1) showed high activity. On the other hand, compounds (EA1) and (APE2) showed moderate activity, compound (AA1) exhibited weak activity. However, compound (APE3) showed no antiquorum-sensing activity. Furthermore, the petroleum ether and methylene chloride fractions showed moderate activity but the ethyl acetate and aqueous extracts showed no activity.

3.4 *Ex-vivo* Antispasmodic Activity

The smooth muscle relaxant effect of the different extracts viz., total methanol extract (TE) petroleum ether fraction (PE), methylene chloride fraction (M.C.) ethyl acetate fraction (EA) and aqueous fraction (Aq) in addition to, the major alkaloids (vasicinone AA1 and vasicinolone EA1) of *Adhatoda vasica* were evaluated using *Ex-vivo* rat ileum method (Figs. 2-12). Atropine (50 µg/mL) used as a positive control substance. The activity of *A. vasica* extracts and isolated alkaloids (Vasicinone and vasicinolone) was presented as % of the highest spasmolytic effect of atropine.

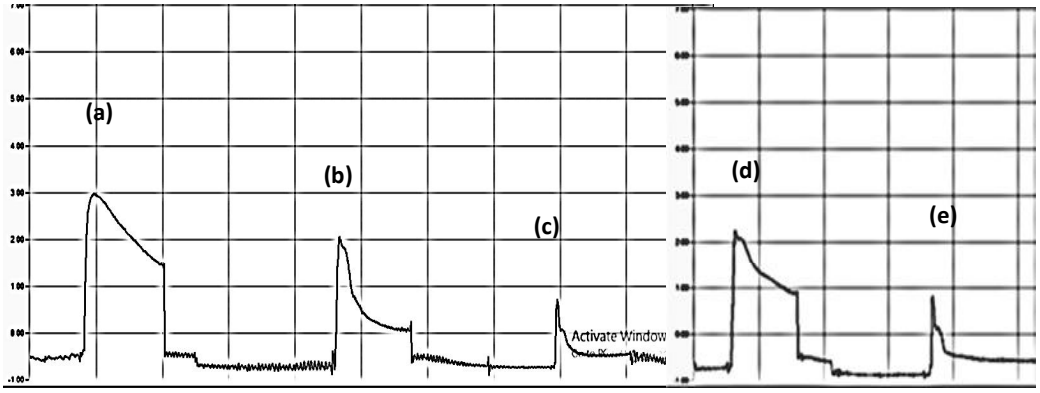

Fig. 2. Effect of different doses of total extract of *A. vasica* (TE) on Ach-induced contraction of rat ileum; (a) Ach alone (50 μg/mL), (b) TE (100 μg/mL)/Ach, (c) TE (200 μg/mL)/Ach, (d) Ach alone, (e) TE (250 μg/mL)/Ach

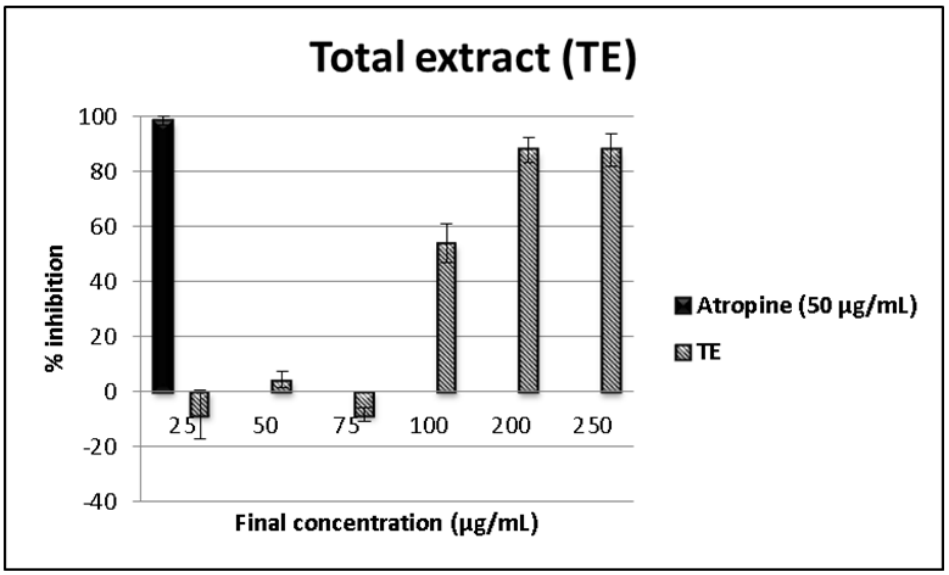

Fig. 3. Percentage inhibition of Ach-induced contraction by total methanol extract (TE) of *A. vasica* at different concentrations as compared with atropine as positive control at 50 μg/mL

