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**ARTEMISIA RUTIFOLIA MEWAH EXTRACTS: PHYTOCHEMICAL
PROFILING, GC-MS ANALYSES, ANTI-BACTERIAL AND ANTIOXIDANT
ACTIVITY**

NADEEM A¹, AHMED B^{1*}, SHAHZAD H², IMRAN M¹ AND CRAKER LE³

1: Department of Biological Sciences, International Islamic University, Islamabad, Pakistan

2: Department of Biochemistry, PMAS Arid Agriculture University, Rawalpindi, Pakistan

3: Department of Plant Biology, Stockbridge school of Agriculture, University of
Massachusetts, Amherst, Massachusetts, USA

*Corresponding Authors: Dr. Bashir Ahmed: E Mail: bashir.ahmad@iiu.edu.pk;

drbashir.iiui@gmail.com

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ABSTRACT

Artemisia rutifolia is extensively grown in Pakistan. In classical and herbal medicine this plant is used extensively to treat various disorders. Present study was designed to perform qualitative and quantitative phytochemical analysis, antioxidant potential, and antimicrobial potential of *Artemisia rutifolia* against gram-positive and negative bacteria. The plant was collected, identified, and processed for Extract preparation in Methanol, Ethanol, Water, Acetone, and Hexane (MEWAH) solvents. Phytochemical Analyses and quantification were done through GCMS. Antibacterial and antioxidant potential of plant extract were identified. *Artemisia rutifolia* reveals a large percentage of alkaloids and Phenols. Water extract exhibited excellent percentage of Flavonoids. However, methanol extracts are considerably rich in Phenols. Total 89 phytochemicals were detected in MEWAH extracts except for water that had none. Medicinally important antitumorigenic, anti-inflammatory and anti-ischemic molecules like thujone, Cis-Verbenol, Phorbol, Gitoxygenin, Stigmasterol, and Eupatorin were identified in *Artemisia rutifolia* extracts. Moderate to excellent Antibacterial activity for *L. Lactis B. Subtilis*, *C. freundii* and *L. monocytogenes*, *E. coli*, and *K. oxytoca* strains was recorded. Acetone extract have highest antioxidant radical scavenging activity. *Artemisia rutifolia* is extremely rich source of phytochemicals and antioxidants functional against bacterial infections, showing radical scavenging activities and anti-tumorigenesis potential.

Keywords: *Artemisia rutifolia*, GC/GC-MS; Plant Biotechnology, Herbal Medicine

INTRODUCTION

Plant tissues produce secondary metabolites while they respond to stress. This may involve bacterial fungal or viral infections and environmental stresses [1]. These metabolites include phenols, flavonoids, Alkaloids, Tannins, Saponins, Triterpenoids, and anthraquinones [2] and have a prominent effect in fighting against infections or managing oxidative stress [1-2]. *Artemisia rutifolia* is relatively understudied but has potential antioxidant and antimicrobial properties [3]. Antibiotic drug resistance in bacteria is a real challenge of today. Naturally extracted metabolites from *Artemisia* species are considered effective and less toxic than synthetic drugs and are considered as a savior against several cancers as per ancient Chinese medicine [4]. Thus, antioxidant

and phytochemical-rich plant extracts could provide an efficient solution to cure chronic illnesses [5]. Phenols and flavonoids are promising antioxidant, antitumorigenic, and cardiac health promoters as found in several *artemisia* species [6] and other plant extracts [7]. The objective of the current study was to prepare *Artemisia rutifolia* extracts from non-polar to polar nature and have a detailed study of *Artemisia* extract as an antibacterial agent for gram-positive and negative bacteria, through various methods. To identify the best solvents for extraction of most phytochemicals, prepare a catalogue of each solvent-based extract via GCMS and identifying their antioxidant activities (Figure 1).

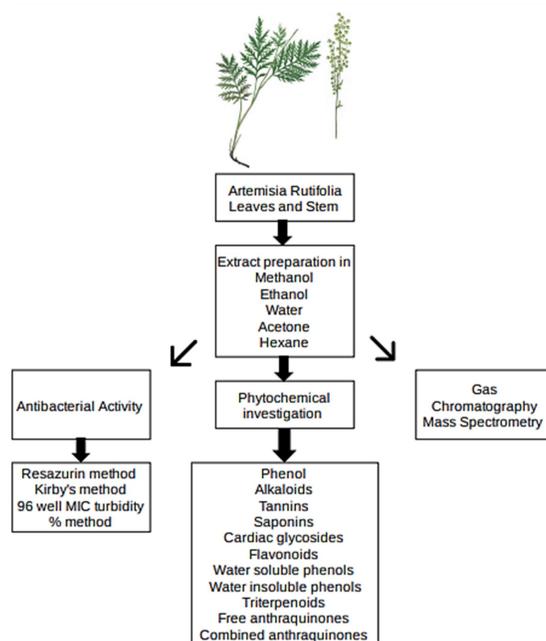


Figure 1: Layout of phytochemical and antibacterial analyses of *Artemisia rutifolia*

MATERIALS AND METHODS

Plant material collection

Artemisia rutifolia was collected from the arid desert mountainous region (Balochistan) of Pakistan. After being identified by the national herbarium it was cleaned, rinsed, and dried at the Antimicrobial Biological Laboratory; AMBL, International Islamic University Islamabad, Pakistan.

Plant Extraction and Filtration

Artemisia rutifolia's stem and leaves were ground in a lab grinder carefully. Methanol, Ethanol, Water, Acetone, and Hexane; MEWAH solvents (polar to non-polar) were used to macerate plant powder at Stockbridge Medicinal and aromatic Lab, University of Massachusetts Amherst, USA. It was performed at room temperature and was put for mixing on a shaking rotator for 48 hours. Post filtration through a Whatmann's No. 41 paper the plant extracts was concentrated via sing rotary evaporator. All the extracts were transferred to glass vials, carefully labelled, and stored at 4°C until further use.

Phytochemical Analysis

Preliminary Qualitative Analysis

Chemical tests were used for identification of Saponin, Phenolic compounds, Water Soluble Phenol, Water Insoluble Phenol, Flavonoids, Polysteroids, Terpenoids, Cardiac Glycosides, Free Anthraquinones, Combined Anthraquinones,

Tannins and Alkaloids presence in all plant extracts as depicted in **Table 1**.

Quantitative Analysis - Phenols

Deionized distilled water 75 μ L (ddH₂O)+ 25 μ L of plant extract/standard +25 μ L Folin C (F-C reagent) was added to each well sequentially. After waiting for 6 minutes 100 μ L of Na₂CO₃ (75 g/L) was added and mixed thoroughly. Later plates were put in dark for 90 minutes. Absorbance readings at 765 nm were taken initially of controls (sample and ddH₂O) in triplicate using spectrophotometric microplate reader. Calibration curve was generated with the readings of Gallic acid as a standard (12.5–400 μ g/mL). Phenols were determined as μ g of gallic acid equivalents / mL which was calculated via formula, $y = 0.6053 x - 0.0567$ (y =absorbance @ 765 nm; x = gallic acid equivalents in μ g/mL) (Andrews, 2001). This experiment was conducted using SPECTRA MAX M2e plate reader (Vesoul & Cock, 2012) [10].

