Section A-Research paper
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ISOLATION, SPECTRAL CHARACTERIZATION AND ANTIBACTERIAL ACTIVITY OF ACTIVE CONSTITUENTS BITA-SITOSTEROL AND DIOSGENIN FROM *AERVA LANATA* FAMILY *AMARANTHACEAE*

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ABSTRACT

Background: Traditionally known as Pashanabheda in India, *Aervalanata* (L) belonging to the family Amaranthaceae, is widely available in Western ghats of India and used as Antiurolithiatic, Antimicrobial, Antiinflammatory, Hepatoprotective drug by Indian traditional system of medicines.

Materials and Method: In the present study this herb is subjected to extraction with Pet. Ether, Chloroform, Ethyl acetate and Methanol followed by fractionation with different solvents of varying polarities such as n-hexane: Acetic acid (9:1) and screened for Antibacterialactivity between Bita-Sitosterol and Diosgenin. Based on the results, the potent fractions were subjected to isolation of active constituents using column chromatography.

Results: The isolated compounds from two fractions n-hexane and Acetic acid were characterized by modern analytical techniques such as UV-visible Spectroscopy, FTIR (Fourier Transform Infrared spectroscopy), NMR Spectroscopy and Mass spectroscopy as Bita-Sitosteol and Diosgenin.

Conclusion: The two compounds isolated from potent fractions based on column chromatographywere characterized as Bita-Sitosterol and Diosgenin by modern analytical techniques. Well Diffusion Assay confirm that Bitasitosterol and Diosgeninhave antibacterial activity and can be used as the source for natural bactericides.

Keywords:*Aervalanata*, Bita-Sitosterol, Diosgenin, isolation

INTRODUCTION

The traditional medicines system is a rich source of valuable medicinal plants but there is no scientific data to establish the activity of these plants. These plants need to be evaluated, based on their biological efficacy and chemical constituents for the drug development. Hence we have selected *Aervalanata* (L) which is traditionally used as Pashanabheda plant (stone dissolving) for antibacterial activity, available from Western Ghats region for our present study. Bioactivity guided fractionation of this plant was carried out in order to investigate the traditional claim.*Aervalanata* contains β-sitosteryl palmitate, α- amyrin and β-sitosterol and tannin. *Aervalanata* is endowed with chemical compounds such as steroids, flavonoids, alkaloids, polysaccharides and saponin (**Chandra S *et al.*, 1990).***Aervalanata* also reported for six alkaloids like Canthin - 6-one and β-Carboline, Hentriacontane, β-sitosterol and its D-

glucoside, α-amyrin and betulin on dry plant material basis (**Zapesochnaya*et al.*, 1992**). Four flavonoid glycosides, betulin, campesterol, Chrysin etc. were reported to present in *Aervalanata* (**Pervykh*et al.*, 1993**). Traditionally *Aervalanata* is used as diuretic, demulcent, in headache and lithiasis. According to Ayurveda, it is also useful in strangury. In ethano-medicine *Aervalanata* is regarded as a valuable medicine for cough, sore throat, indigestion, wounds and as a vermifuge for children. It is also used for diabetics **(Kirtikar*et al.*, 1996**). The flowers are used for the removal of kidney stones and in gonorrhoea (**Chopra *et al.*, 1993**). The herb has been used for its therapeutic effects in renal diseases by some Unani physicians and to treat urinary calculi (**Amin *et al.*, 1994**). *Aervalanata* has been documented for its urolithiasis effects (**Soundararajan*et al.*, 2006**), antimicrobial (**Sabahi*et al.*, 1987**), cytotoxicity (**Nevin*et al.*, 2003**), antidiabetic (**Vetrichelvan*et al.*, 2002**), nephroprotective (**Shirwaikar*et al.*, 2004**), immunomodulatory (**Nevin*et al.*, 2006**), diuretic and its anti-inflammatory activities (**Vetrichelvan*et al.*, 2000**).Medicinal plants have been used for centuries as remedies for microbial diseases by native cultures around the world **(M.J *et al.*, 2005; A. Gurib 2006**) and have the potential for providing effective treatments for antibiotic-resistant infections (**D. Senthil*et al.*,2013; P.G *et al.*,2008; M. G *et al.*,2015)**, flavoring food, as traditional medicine, and as preservatives A high percent (about 80%) of persons from developed countries are using plant derived products for traditional medicine (**G.G.F *et al.*, 2004**) or as food flavoring or preservatives, and therefore, such plants should be investigated to better understand their properties, safety and efficiency. Since in the scientific literature there is reported the isolation of bacteria sensitive to routinely prescribed drugs and that became multiresistant to available products, it seems that bacterial pathogens own the genetic ability to acquire and transmit resistance to current antibacterials**(N.C.C *et al.*, 2010).**

Materials and methods

Chemical reagents

All the chemicals used in this study were obtained from Hi Media Laboratories Pvt. Ltd. (Mumbai, India), SD Fine-Chem Chem. Ltd. (Mumbai, India) and SRL Pvt. Ltd. (Mumbai, India).All the chemicals used in this study were of analytical grade.