The results are expressed as the mean ± standard deviation (SD) calculated from three independent experiments. The results are considered as statistically significant at p ≤ 0.05

The obtained results indicated that the total methanol extract of *A. vasica* (TE) showed remarkable inhibition of Ach-induced contraction of rat ileum at concentrations of 100, 200 and 250 μg/mL (Figs. 2 and 3). The maximum inhibition (87.8%) of the TE was obtained at a concentration of 200 μg/mL.

Further investigation of the different fractions of *A. vasica* indicated that the petroleum ether fraction (PE) showed a dose-dependent inhibition with the highest activity among the tested fractions and compounds (Figs. 4 and 5). It exhibited remarkable inhibitions (89.5 and 95.2%) of Ach-induced contraction at a total concentration of 200 and 250 μg/mL, respectively.

Similarly, the methylene chloride fraction (MC) of *A. vasica* showed good antispasmodic activity (Figs. 6 and 7). This was revealed from the dose-response inhibition of Ach-induced contraction at 25, 50, 75 and 150 μg/mL. A maximum percentage inhibition of 97.6% for MC was observed at a total concentration of 150 μg/mL.

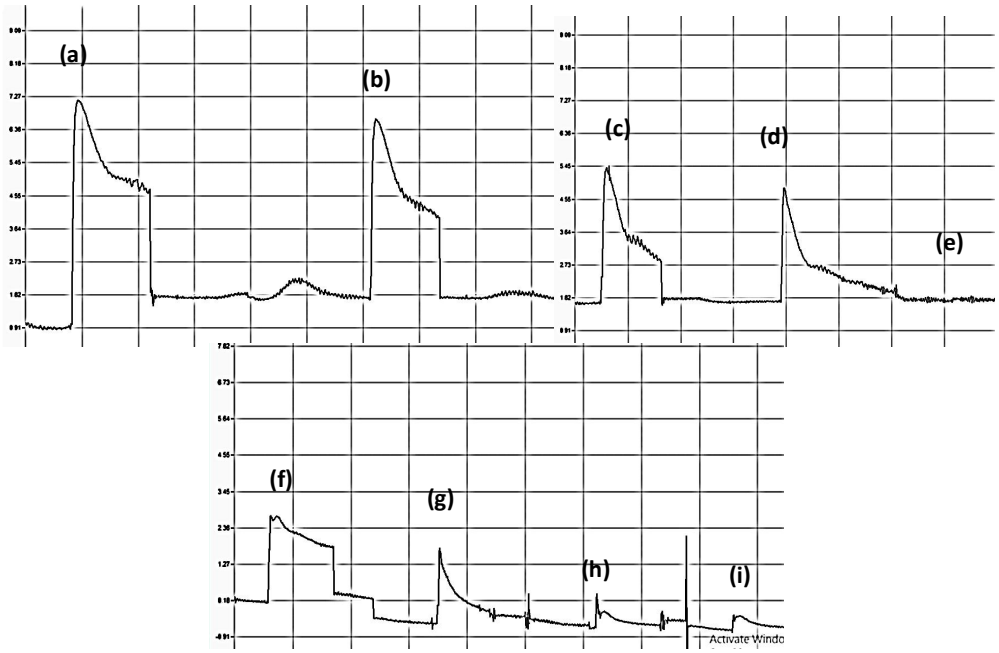

Fig. 4. Effect of different concentrations of petroleum ether fraction (PE) of *A. vasica* on Ach-induced contraction of rat ileum; (a) Ach alone (50 µg/mL), (b) PE (25 µg/mL)/Ach, (c) PE (50 µg/mL)/Ach, (d) PE (75 µg/mL)/Ach, (e) PE (150 µg/mL)/Ach cumulative inhibition, (f) Ach alone (50 µg/mL), (g) PE (100 µg/mL)/Ach, (h) PE (200 µg/mL)/Ach, (i) PE (250 µg/mL) complete inhibition