Quantitative Analysis - Flavonoids

100 μ L ddH₂O + 10 μ L of NaNO₂ (50 g/L) + 25 μ L of standard / plant extract sample solution were put in each of 96 wells of the plate followed by incubation for 5 minutes at room temperature. 15 μ L of AlCl₃ (100 g/L) was added to the wells and the plate was again set for incubation for 6 minutes. 50 μ L of NaOH (1 mol/L) + 50 μ L of ddH₂O were poured in all wells

and the plate was set up for shaking incubation for approximately 45 seconds before measuring absorbance @510 nm. Catechin standard (5–500 µg/mL) was used for generation of calibration curve and flavonoids content of plant extract was expressed as Catechin equivalents (ug/ mL) and were calculated by the formula, $y = 0.5377 x + 0.316$ ($y =$ absorbance @ 510 nm; $x =$ Catechin equivalents - µg/mL) [8, 9]. This experiment was conducted using SPECTRA MAX M2e plate reader [10].

Gas Chromatography-Mass Spectrometry (GC/MS) Analysis

GC-MS equipment (Bruker Scion 456 GC, EVOQ triple quadrupole GC-MS/MS) was used to investigate the phytochemicals of plant extracts. Experimental conditions followed in GC-MS system were as follows: Column length = 15m, Column inner diameter = 0.25mm, film thickness = 0.25mm. Helium was used as a carrier gas with flow rate of 1.5mL/min. In the gas chromatography part, temperature

programming 45°C for 3 min, 250°C at 8°C/min for 10 min. Injection volume was 1 µL using varying split ratio (5:1/ 15:1/ 20:1). Range of 45-350 m/z was used to run the plant extracts and the results were recorded. Software MSWS 8, Automated Mass Spectral Deconvolution and Identification System (AMDIS) for GC-MS and NIST library were used for compilation of all results.

Antibacterial activity

Culture Media and Bacterial Inoculum Preparation

Tryptic Soy Broth (TSB) medium (Thermo Fisher Scientific, USA) was used for growth of all bacterial strains separately (**Table 1**). Culture preparation was done by dissolving 30g of medium in 1000 mL ddH₂O. TSB medium was autoclaved (Temp. 121 °C, Pressure 15 *psi*, 15 minutes) and stored at -4 °C until further use. This was done at the Food Sciences department at University of Massachusetts Amherst, USA.

Table 1: catalogue of scientific names, accession number and gram strain of bacterial strains used in antibacterial testing

MICROORGANISM	ACCESSION NUMBER	GRAM STRAIN
<i>Bacillus subtilis</i>	(ATCC_6051)	Gram positive
<i>Escherichia coli</i>	(ATCC_25922)	Gram negative
<i>Klebsiella Oxytoca</i>	(ATCC_43863)	Gram negative
<i>Lactococcus lactis</i>	(ATCC_LMO230)	Gram positive
<i>Listeria monocytogenes</i>	(ATCC_LM21)	Gram positive
<i>Micrococcus luteus</i>	(ATCC_4698)	Gram positive
<i>Salmonella enterica</i>	(ATCC_14028)	Gram negative
<i>Shigella sonnei</i>	(ATCC_25931)	Gram negative
<i>Staphylococcus aureus</i>	(ATCC_25923)	Gram positive

Determination of Minimum Inhibitory Concentrations (MIC) / antibacterial activity

The antibacterial activity against mentioned strains was evaluated via three different methods. It included 96 Well test, Kirby-Bauer Disk Diffusion and Resazurin based Well Plate Microdilution Method [11].

i) 96 Well Plate method

Microtitre tray of 96 wells was used to perform antibacterial assay. Tryptic soy broth growth medium and plant extracts were added 100uL each to each well. Five serial dilution levels were prepared and each plant extract was checked at all these levels for maximum efficacy (1000 µg, 500 µg, 250 µg, 125 µg and 62.5 µg). Bacterial culture (10^5 to 10^6 cfu/ml concentration) in 50µL amount was added to all wells. Plant extracts and culture of bacteria were not added to the Double negative control wells and only TSB medium was added. Sterility of the medium was ensured via double negative control. Single negative control wells lacked only Plant extract so that bacterial growth trends could be observed on average scale. Microtitre plates properly covered to avoid any contamination and incubated for 24 hours. Readings of absorbance of all microtitre plates were observed at 570 nm (Elx 800 plate reader). Following formula was used for calculation

of bacterial inhibition $[(\text{OD in control}-\text{OD in treatment}) / \text{OD in control}] \times 100$.

ii) Kirby-Bauer Disk Diffusion Method

Bacterial inoculum was poured on solidified agar plates. Plant extracts were taken in 20uL amount and paper discs (10mm) were soaked in it. Paper discs were placed on prepared culture plates and were incubated at 26 °C for 24 hours. All the steps were done in the laminar flow to maintain aseptic conditions. Zone of inhibition or no growth area in the vicinity of plant extract disc was measured in millimetres. A negative control plate was maintained with discs soaked in distilled water to allow maximum growth of bacteria [12]. All extracts were tested in triplicates and results were represented as average values of inhibition zone in mm \pm standard deviation.

iii) Resazurin based Well Plate Microdilution Method

Resazurin (7-Hydroxy-3H-phe-noxazin-3-one 10-oxide) solution was prepared by adding of Resazurin powder (121.5 mg) was mixed thoroughly in ddH₂O (18 mL) using vortex mixer for approximately one hour and pH adjustment at 7.4 was achieved via Phosphate buffer saline. Wells were added with TSB liquid medium (100µL) and plant extracts were also added in serial dilution to separate wells [13]. Each well was then incorporated with

individual bacterial inoculum (10^6 CFU/mL). To ensure sterility of the medium TSB medium was separately added to a well labelled as -/- double negative control. TSB media along with bacterial inoculum were added to see normal growth trend of each bacterial strain. This was labelled as – Single negative control. Overnight incubation was given to the trays at room temperature and then resazurin (20 μ L) was added to each well. This was set for 2nd incubation for approximately 4 hours and the spectrophotometer absorbed colour intensity readings at 550-590 nm. This experiment was conducted using SPECTRA MAX M2e plate reader [13].

DPPH Antioxidant assay

Antioxidant activity of plant extract was checked via Bersuder [14] method of DPPH radical scavenging assay was used to determine. DMSO was used to dissolve all solvent extracts of one plant and DPPH ethanol reagent was made separately. Plant-DMSO mix (25 μ L in 200 μ L) was reacted DPPH (ethanol prepared) mix for approximately six hours. Standard calibration curve was generated of ascorbic acid prepared in DMSO at 50–500 μ mol/L concentrations and 517nm absorbance value. This was considered as a negative control and absorbance at 517nm was absorbed for all. This experiment was also

conducted using SPECTRA MAX M2e plate reader [13].

RESULTS

Artemisia rutifolia hosts various phytochemicals that act against abdominal pain, tumorigenesis, respiratory tract inflammations, asthma, fever and other malicious health conditions and antimicrobial action [15, 16].

Preliminary Phytochemical Analysis

Plant extracts were prepared in MEWAH solvents i.e. Methanol, Ethanol, Water, Acetone and Hexane. All of these extracts were investigated for various phytochemicals including Phenol, Alkaloids, Tannins, Saponins, Cardiac glycosides, Flavonoids, Water soluble phenols, Water insoluble phenols, Triterpenoids, Freeand Combined anthraquinones. Methanol extract remained richest in concentrations of all phytochemicals whereas least number of phytochemicals were extracted in hexane extract. Alkaloids and phenols were extracted in almost all of the extracts (Table 2).