Isolation of column chromatography:-

Extract was subjected to silica gel column chromatography for isolation of bioactive components from *Aervalanata*extract. Gradient elusion technique was followed for column chromatography. The column was eluted with n-hexane: Acetic acid (9:1)and number of elutes were collected **(Srivastava*et al.*, 2021)**.

Characterization-

UV-visible Spectroscopy

The isolated fraction of sample was diluted with the same solvent. The extract was scanned from 200 to 800 nm wavelength using UV-Visible Spectrophotometer (Shimadzu UV-1800) and the characteristic peaks were detected and recorded **(Perkampus.*et al.*, 2013)**.

FT-IR Spectroscopy

To establish the presence of the functional groups, FT-IR spectroscopy was performed using Perkin Spectrum BX spectrophotometer.**(Lucien*et al.*, 2008).**

NMR Spectroscopy

NMR spectroscopy was performed for the isolated fraction to identify the structure of the compound present in the isolated fraction. 1H and ^{13}C-NMR spectra were recorded on a FT-NMR Cryomagnet Spectrometer 400 MHz (Bruker) using TMS as an internal standard. The solvents used were Hexane: Acetone (8: 2) and DMSO. Chemical shifts ware shown in δ values (ppm) with TMS as an internal reference. **(Eswaraiah*et al.*, 2011)**

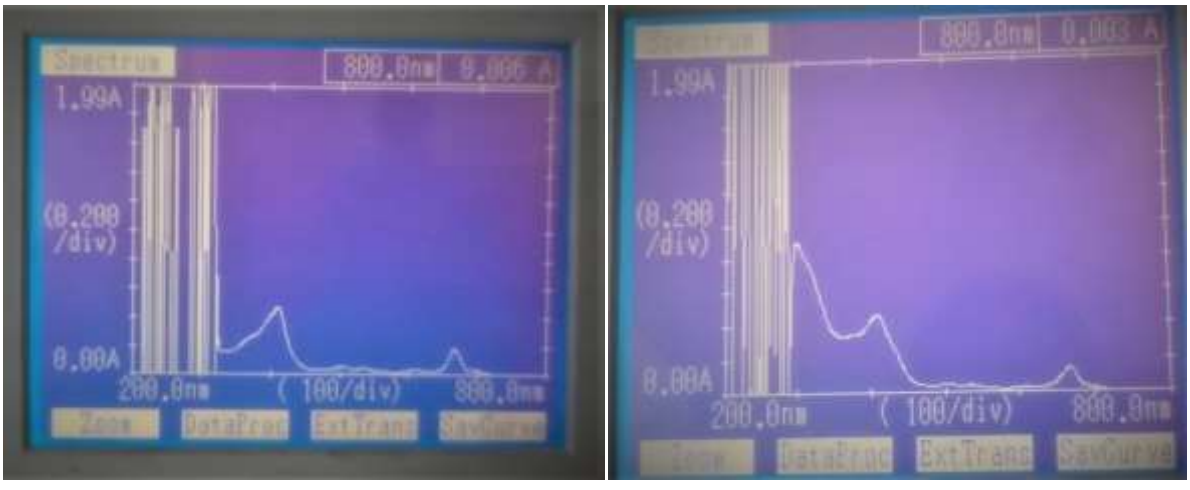
Mass Spectroscopy

The mass spectrometer used for the identification of the molecular weight of the compound was Bruker Daltonik, Benchtop easy-to-use, high performance Electrospray Ionization Quadrupole time-of-flight LC MS spectrometer **(Wiley *et al.*, 1995).**

Antibacterial Activity (Well Diffusion Assay)

Culture of bacterial strains *(E. coli MTCC 42, S. mutans MTCC 389, P. aeroginosa MTCC 8076 and S. aureus MTCC 10787*) was spread on the Nutrient agar media (NAM). The bacterial suspension was standardized to 108 CFU/ml of bacteria and kept into the shaker. Then, 100µl of the inoculum from the broth (containing 108 CFU/ml) was taken with a micropipette and then transferred to fresh and sterile solidified Agar Media Plate (**Mohammadi-Sichani et al., 2012**). Four wells of 6 mm were bored in the inoculated media with the help of sterile cork-borer. Each well was filled with different concentration (25µg/ml, 50µg/ml, 75µg/ml, and 100µg/ml) of samples. It was allowed to diffuse for about 30 minutes at room temperature and incubated for 18-24 hours at 37o C. After incubation, plates were observed for the formation of a clear zone around the well which corresponds to the antimicrobial activity of tested compounds(**Manandhar et al., 2019**).