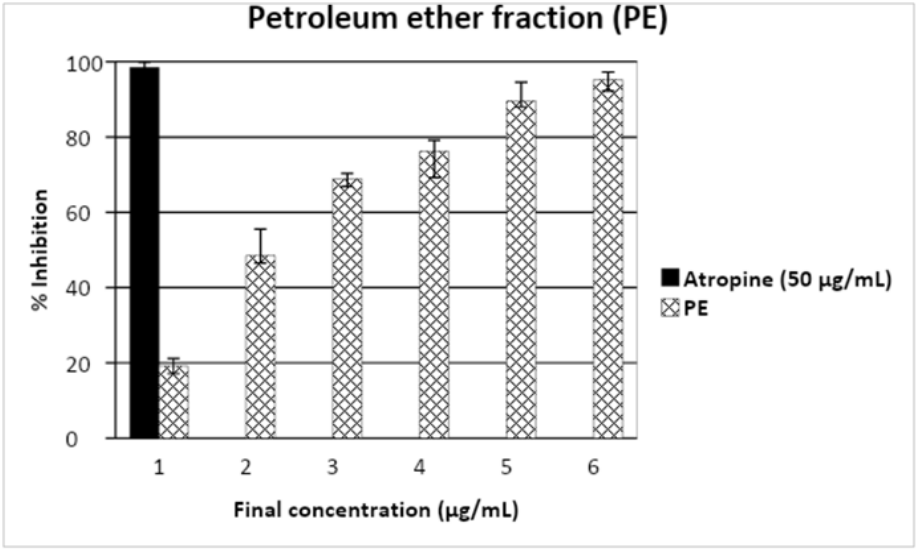

Fig. 5. Percentage inhibition of Ach-induced contraction by petroleum ether fraction (PE) of *A. vasica* at different concentrations as compared to atropine as positive control at 50 µg/mL

The results are expressed as the mean ± standard deviation (SD) calculated from three independent experiments. The results are considered as statistically significant at p ≤ 0.05

The ethyl acetate (EA) and the aqueous (Aq) fractions showed moderate antispasmodic effect as indicated from the results represented in Figs. (8-11), that showed maximum

percentage of inhibitions equal 53.5% and 42.2%, respectively at a total concentration 200 µg/mL.

The isolated alkaloids (AA1and EA1), showed very weak antispasmodic activity at low concentrations (25, 50 and 75 µg/mL). However, a marked inhibition of Ach-induced contraction was observed at a cumulative concentration of 150 µg/mL. Vasicinone (AA1) showed an inhibition of 89.9%, while vasicinolone (EA1) showed an inhibition of 84.8% (Fig. 12). The moderate effect of the EA and Aq fractions could be explained by the concentration of AA1 and EA1 in the crude extracts fractions. These findings indicated that vasicinone and vasicinolone may contribute in the reported antispasmodic effect of *A. vasica*.

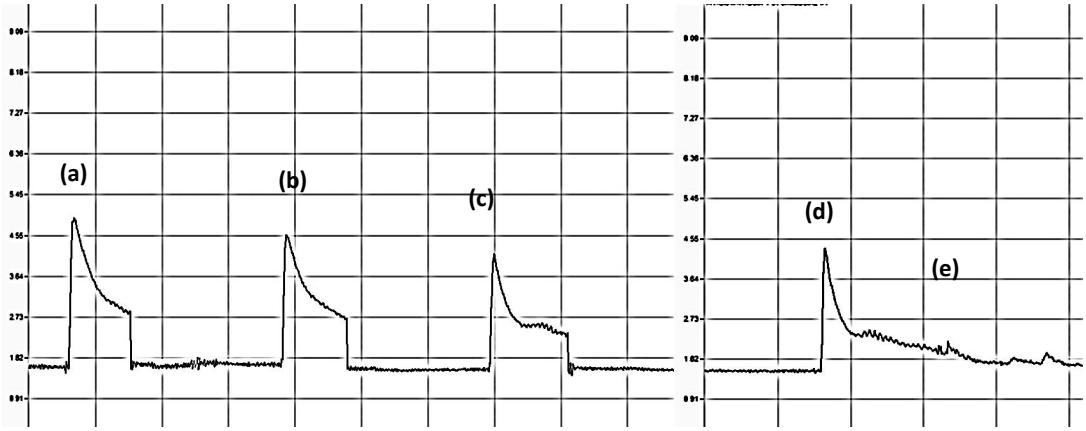

Fig. 6. Effect of different concentrations of methylene chloride fraction (MC) of *A. vasica* on Ach-induced contraction of rat ileum; (a) Ach alone (50 µg/mL), (b) MC (25 µg/mL)/Ach, (c) MC (50 µg/mL)/Ach, (d) MC (75 µg/mL)/Ach, (e) MC (150 µg/mL)/Ach cumulative