Total flavonoid and phenol content

Water, hexane and acetone extract contained highest flavonoid content. Methanol ethanol and acetone extracts contain high extent of phenols. Overall, very high percentage of total flavonoids

was indicated in all extracts of *Artemisia rutifolia* (Figure 2).

Gas Chromatography-Mass Spectrometry (GC/MS) Analysis

Artemisia rutifolia extracts were carried out for GCMS analyses and results of phytochemical profile for each solvent is shown in table. The empirical formulas, molecular weight, area percentages and standard deviation for molecules that are extracted in more than one solvent are also shown in table. Methanolic extract of *Artemisia rutifolia* exhibited 16 phytochemicals (Figure 3, Table 3); ethanolic extracts exhibited 41 phytochemicals (Figure 4, Table 3), acetone extract exhibited 16 phytochemicals as hexane extract 24 phytochemicals were detected (Figure 5, Table 3). No phytochemical was detected in water extract. Approximately 40 minutes running time was practiced for each column of each extract separately.

Determination of Antibacterial activity

Antimicrobial potential of *Artemisia rutifolia* was characterized using Minimum inhibitory concentration-based Kirby's disc diffusion and spectrophotometric techniques viz. 96 well method (mono-solvent) and resazurin absorption (total extract). Various methodologies used explains the dynamics of plants extracts efficacy against pathogenic strains in versatile aspects i.e. using single solvent

and spectrophotometry, single solvent and classical culture plate, total mixture using all solvents and photometric analyses. Antibacterial activity was checked for all bacteria listed in Table 1.

i) Percentage Growth inhibition by 96 Well Method

Ethanol and Methanol extracts of *Artemisia rutifolia* exhibited most antibacterial potential as shown in the Figure 8. *L. lactis* impressively exhibited growth inhibition via ethanol and methanol extracts used in 500ug and 1000ug dilutions respectively. *B. subtilis*, *C. freundii* and *L. monocytogenes* also exhibited notable growth inhibition via both extracts.

ii) Minimum Inhibitory Concentrations (MIC) of bacterial growth by Kirby-Bauer Disk Diffusion Method.

Bacterial growth inhibition was studied via Kirby disc diffusion method. It was performed in triplicates and average mic was recorded along with the standard deviation in three readings and is depicted in table. Chloramphenicol was used as a standard antibiotic and referral inhibitory concentration (mm) is recorded for it. All the extracts of *Artemisia* showed significant antibacterial activity against gram positive and negative bacteria. It was observed that ethanol, acetone and methanol extracts showed best overall antibacterial activity in

regard to the zone of inhibition. Comparatively, water extract remained least effective in inhibiting bacterial growth. Overall bacterial inhibition was promising against *C. freundii*, *K. oxytoca*, *S. sonnei*, *S. enterica* and *B. subtilis* as indicated by ANOVA analysis (Table 4a and Table 4b, Figure 9).

iii) Resazurin based Well Plate Microdilution Method

All plant extracts were dissolved in DMSO and a total extract was prepared for evaluating combined effect against bacterial growth. This experiment was conducted at different serial dilution concentrations i.e. 100, 50, 25, 12.5 and 6.25. Chloramphenicol ($\mu\text{g mL}^{-1}$) was used as a standard antibiotic for reference

growth inhibition in same serial dilution concentrations as total extract. Overall maximum growth inhibition was exhibited by 100% total extract as expected. *L. lactis*, *E. coli* and *K. Oxytoca* exhibited better inhibition through total extract as compared to the standard chloramphenicol (Figure 10).

DPPH antioxidant activity

We used DPPH for inquiring the antioxidant potential of plant extract (Sharopov *et al.*, 2015). The antioxidant potential trend of various extracts of *Artemisia rutifolia* was Acetone > water > hexane > methanol whilst ethanol being least active as antioxidant agent (Figure 11).

Table 2: Qualitative presence catalogue of Phytochemicals of *Artemisia rutifolia* in the plants extract in different solvents

Phytochemical molecules	Water	Methanol	Ethanol	Acetone	Hexane
Phenol	+	+	+	+	-
Alkaloids	-	+	+	+	+
Tannins	-	+	+	+	-
Saponins	+	-	-	-	-
Cardiac glycosides	-	+	+	-	-
Flavonoids	-	+	+	+	-
Water soluble phenols	+	+	-	-	-
Water insoluble phenols	-	+	+	+	-
Triterpenoids	+	-	+	+	+
Free anthraquinones	-	+	-	-	-
Combined anthraquinones	+	-	-	-	-

Table 3: GCMS profiling of Methanol, Ethanol, Water, Acetone and Hexane extracts of *Artemisia rutifolia*

S. No.	Compound	Mol. formula	Amount in percentage	Mol. weight	Retention Time	Solvent
1	Bicyclo [3.1.0] hexan-3-ol, 4-	C ₆ H ₁₀ O	0.708	98.14 g/mol	7.661	Methanol
2	n-Hexadecanoic acid	C ₁₆ H ₃₂ O ₂	7.351	256.4241 g/mol	20.352	Methanol
3	5,8,11-Eicosatriynoic acid,	C ₂₀ H ₂₈ O ₂	3.655	300.4 g/mol	21.678	Methanol
4	9,12,15-Octadecatrienoic aci	C ₁₈ H ₃₀ O ₂	5.275	278.43 g/mol	22.315	Methanol
5	Achillicin	C ₁₇ H ₂₂ O ₅	3.347	306.4 g/mol	22.717	Methanol
6	Retinal	C ₂₀ H ₂₈ O	2.756	284.436 g/mol	23.26	Methanol
7	Phorbol	C ₂₀ H ₂₈ O ₆	5.978	364.44 g/mol	23.476	Methanol
8	1-Heptatriacotanol	C ₃₇ H ₇₆ O	5.521	537 g/mol	23.575	Methanol
9	Bicyclo [3.1.0] hexan-3-ol, 4-	C ₆ H ₁₀ O	6.699	98.14 g/mol	23.713	Methanol
10	Fumaric acid, dimyrtenylest	-	5.897	-	24.152	Methanol
11	Androstan-17-one, 3-ethyl-3-	C ₂₁ H ₃₄ O ₂	3.003	318.5 g/mol	24.401	Methanol
12	Grossmisine	C ₁₅ H ₁₈ O ₄	3.972	262.3 g/mol	24.561	Methanol
13	1H-2,8a-Methanocyclopenta[a]	-	12.13	-	24.821	Methanol
14	3-Buten-2-one, 4-(3-hydroxy-	-	2.563	-	24.908	Methanol
15	Retinal	C ₂₀ H ₂₈ O	1.986	284.436 g/mol	25.341	Methanol
16	Androstan-17-one, 3-ethyl-3-	C ₂₁ H ₃₄ O ₂	2.581	318.5 g/mol	25.554	Methanol
17	Columbin	-	1.127	-	25.61	Methanol
18	Stigmasterol	C ₂₉ H ₄₈ O	3.634	412.69 g/mol	32.086	Methanol
19	Eupatorin	C ₁₈ H ₁₆ O ₇	1.924	344.3 g/mol	32.334	Methanol
20	beta. -Sitosterol	C ₂₉ H ₅₀ O	5.642	414.71 g/mol	32.547	Methanol
21	No Match	-	3.949	-	31.479	Methanol
22	No Match	-	7.708	-	32.426	Methanol
23	No Match	-	2.593	-	33.107	Methanol
1	p-Cymen-7-ol	C ₁₀ H ₁₄ O	0.49	150.2176 g/mol	9.688	Ethanol
2	2-Cyclohexen-1-ol, 2-methyl-	C ₇ H ₁₂ O	1.477	112.170 g/mol	11.398	Ethanol
3	(1S,2S,4S)-Trihydroxy-p-ment	C ₁₀ H ₂₀ O ₃	1.116	188.2640 g/mol	12.817	Ethanol
4	n-Hexadecanoic acid	C ₁₆ H ₃₂ O ₂	6.397	256.4241 g/mol	19.339	Ethanol
5	Hexadecanoic acid, ethyl est	C ₁₈ H ₃₆ O ₂	0.89	284.4772 g/mol	19.716	Ethanol
6	Cycloisolongifolene, 8,9-deh	C ₁₅ H ₂₂	3.252	202.33 g/mol	20.671	Ethanol
7	Phytol	C ₂₀ H ₄₀ O	1.333	128.1705 g/mol	21.001	Ethanol
8	9,12,15-Octadecatrienoic aci	C ₁₈ H ₃₀ O ₂	8.118	278.4 g/mol	21.302	Ethanol
9	Ethyl 9,12-hexadecadienoate	C ₁₈ H ₃₂ O ₂	0.371	280.4 g/mol	21.557	Ethanol
10	Docosahexaenoic acid, 1,2,3-	-	2.772	-	21.638	Ethanol
11	Achillicin	C ₁₇ H ₂₂ O ₅	3.54	306.4 g/mol	21.709	Ethanol