RESULTS



A B

Figure 1: (a)Active constitutes estimation By UV- Spectra of A fraction of ALE (EA) extract after column chromatography (B) Active constitutes estimation By UV- Spectra of D fraction of ALE (M) extract after column chromatography

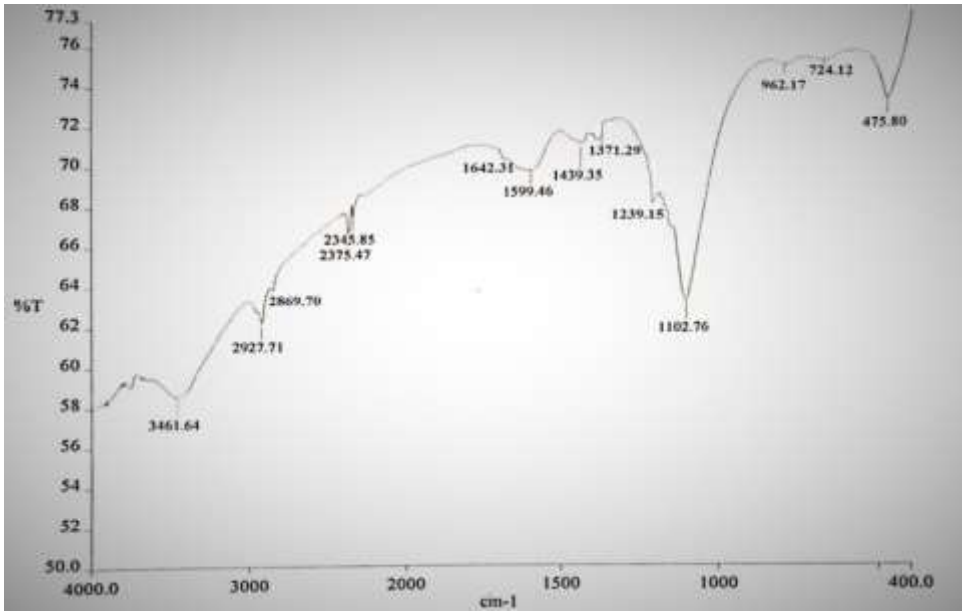


Figure 2:IR spectra of the isolated compound (Fraction A) of ALE (EA)

Table 1: FTIR- Spectrum Frequency Range of the isolated compound (Fraction D) of ALE (M)

Sr. No.	Fraction	Frequency Range	Group Absorption (cm^{-1})	Appearance	Group	Compound Class
1.	A Mobile Phase (8:2)	4000- 3000 (cm^{-1})	3461.64	strong, broad	O-H stretching	Hydroxyl Group
		1738- 1638 (cm^{-1})	1642.31	Weak	C=C stretching	Alkene
		3000- 2500 (cm^{-1})	2927.71	Medium	C-H stretching	Methyl Group
		3000- 2500 (cm^{-1})	2869.70	Medium	C-H stretching	Methylene Group
		1500- 1300 (cm^{-1})	1439.35	Medium	C-H bending	Methylene Group
		1385-1370 (cm^{-1})	1371.29	Medium	C-H bending	dimethyl group
		980-960 (cm^{-1})	962.17	strong	C=C bending	disubstituted
		800 -700 (cm^{-1})	724.12	strong	C-H bending	mono substitution in aromatic ring
		1000- 1400 (cm^{-1})	1239.15	Weak	C-C stretching	Alkane
		1200-1000 (cm^{-1})	1102.76	Medium	C-O stretches	C-O-H Group

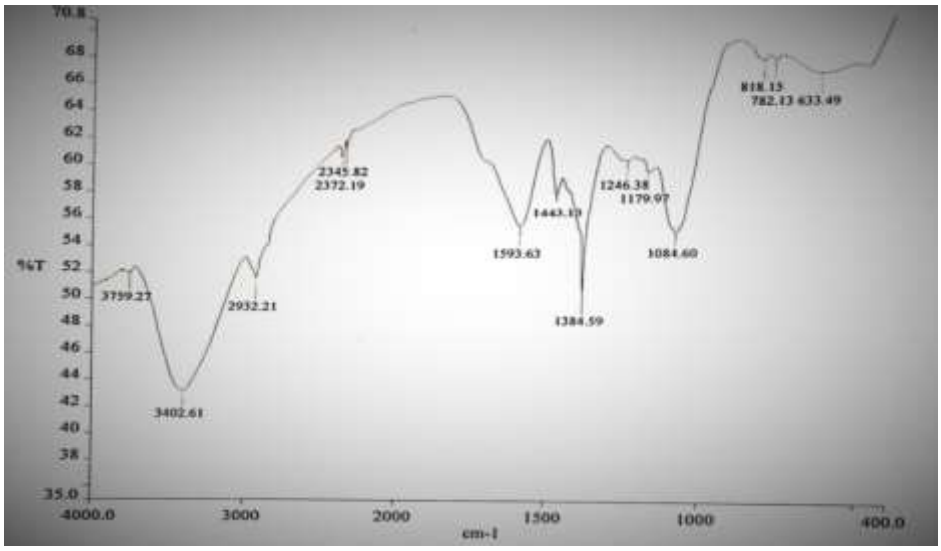


Figure 3: IR spectra of the isolated compound (Fraction D) of ALE (M)

Table 2: FTIR- Spectrum Frequency Range of the isolated compound (Fraction D) of ALE (M)