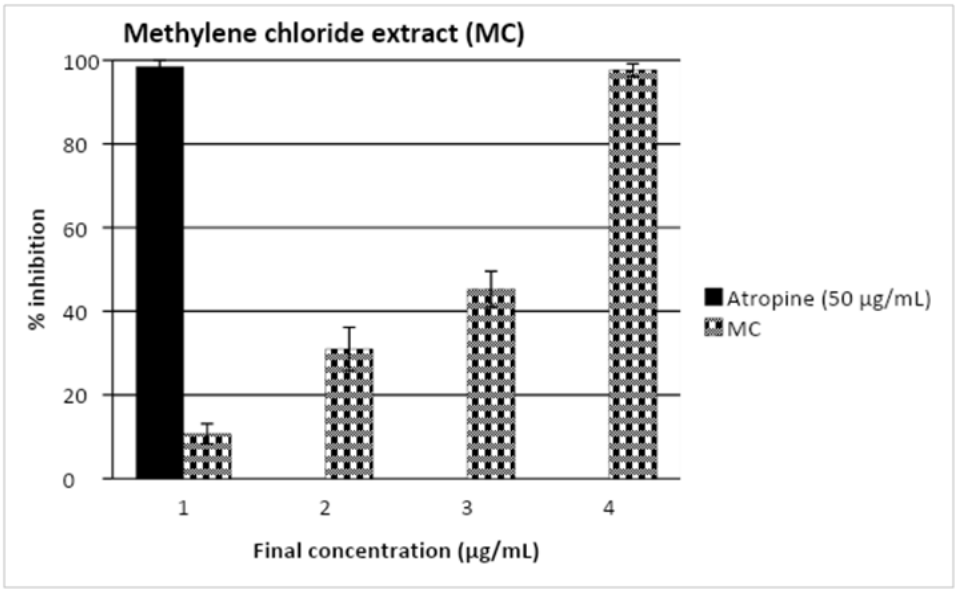

Fig. 7. Percentage inhibition of Ach-induced contraction by the methylene chloride fraction (MC) of *A. vasica* at different concentrations as compared to atropine as positive control at 50 µg/mL

The results are expressed as the mean ± standard deviation (SD) calculated from three independent experiments. The results are considered as statistically significant at $p \leq 0.05$

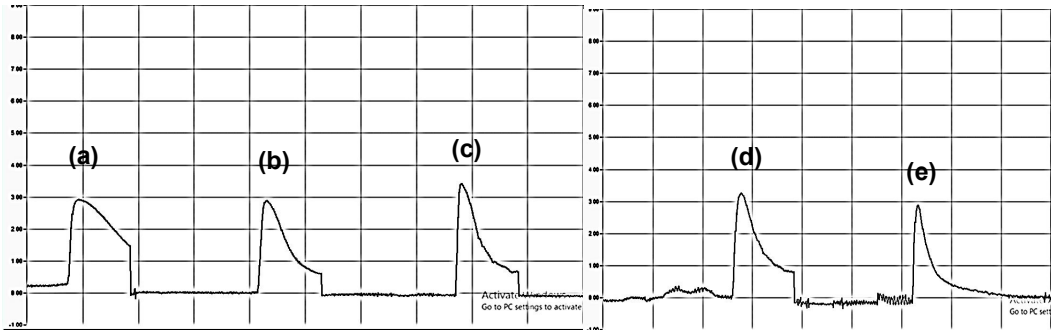

Fig. 8. Effect of different concentrations of the ethyl acetate fraction (EA) of *A. vasica* on Ach-induced contraction of rat ileum; (a) Ach alone (50 µg/mL), (b) EA (25 µg/mL)/Ach, (c) EA (50 µg/mL)/Ach, (d) EA (75 µg/mL)/Ach, (e) EA (200 µg/mL)/Ach