12	n-Propyl 5,8,11,14,17-eicosa	-	1.564	-	22.25	Ethanol
13	Methyl 4,7,10,13,16-docosape	C ₂₃ H ₃₆ O ₂	1.047	344.5 g/mol	22.476	Ethanol
14	beta. -D-Mannofuranoside, fa	-	3.733	-	22.589	Ethanol
15	Bicyclo [3.1.0] hexan-3-ol, 4-	C ₆ H ₁₀ O	4.932	98.14 g/mol	22.686	Ethanol
16	1H-2,8a-Methanocyclopenta[a]	-	0.771	-	22.793	Ethanol
17	Androstan-17-one, 3-ethyl-3-	C ₂₁ H ₃₄ O ₂	3.554	318.5 g/mol	23.037	Ethanol
18	Grossmisine	C ₁₅ H ₁₈ O ₄	3.564	262.3 g/mol	23.548	Ethanol
19	4,8,13-Cyclotetradecatriene-	C ₂₀ H ₃₄ O ₂	4.113	306.5 g/mol	23.758	Ethanol
20	2- [4-methyl-6-(2,6,6-trimeth	C ₂₃ H ₃₂ O	2.418	324.5 g/mol	24.33	Ethanol
21	i-Propyl 5,8,11,14,17-eicosa	-	0.796	-	24.601	Ethanol
22	2- [4-methyl-6-(2,6,6-trimeth	C ₂₃ H ₃₂ O	0.904	324.500 g/mol	25.005	Ethanol
23	Stigmasterol	C ₂₉ H ₄₈ O	3.307	412.69 g/mol	30.85	Ethanol
24	Eupatorin	C ₁₈ H ₁₆ O ₇	1.752	344.3 g/mol	31.064	Ethanol
25	beta. -Sitosterol	C ₂₉ H ₅₀ O	5.437	414.71 g/mol	31.251	Ethanol
26	9,19-Cycloergost-24(28)-en-3	C ₃₂ H ₅₂ O ₂	1.054	468.7541 g/mol	32.083	Ethanol
27	Ethanol, 2-(9-octadecenyloxy	C ₂₀ H ₄₀ O ₂	1.537	312.5 g/mol	32.924	Ethanol
28	No Match	-	2.235	-	11.026	Ethanol
29	No Match	-	1.101	-	23.113	Ethanol
30	No Match	-	0.783	-	23.299	Ethanol
31	No Match	-	0.945	-	23.663	Ethanol
32	No Match	-	1.742	-	23.899	Ethanol
33	No Match	-	3.491	-	30.308	Ethanol
34	No Match	-	6.963	-	31.14	Ethanol
35	No Match	-	2.56	-	31.732	Ethanol
1	Phosphonic acid, (p-hydroxyp	-	11.887	-	4.974	Water
2	Phenol, 2-methoxy-	C ₇ H ₈ O ₂	11.79	124.1372 g/mol	6.597	Water
3	(E)-2,6-Dimethylocta-3,7-die	C ₁₀ H ₁₈ O ₂	7.545	170.25 g/mol	8.097	Water
4	Methyl 12,13-octadecadienoat	-	13.534	-	9.212	Water
5	cis-Verbenol	C ₁₀ H ₁₆ O	12.441	152.24 g/mol	9.906	Water
6	13-Octadecenoic acid, methyl	-	29.388	-	18.714	Water
7	No Match	-	13.416	-	9.852	Water
1	Oxime-, methoxy-phenyl-	C ₈ H ₉ NO ₂	12.314	151.16 g/mol	3.689	Acetone
2	Bicyclo [3.1.0] hexan-3-ol, 4-	C ₆ H ₁₀ O	2.261	98.14 g/mol	5.866	Acetone
3	Benzoic acid, 4-ethoxy-, eth	C ₁₁ H ₁₄ O ₃	6.663	194.23 g/mol	10.392	Acetone
4	No Match	-	2.897	-	5.611	Acetone
1	3-Isopropoxy-1,1,1,7,7-hex	C ₁₈ H ₃₂ O ₇ Si ₇	11.149	577.2 g/mol	15.293	Hexane
2	Diethyl Phthalate	C ₁₂ H ₁₄ O ₄	9.136	222.24 g/mol	16.83	Hexane
3	1,2-Benzenedicarboxylic acid	C ₁₆ H ₂₀ O ₄	3.16	276.33 g/mol	20.451	Hexane
4	Dibutyl phthalate	C ₁₆ H ₂₂ O ₄	2.566	278.34 g/mol	21.613	Hexane
5	Sulfurous acid, octadecyl pe	-	3.233	-	31.103	Hexane
6	No Match	-	2.861	-	7.713	Hexane

7	No Match	-	2.384	-	7.822	Hexane
8	No Match	-	1.595	-	8.339	Hexane
9	No Match	-	4.143	-	12.39	Hexane
10	No Match	-	4.88	-	12.537	Hexane
11	No Match	-	3.908	-	12.676	Hexane
12	No Match	-	2.809	-	12.769	Hexane
13	No Match	-	1.915	-	15.452	Hexane
14	No Match	-	1.62	-	15.856	Hexane
15	No Match	-	4.203	-	16.131	Hexane
16	No Match	-	2.413	-	16.269	Hexane
17	No Match	-	2.463	-	19.052	Hexane
18	No Match	-	6.667	-	19.712	Hexane
19	No Match	-	2.981	-	21.534	Hexane
20	No Match	-	2.252	-	23.206	Hexane
21	No Match	-	1.905	-	32.706	Hexane
22	No Match	-	0.478	-	35.933	Hexane
23	No Match	-	1.009	-	37.034	Hexane
24	No Match	-	0.586	-	38.811	Hexane