Sr. No.	Fraction	Frequency Range	Group Absorption (cm^{-1})	Appearance	Group	Compound Class
1.	**A Mobile Phase (8:2)**	4000- 3000 (cm^{-1})	3402.61	strong, broad	O-H stretching	Hydroxyl Group
		3000- 2500 (cm^{-1})	2932.21	Medium	C-H stretching	Methyl Group
		1500- 1300 (cm^{-1})	1443.13	Medium	C-H bending	Methylene Group
		1385-1370 (cm^{-1})	1384.59	Medium	C-H bending	dimethyl group
		800 -700 (cm^{-1})	782.13	strong	C-H bending	mono substitution in aromatic ring
		1000- 1400 (cm^{-1})	1246.38	Weak	C-C stretching	Alkane
		1200-1000 (cm^{-1})	1179.97	Medium	C-O stretches	C-O-H Group
		1200-1000 (cm^{-1})	1084.60	Medium	C-O stretches	C-O-H Group

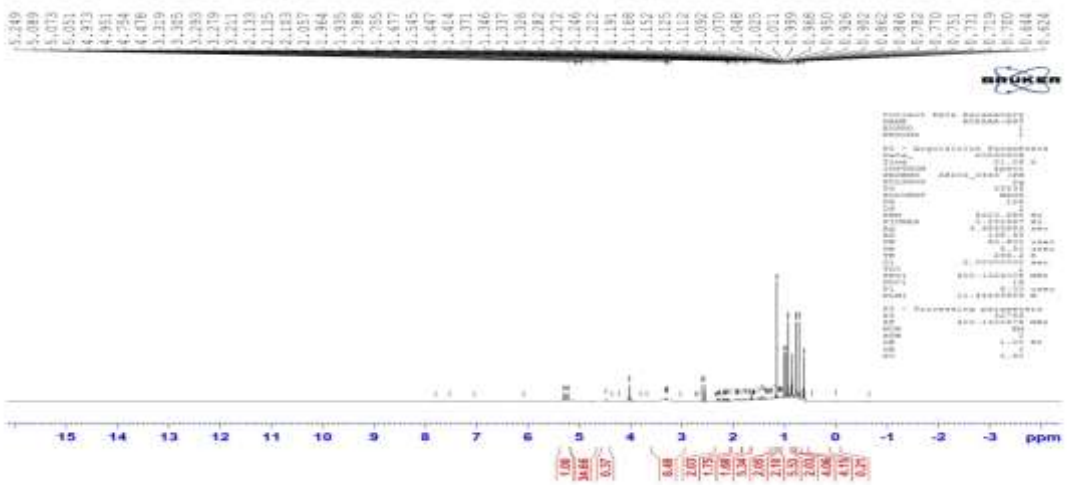


Figure 4: ^{1}H NMR spectra of the isolated Fraction(Fraction A) of ALE (EA)

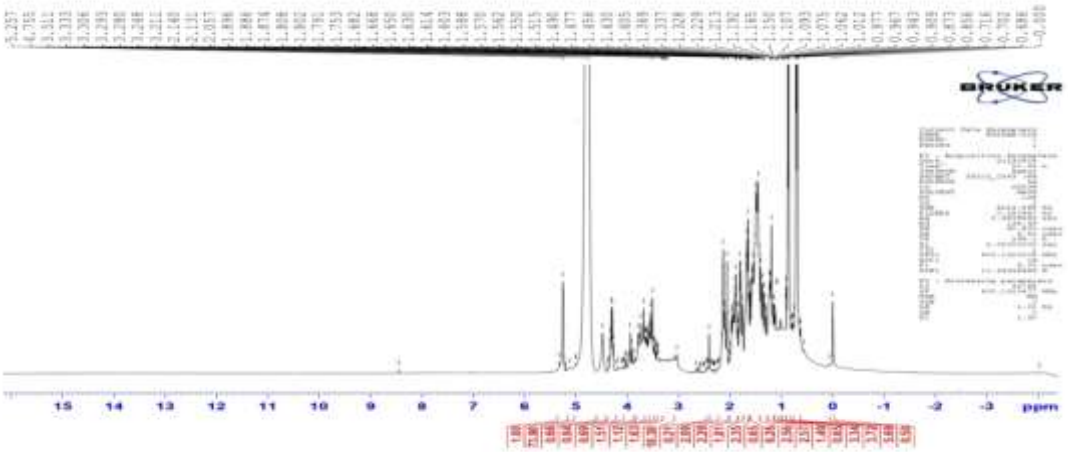


Figure 5: ^{1}H NMR spectra of the isolated Fraction(Fraction A) of ALE (EA)