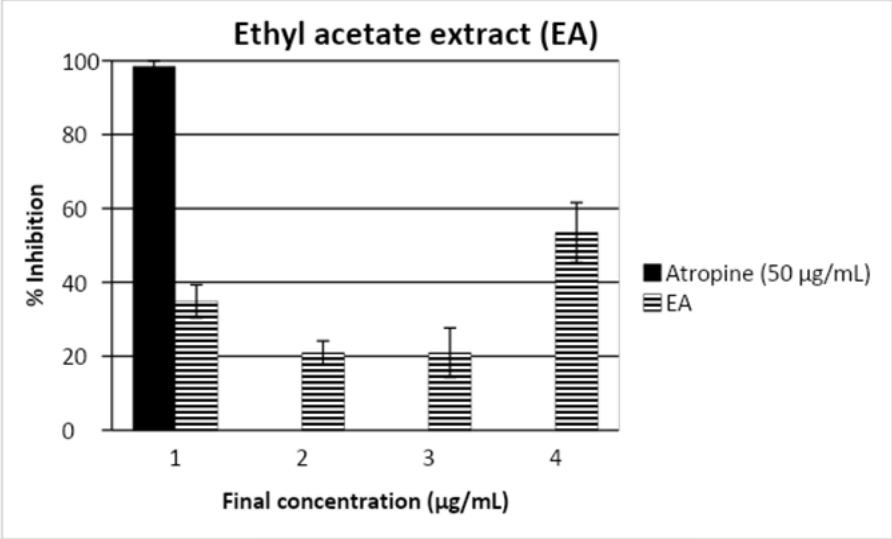

Fig. 9. Percentage inhibition of Ach-induced contraction by the ethyl acetate fraction (EA) of *Adhatoda vasica* at different concentrations as compared to atropine as positive control at 50 µg/mL

The results are expressed as the mean ± standard deviation (SD) calculated from three independent experiments. The results are considered as statistically significant at p ≤ 0.05.

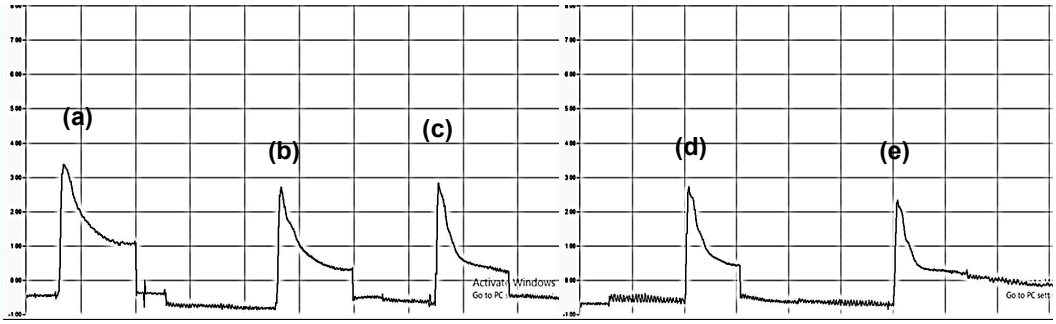

Fig. 10. Effect of different concentrations of the aqueous fraction (Aq) of *A. vasica* on Ach-induced contraction of rat ileum; (a) Ach alone (50 µg/mL), (b) AqE (25 µg/mL)/Ach, (c) AqE (50 µg/mL)/Ach, (d) AqE (75 µg/mL) /Ach, (e) AqE (200 µg/mL)/Ach