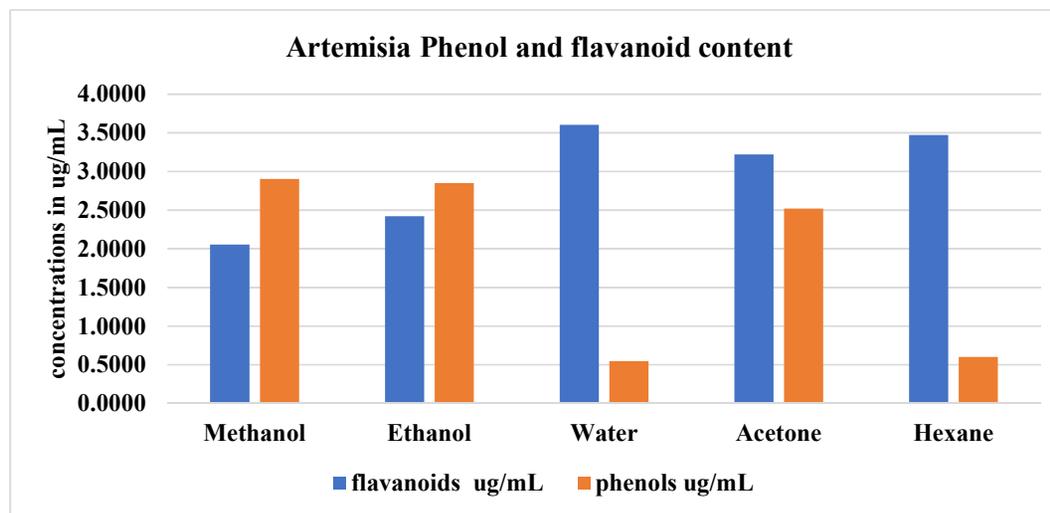


Figure 2: Total flavonoid and phenol estimation in Methanol, ethanol, water, acetone and hexane extracts of *Artemisia rutifolia*

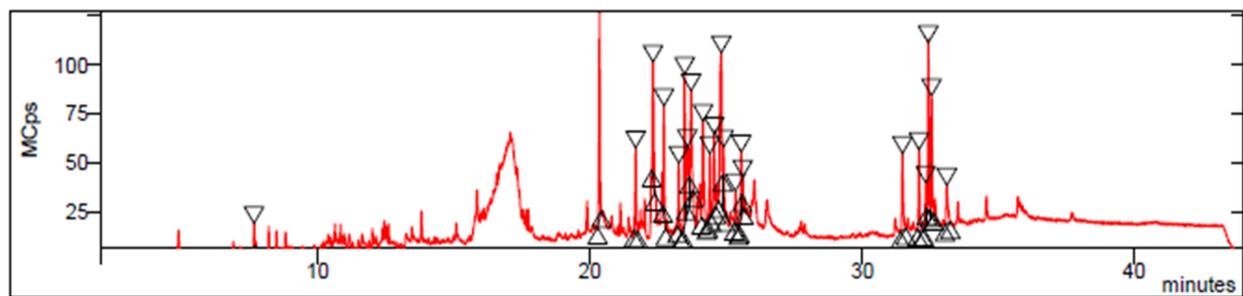


Figure 3: GCMS Spectral Chromatogram of Methanolic Extract of *Artemisia rutifolia*