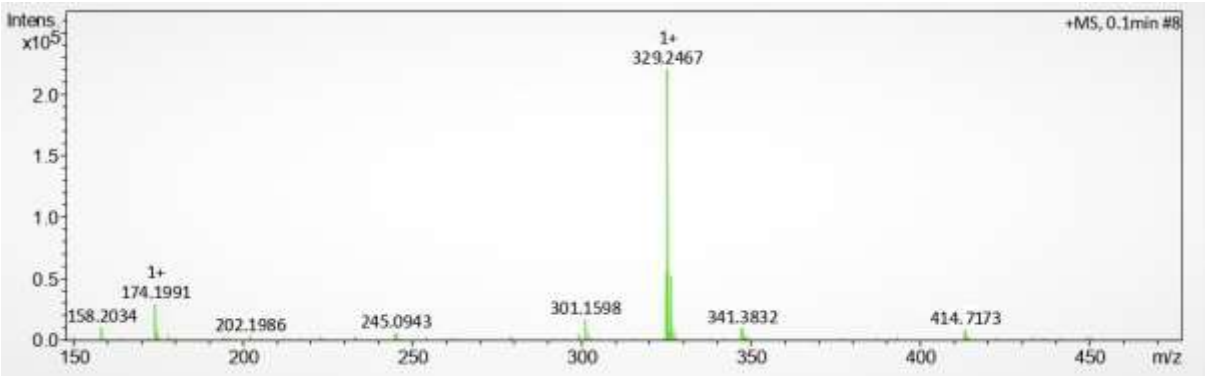


Figure 6: Mass spectra of theisolated compound (Fraction D) of ALE (M)

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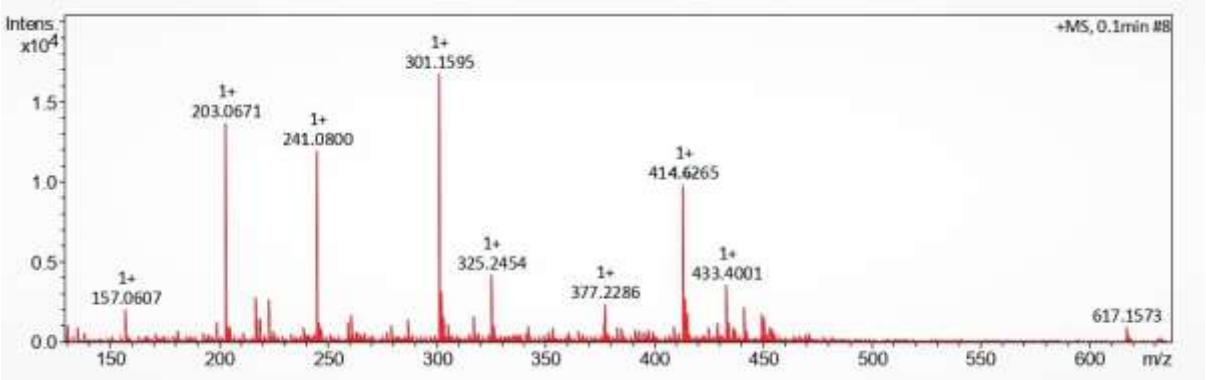

Figure 7: Mass spectra of theisolated compound (Fraction D) of ALE (M)

Table 3 Antimicrobial activity of Bita-Sitosterol

Bacteria	Zone of Inhibition at 100µg
E. coli	13.33±0.577
P. aeruginosa	11.66±0.577
S. aureus	20.66±2.516
S. mutans	22±1

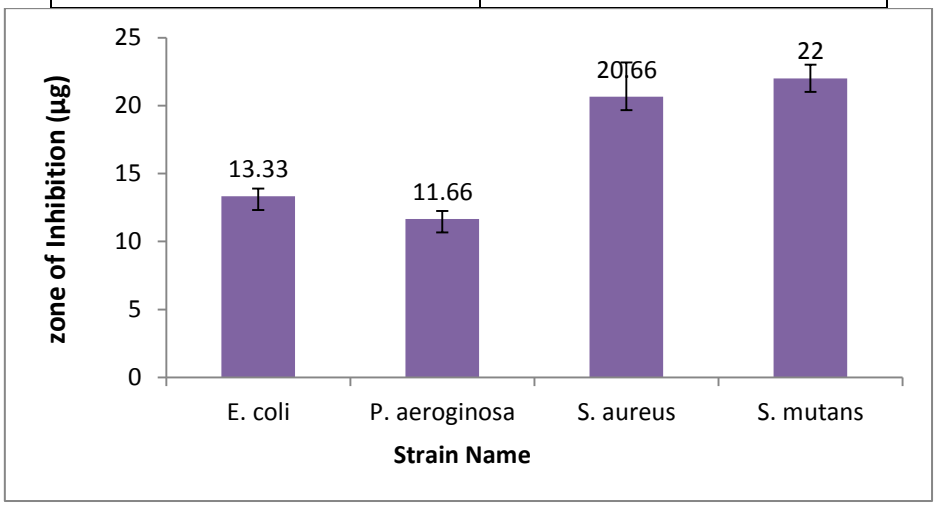

Figure 8: Graph represent Antimicrobial activity of Bita-Sitosterol

Table 4:Antimicrobial activity of Diosgenin

Bacteria	Zone of Inhibition at 100µg
E. coli	10.66±1.154
P. aeruginosa	12.33±0.577
S. aureus	11.66±0.577
S. mutans	12.33±0.577