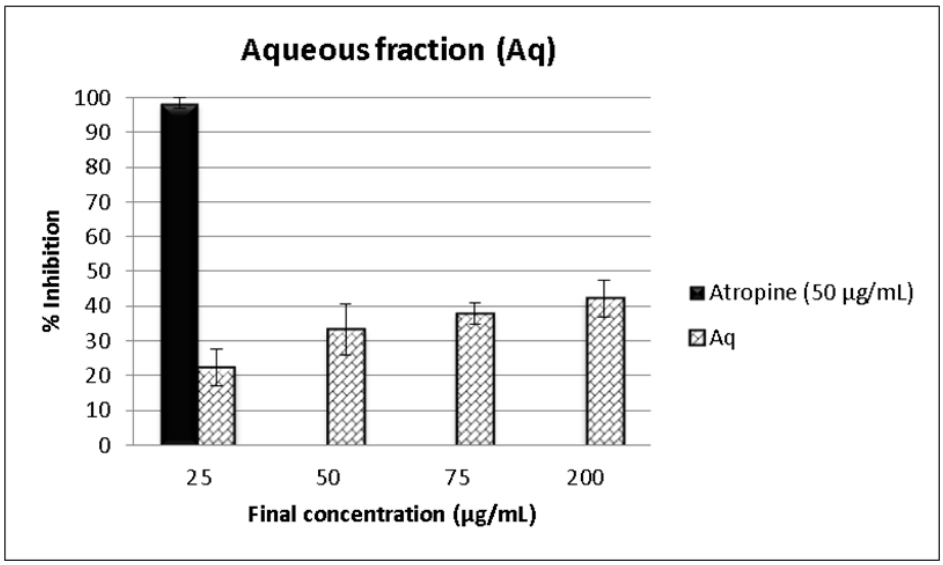

Fig. 11. Percentage inhibition of Ach-induced contraction by the aqueous fraction (Aq) of *Adhatoda vasica* at different concentrations as compared to atropine as positive control at 50 µg/mL

The results are expressed as the mean ± standard deviation (SD) calculated from three independent experiments. The results are considered as statistically significant at p ≤ 0.05

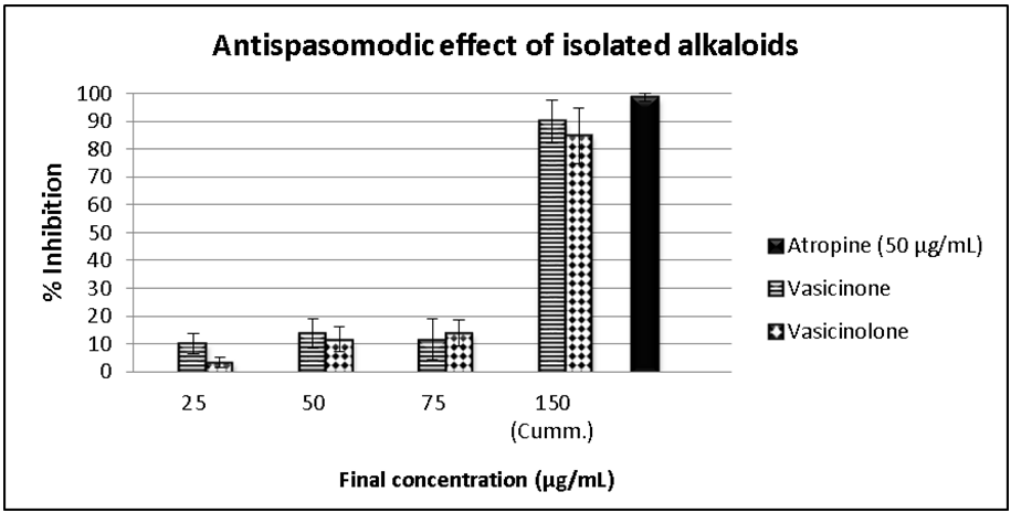

Fig. 12. Percentage inhibition of Ach-induced contraction by the alkaloids isolated from *A. vasica* viz., vasicinone (AA1) and vasicinolone (EA1), at different concentrations as compared to atropine as positive control at 50 µg/mL

The results are expressed as the mean ± standard deviation (SD) calculated from three independent experiments. The results are considered as statistically significant at p ≤ 0.05

4. CONCLUSION

In this study, the phytochemical constituents of *Adhatoda vasica* have been investigated. Seven known compounds have been isolated from the stems of *A. vasica*, three of them (Monopalmitin, Vanillin, and vanillic acid) are reported in the titled plant for the first time. The various extracts and compounds isolated from *A. vasica* have been tested for their antimicrobial, antiquorum-sensing and antispasmodic activities. Most of the tested *A. vasica* samples showed pronounced

antibacterial activities with remarkable inhibitions of Ach-induced spasm in isolated rat ileum. This work provides scientific evidences for the reported traditional medicinal uses of *A. vasica* with contribution of some of the identified and tested phytoconstituents in the obtained biological effects.

CONSENT

It is not applicable.

ETHICAL APPROVAL

The protocol of the current research has been approved by the Bioethical Research Committee (BERC), College of Pharmacy, Prince Sattam Bin Abdulaziz University, KSA (IRB No.: BERC-003-12-19).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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