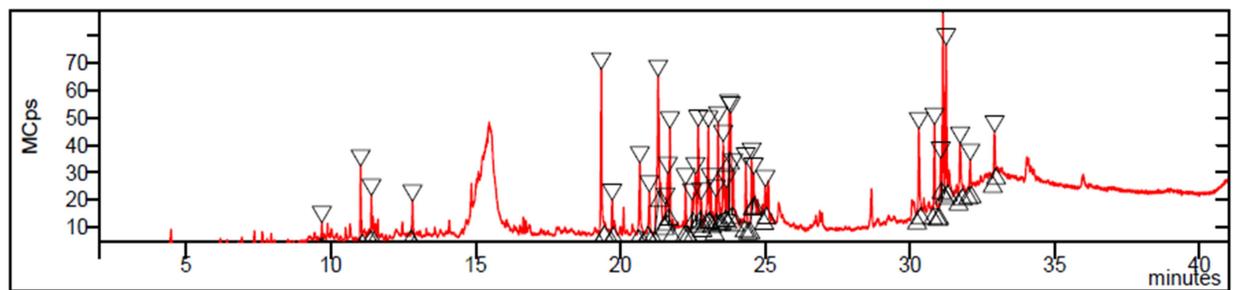


Figure 4: GCMS Spectral Chromatogram of Ethanol Extract of *Artemisia rutifolia*

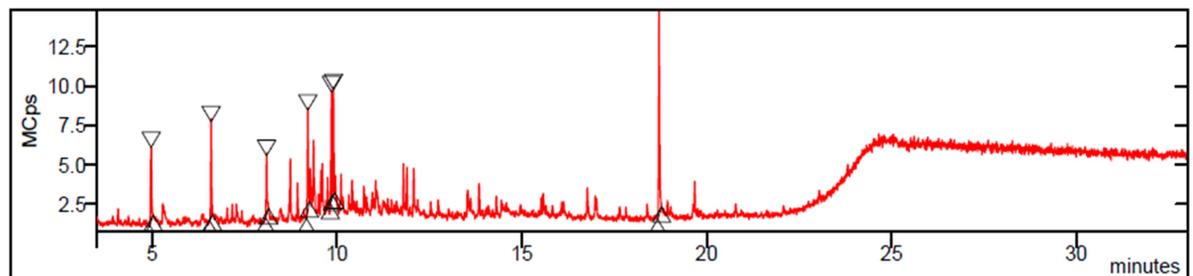


Figure 5: GCMS Spectral Chromatogram of Water Extract of *Artemisia rutifolia*

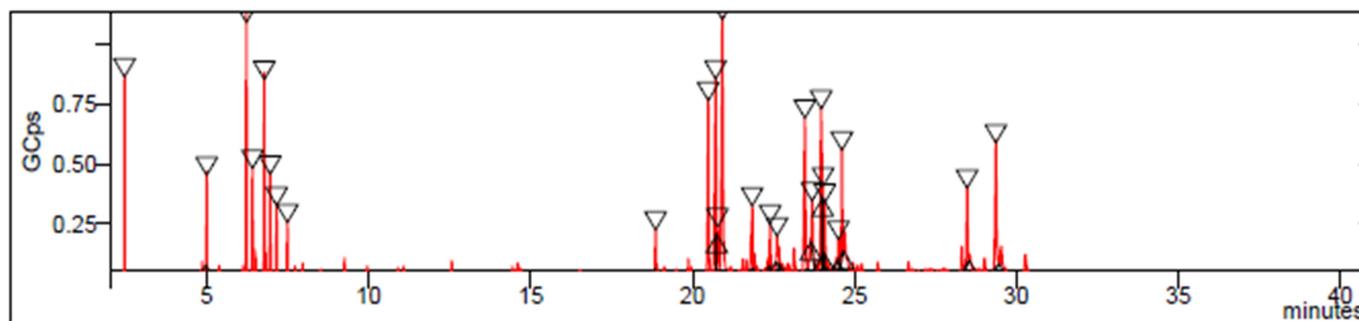


Figure 6: GCMS Spectral Chromatogram of Acetone Extract of *Artemisia rutifolia*

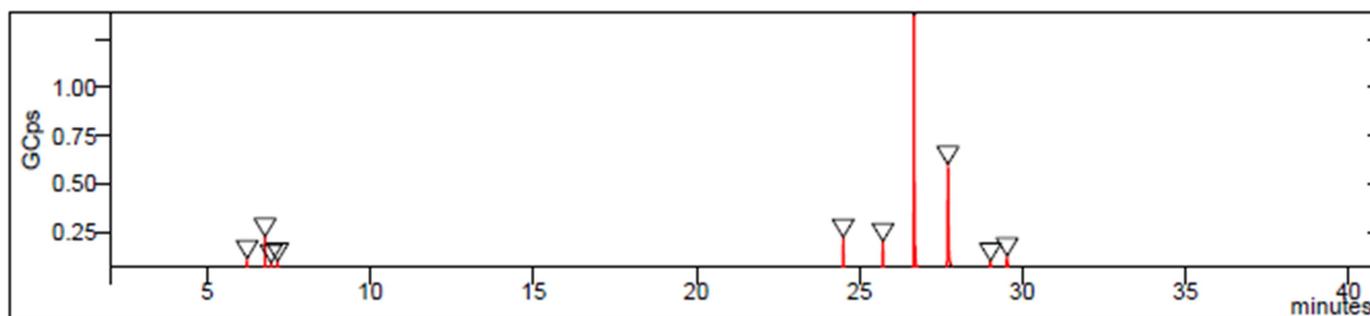


Figure 7: GCMS Spectral Chromatogram of Hexane Extract of *Artemisia rutifolia*

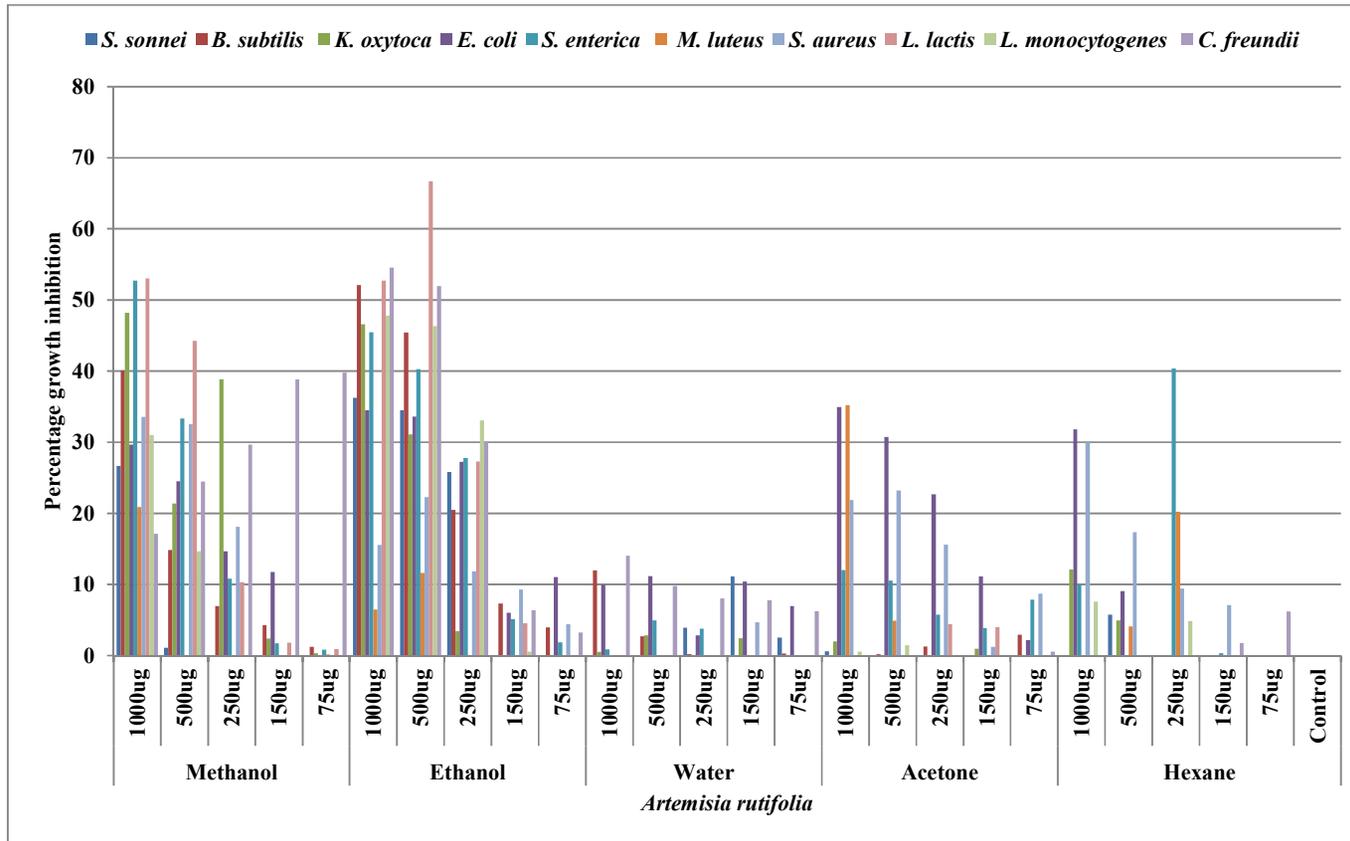


Figure 8: Determination of Minimum Inhibitory Concentrations (MIC) for Gram positive and negative bacterial strains in response to *Artemisia rutifolia* extracts in various solvents (methanol, ethanol, water, acetone and hexane)

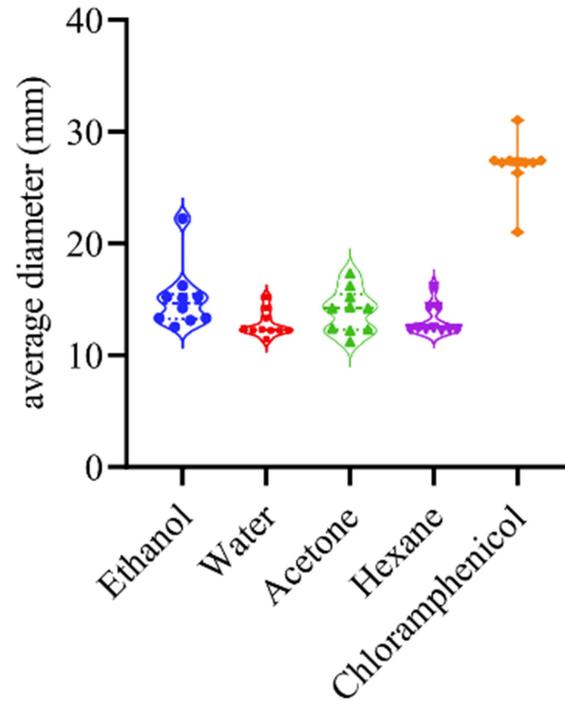
Table 4: (a) Minimum Inhibitory Concentration (MIC) of *Artemisia rutifolia* Against Gram positive and negative bacteria, expressed as average diameter (mm) in the test culture plates. It was recorded in triplicate and mean value \pm standard deviation of diameter has been presented in table. (b) statistically significant difference ($p < 0.001$) detected between the different bacterial strains and their growth termination by plant extracts after tested by ANOVA

Table 4 (a)