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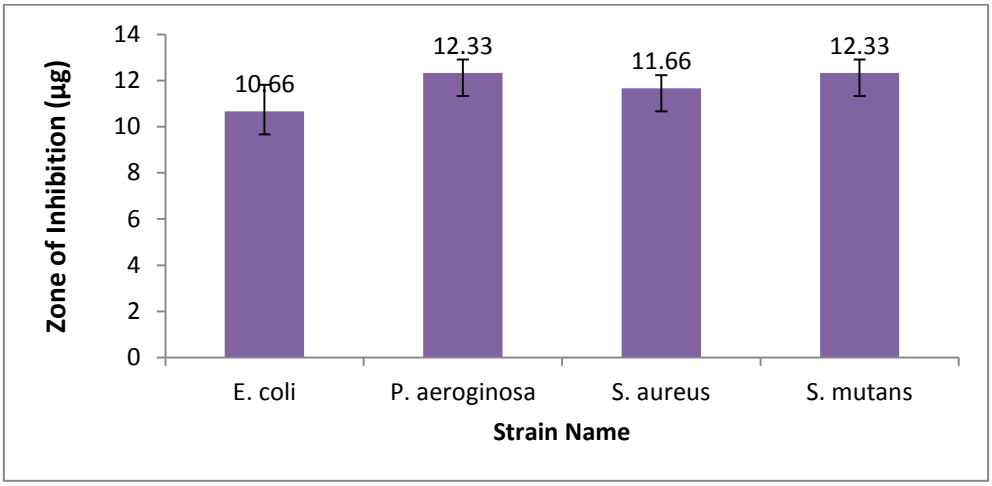

Figure 9: Graph represent Antimicrobial activity of Diosgenin

Table5 : Antibacterial activity of the different concentrations of Elute B (Ethyl acetate extract) of *Aervalanata*

Strain name	100%	75%	50%	25%
Escherchia.coli	13.33±0.577	10.66±0.577	8.33 ± 0.577	7±0
Pseudomonas aeruginosa	11.66±0.577	8.66±0.577	7±0	0±0
Staphylococcus aureus	20.66±2.516	15.66±1.527	13±1	10±1
Sterptococcus Mutans	22±1	17.33±0.577	13±0	10.6667±1.154

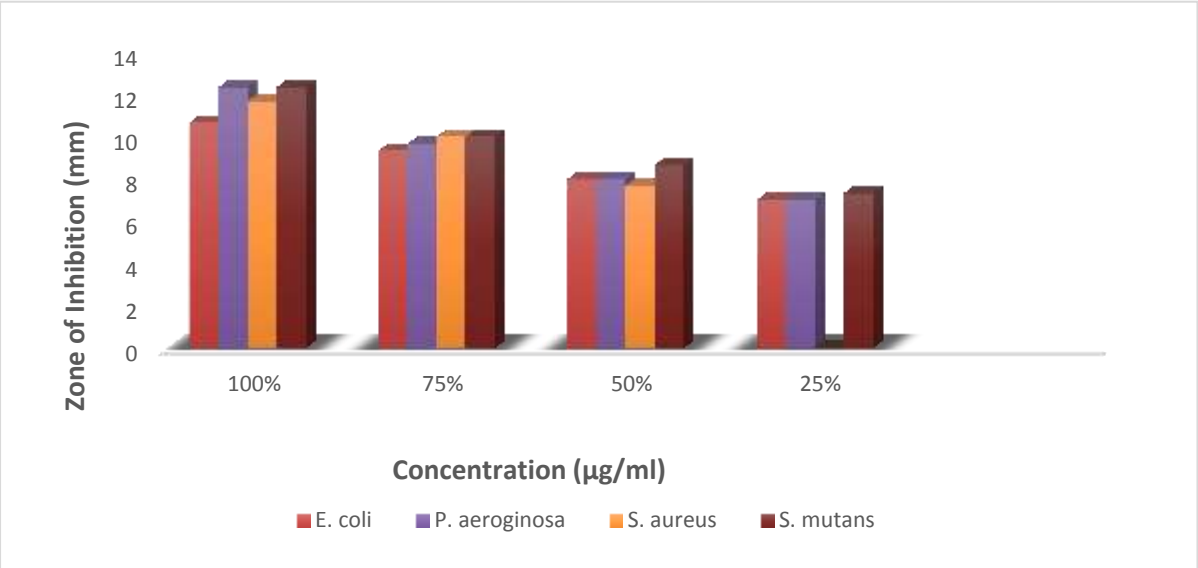

Figure 10 :Graphical representation of Antibacterial activity of the different concentrations of Elute B (Ethyl acetate extract) of *Aervalanata*

Table 6: Antibacterial activity of the different concentrations of Elute D (Methanol extract) of *Aervalanata*

Strain name	100%	75%	50%	25%
Escherchia.coli	10.66±1.154	9.33±0.577	8± 0	7±0
Pseudomonas aeruginosa	12.33±0.577	9.66±0.577	7±0	8±0
Staphylococcus aureus	11.66±0.577	10±0	7.66±0.577	0±0
Sterptococcus Mutans	12.33±0.577	10±1	8.66±0.577	7.33±0.577

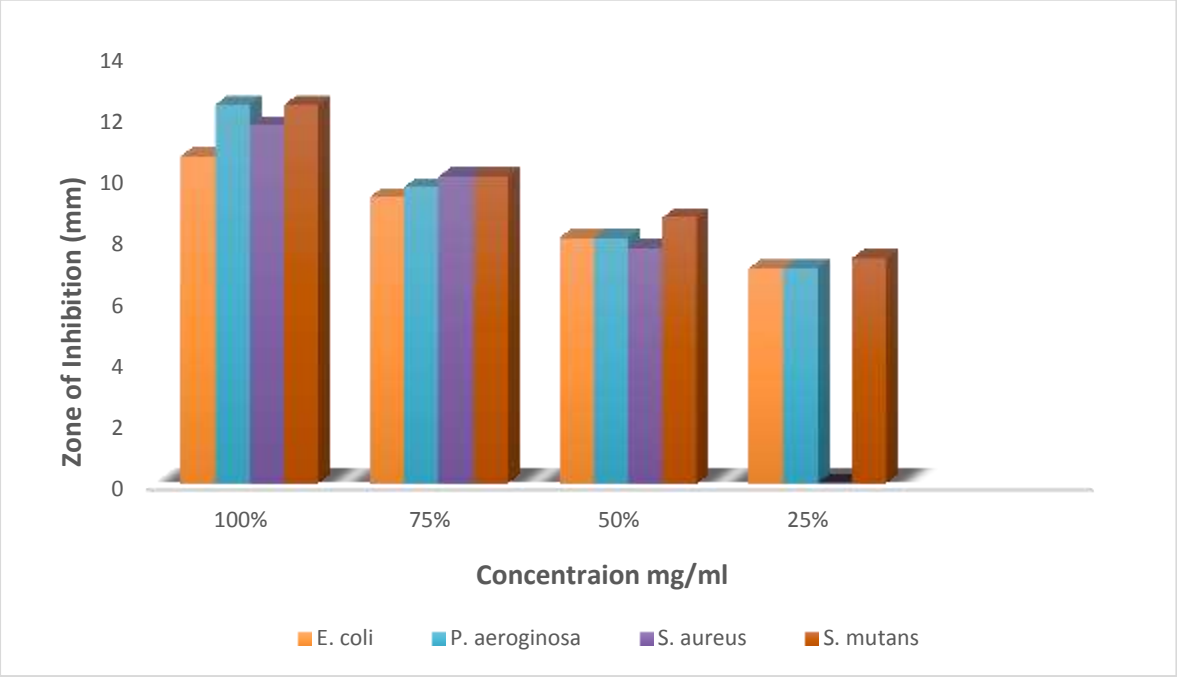

Figure 12 :Graphical presentation ofAntibacterial activity of the different concentrations of Elute D (Methanol extract) of *Aervalanata*

Discussion

TLC performed in n-hexane: Acetic acid (9:1) and n-hexane: Acetone (8:2) were taken as Mobile phase for column chromatography. Active constitutes are isolated from column chromatography with the mobile phase of n- Hexane: acetone (8:2) for ALE (C, EA) to obtained ALE (C) Fractions 01-05 (A1, A2, A3, A4, A5), 06 (B), 07 (C), ALE (EA) Fractions 01 (A), 02 (B), 03-04 (C1, C2) and n- Hexane: Acetic acid (9:1) for ALE (EA, M) to obtained ALE (EA)Fractions 01 (A), 02-03 (B1, B2), 04 (C), 05-06 (D1, D2), 07 (E), 08-