Microorganisms	Methanol	Ethanol	Water	Acetone	Hexane	Chloramphenicol
<i>Shigella sonnei</i>	14.1 \pm 0.1	13.3 \pm 0.3	12.2 \pm 0.2	16.2 \pm 0.2	14.3 \pm 0.3	27.4 \pm 0.51
<i>Bacillus subtilis</i>	17.2 \pm 0.2	15.1 \pm 0.1	12.2 \pm 0.2	12.2 \pm 0.2	12.3 \pm 0.3	31.0 \pm 1
<i>Klebsiella oxytoca</i>	13.2 \pm 0.2	15.2 \pm 0.2	15.2 \pm 0.2	14.3 \pm 0.3	16.1 \pm 0.1	27.2 \pm 0.68
<i>Escherichia coli</i>	15.2 \pm 0.2	14.2 \pm 0.2	14.2 \pm 0.2	11.2 \pm 0.2	12.2 \pm 0.2	27.2 \pm 0.68
<i>Salmonella enterica</i>	14.3 \pm 0.3	13.1 \pm 0.2	12.4 \pm 0.3	17.3 \pm 0.3	12.3 \pm 0.3	26.3 \pm 0.64
<i>Micrococcus luteus</i>	11.9 \pm 0.8	16.2 \pm 0.2	12.3 \pm 0.3	14.2 \pm 0.2	14.2 \pm 0.2	27.4 \pm 0.69
<i>Staphylococcus aureus</i>	13.3 \pm 0.3	15.1 \pm 0.1	11.4 \pm 0.5	12.3 \pm 0.3	12.3 \pm 0.3	21.0 \pm 1.25
<i>Lactococcus lactis</i>	13.3 \pm 0.3	13.3 \pm 0.3	12.2 \pm 0.3	14.2 \pm 0.3	12.4 \pm 0.3	27.2 \pm 0.68
<i>Listeria monocytogenes</i>	12.3 \pm 0.3	12.5 \pm 0.4	12.2 \pm 0.2	12.4 \pm 0.4	12.3 \pm 0.4	27.4 \pm 0.75
<i>Citrobacter freundii</i>	12.2 \pm 0.3	22.2 \pm 0.2	13.3 \pm 0.3	15.2 \pm 0.2	12.4 \pm 0.3	27.2 \pm 0.68

Table 4 (b)

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between extracts)	1430	4	357.4	F (2.832, 25.49) = 95.96	P<0.0001
Individual (between bacterial strains)	50.48	9	5.609	F (9, 36) = 1.506	P=0.1832
Residual (random)	134.1	36	3.724		
Total	1614	49			



Minimum Inhibitory Concentration (MIC) of *Artemisia rutifolia* Against Gram positive and negative bacteria

Figure 9: Minimum Inhibitory Concentration (MIC) of *Artemisia rutifolia* Against Gram positive and negative bacteria, expressed as average diameter (mm) in the test culture plates

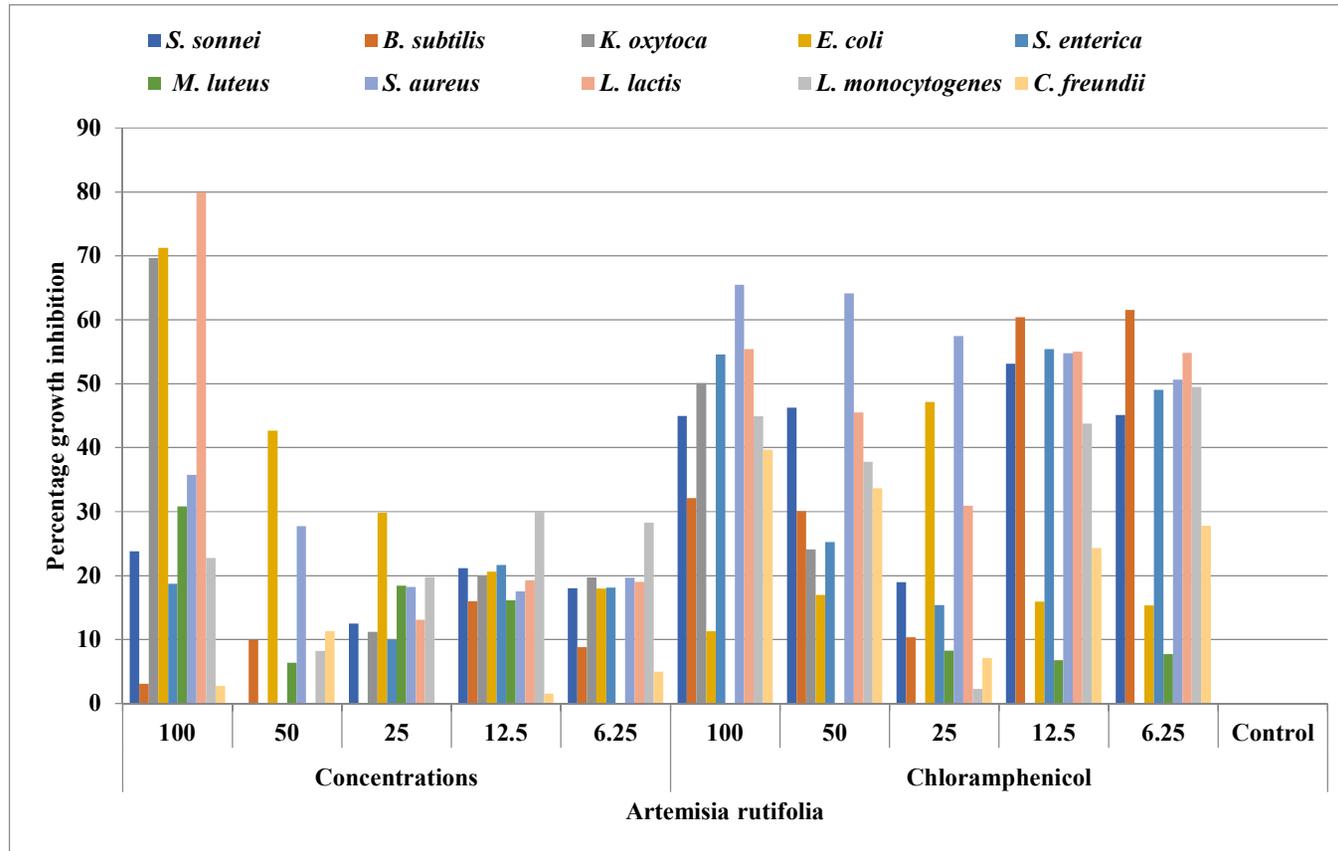


Figure 10: Antimicrobial Efficacy of *Artemisia rutifolia* total Plant Extract dissolved in DPPH Against Bacterial Isolates at different concentrations (100ul/ml, 50ul/ml, 25ul/ml, 12.5ul/ml and 6.25 ul/ml)