11(F1, F2, F3, F4), ALE (M) Fractions 01 (A), 02 (B), 03 (C), 04 (D).UV spectra of the isolated compounds were recorded over a scanning range of 200-800 nm and λmax of compounds were determined and the wavelength of ALE (EA) A fraction and ALE (M) D fraction were found out 397 and 285 nm.(**Fig 1**) In IR spectrum of isolated Fraction A of ALE (EA), O-H bond vibrations of hydroxyl group broad peak was observed at 3461.64 cm^{-1}, C=C vibrations peak at 1642.31 cm^{-1}, C-H peak at 2927.71 and 2869.70 cm^{-11}, C-O peak at 1102.76 cm^{-1} and C-C vibration peak at 1105.50 cm^{-1}.The IR Spectrum of isolated compound fraction A of ALE (EA) showed broad absorption peak at 3461.64 indicating the presence of -OH group. The peak at 2927.71 and 2869.70 cm-1 indicated the presence of aliphatic C-H stretching. The absorption peak at 1642.31 cm-1 indicated the unsaturation that is C=C absorption peak and peak at 1102.76 cm-1 indicated the presence of C-O stretches. The absorption band at 1439.35 cm-1 indicated the presence of C-H bending of CH2 and absorption band at 1371.29 cm-1 is due to C-H bending of dimethyl group. The absorption peak at 962.17 cm-1 is due to C=C-H group. The absorption peak at 1239.15 cm-1is due to C-C stretching and peak at 724.12 cm-1 indicated mono substitution in aromatic ring(**Fig 2**).The IR Spectrum of isolated compound fraction D of ALE (M) showed broad absorption peak at 3402.61 indicating the presence of -OH group. The peak at 2932.21 indicated the presence of aliphatic C-H stretching. The absorption peak at 1179.97 and 1084.60 cm-1 indicated the presence of C-O stretches. The absorption band at 1443.13 cm-1 indicated the presence of C-H bending of CH2 and absorption band at 1384.59 cm-1 is due to C-H bending of gem-dimethyl group. The absorption peak at 1246.38 cm-1is due to C-C stretching and peak at 782.13 cm-1 indicated mono substitution in aromatic ring(**Fig 3**). In ^{1}H-NMR spectrum isolated fraction (Fraction A) of ALE (EA), H-19 proton appeared at 0.60-1.00 ppm (19H, 0.62 (td), 0.62 (td), 0.64 (td), 0.64 (td), 0.75 (d), 0.75 (d), 0.84 (t), 0.90 (s), 0.95 (d) ppm), H-6 proton appearedat 1.13-1.27 (6H, 1.19 (tq), 1.21 (quint), 1.21 (quint), 1.21 (s)), H-18 proton appearedat 1.28-1.99 (18H, 1.34 (ddd), 1.37 (o), 1.41 (tqd), 1.44 (dtd), 1.44 (ddd), 1.53 (dt), 1.55 (ddd), 1.55 (dddd), 1.67 (dddd), 1.75 (ddd), 1.78 (ddd), 1.82 (dtd), 1.93 (ddt), 1.96 (ddd)), H-3 proton appeared at 2.05-2.36 (3H, 2.10 (ddd), 2.24 (dd), 2.28 (ddd)), H-1 proton appeared at2.54 (1H), 4.04 (1H, dtd), 5.24 (1H) and 5.24 (1H)(**Fig 4**).In ^{1}H-NMR spectrum isolated fraction (Fraction D) of ALE (M), H-9 proton appeared at 0.87-1.01 (9H, 0.90 (d), 0.90 (d), 0.94 (d), 0.94 (d), 0.96 (s), 0.96 (s)), H-3 proton appeared at 1.18-1.28 (3H, 1.22 (s), 1.22 (s)), H-22 proton appeared at 1.33-2.33 (22H, 1.43 (dddd), 1.45 (ddd), 1.47 (dddd), 1.51 (ddd), 1.58 (ddd), 1.57 (ddd), 1.58 (dddd), 1.60 (dtd), 1.66 (ddd), 1.66 (dddd), 1.75 (dddd), 1.68 (ddd), 1.71 (tqdd), 1.73 (ddd), 1.75 (ddd), 1.88 (dddd), 1.89 (ddd), 1.89 (ddd), 1.98 (ddd), 2.00 (dd), 2.11 (ddd), 2.14 (dq)), H-2 proton appeared at 2.45-2.62 (2H, 2.52 (dd,), 2.55 (dd)), H-2 proton appeared at 3.75-3.97 (2H, 3.51 (dd), 3.90 (dd)), H-1 proton appeared at 4.03 (1H), H-1 proton appeared at 4.18 (1H, ddd) and H-1 proton appeared at 5.25 (1H, dd)(**Fig 5**).Mass spectrum of isolated fraction (Fraction A) of ALE (EA) and (Fraction D) of ALE (M)showed parent molecular ion [M^{+}] peaks at mlz 414.7173and 414.6265 which corresponds to the molecular formula $C_{29}H_{50}O$ and $C_{27}H_{42}O_3$ according to their fragments (**Fig 6 and 7**)From these physical, chemical and spectral evidences was confirmed as Bita-Sitosteolin fraction A of ALE (EA) and Diosgeninin fraction D of ALE (M). These two compounds were also studied by anti bacterial activity. As shown in Table 4

and 5, the antibacterial activities of compounds Bita-Sitosterol and Diosgenin were tested against four bacterial pathogens(*E. coli MTCC 42, S. mutans MTCC 389, P. aeroginosa MTCC 8076 and S. aureus MTCC 10787)* by using agar well diffusion assay.Bitasitosterol displayed maximum antibacterial activities as compare to diosgenin compound.Compounds showed increase in zone of inhibition with the increase in concentration. 100µg/ml concentration showed highest zone of inhibition against the test organisms. Bitasitosterol showed highest zone of inhibition of 22mm against S. mutans that showed high sensitivity even at 25µg/ml, followed by *S. aureus(*20.66*)*>*E. coli*(13.33)>*P. aeruginosa* (11.66).On the other hand, Diosgenin showed maximum zone of inhibition at 100µg/ml against *P. aeroginosa(12.33),* followed by*S. mutans*(12.33)> *S. aureus*(11.66)>*E. coli*(10.66).

Conclusion

The phytochemical examination of the parts of *Aervalanata*belonging to the family *Amaranthaceae*was effectively carried out. From these physical, chemical and spectral evidences was confirmed as Bita-Sitosterolin fraction A of ALE (EA) and Diosgeninin fraction D of ALE (M). Our study reveals that, *Aervalanata's*phytoconstituent, Bitasitosterol and Diosgenin, have the ability to inhibit the bacterial growth and can be used as the source for natural bactericides.

Conflict of interest

Regarding this inquiry, there are no conflicts of interest.

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