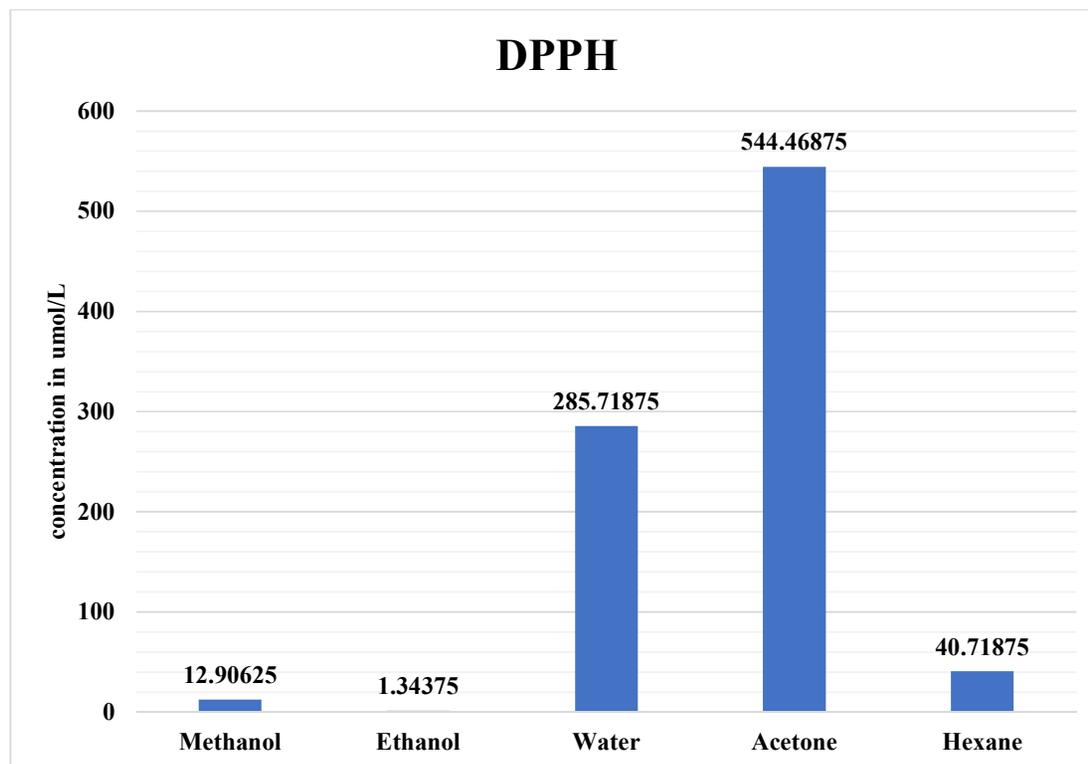


Figure 11: Concentration of DPPH in Plant Extracts of *Artemisia rutifolia* prepared in Different Solvents

DISCUSSION

Artemisia rutifolia extensively present in Pakistan, China, Tajikistan, Russia and India etc. Prominent uses of *Artemisia* genus include bactericidal potential, pain control, analgesics, diuretics, anti-nociceptive activity, asthma and arthritis control, antifungal, anti-inflammatory, and antiviral activities [13, 15]. Several other studies claim its use for treatment of cardiac pain, tooth pain, cardiovascular disorders, and some digestive problems [17, 18]. Essential oils of *Artemisia rutifolia* possess phytochemicals like 1,8-Cineol, camphor, terpenen-4-ol, and 4-isobutylphenol. However mass level spectrophotometric profiling of vital phytochemicals from *Artemisia rutifolia* leaves and stem is still missing [19].

Most phytochemicals were extracted in the methanol extract and all extracts were rich in alkaloid and phenol contents. Other studies have also indicated excellent percentage of alkaloids and phenols in methanol extracts of *Artemisia sp* [3]. Beside methanol extract, ethanol and acetone extracts were also rich in total phenols which directly enhance the antioxidant potential of plant. Our results exhibited that flavonoids were higher in water, hexane and acetone extracts of *Artemisia rutifolia* which is highlighted from other studies too [20].

Total of eighty-nine phytochemicals were detected in all the extracts of *Artemisia rutifolia* except water extract that had none. Impressive percentages of medicinally important molecules were found. Thujone was found in acetone and hexane extracts of *Artemisia rutifolia*. Promising antitumorigenic potential of thujone variants is exhibited in several studies [21].

Human placental choriocarcinoma and malignant glioblastoma have also shown sufficient downregulation because of thujone [22, 23]. Cis-Verbenol was found in the methanol extract and is known for its anti-inflammatory and anti-ischemic properties along with imparting healthier growth effects on neurons. Studies report down-expression of proinflammatory cytokines via cis verbenol [24]. Phorbol was also indicated in GC spectrum of *Artemisia rutifolia* extracts and is potential transcription regulator and acts in cell signalling mechanisms during tumorigenesis [25, 26]. Arglabin was also identified in *Artemisia* extract. It possesses anti-inflammatory and antiatherogenic effects [27]. Evidences of DNA synthesis alteration in leukaemia are reported when tested *in vivo* [28]. Gitoxigenin extracted in ethanol extract possess significant anticancer activity [29]. Terpeneol extracted in ethanol extract possess anti-diarrheal properties [30] Stigmasterol possess the

tumor angiogenesis suppression properties [31]. Nano particle mediated Eupatorin delivery has shown promising results in suppression of tumor growth just like some traditional methods and medicines [32]. Other studies have indicated presence of eminent compounds like gallic acid, caffeic acid, chlorogenic acid, syringic acid, sinapic acid, p-coumaric acid, m-coumaric acid, ferulic acid, vanillic acid, myricetin, and querce [20, 33].

Microorganisms like viruses and bacteria attack plants and get evaded via its defence system. This is done via utilization of secondary metabolites to ensure herbivore, microorganism and other threat evasion or to minimize harm. Plants extracts have therefore potential capacity to ensure antibacterial action and survival of its own [34]. Screening and reports of antimicrobial action of *A. rutifolia* is not much prevalent when compared to other *Artemisia* species. Present study is therefore one of its own kind of broad-spectrum analysis.

Ethanol and Methanol extracts of *Artemisia rutifolia* exhibited most antibacterial potential like other studies [20]. Notable growth inhibition against several bacteria specifically: *L. Lactis*, *B. subtilis*, *C. freundii* and *L. monocytogenes* was observed via 96 well method. Methanol extracts of *Artemisia* species are known for excellent antibacterial action

against *E. coli* and *B. subtilis* [35]. Total plant extract showed maximum efficacy against *L. lactis*, *E. coli* and *K. oxytoca* as compared to the standard chloramphenicol. Possible reason of exemplary antibacterial spectrum lies in rich phenolic content of methanol extract.

We used DPPH for inquiring the antioxidant potential of plant extract [36]. Acetone and water extracts of *Artemisia rutifolia* possessed best antioxidant activities whilst ethanol being least active as antioxidant agent. The GCMS spectrum revealed compounds like Cis verbenol which may contribute to good antioxidant potential [24].

CONCLUSION

Current study identifies potential herbal and pharma-medical features offered by *Artemisia rutifolia* which remains a relatively less studied *Artemisia* specie. Intensive phytochemical profiling revealed best phytochemical extraction in methanol extract. Methanol extract was also rich in total phenols whereas flavonoids were higher in water extract. Approximately ninety phytochemicals were identified via GCMS analysis and gives reason for immaculate antitumorigenic, antibacterial and antioxidant potential. Detailed profiling of *A. rutifolia* extracts will help provide basis for pharmaceutical solutions for cancers and oxidative stress. Moreover, the comparisons of various solvents give

overall idea about extract dynamics and its practical applications in various health fields. Cell culture and Animal studies for disease infections models must be proceeded as future prospect for further validation of anti-microbial and antioxidant potential of *Artemisia rutifolia* extract.

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