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Screening antibacterial efficacy and anticancer studies of *A. vasica* leaf crude extracts for formulation of potential herbal drug

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Abstract
The study was conducted to investigate the medicinal properties and chemical composition of various solvent extracts of *Adhatoda vasica* (leaves). The in vitro antibacterial activity was assessed by Agar well diffusion method and radical scavenging activity was measured by FRAP assay. The anticancer potential of leaf extracts of *A. vasica* on human lung epithelial adenocarcinoma cell line (HCC- 827) were tested by MTT assay. In order to find out the compounds responsible for the medicinal activities, GC-MS analysis was performed. Out of the three extracts subjected to determine the antibacterial activity against the pathogens used in the study, aqueous extract was found to be the best antibacterial agent at the concentration of 0.5mg/100µl. The MIC was tested at various concentrations from 0.5-0.0156 mg/ml for all the plant extracts. MIC/MBC index was also determined and values were less than 4 so all the extracts were bactericidal in nature. The antioxidant powers of all the extracts were assessed and it was concentration dependent. The potency of plant extracts to inhibit the growth of cancerous cell line was recorded in terms of decrease in viable cell count as compared to the control value and it was found to be dose dependent and time dependent. The Phalloidin staining was performed to check the structure of cytoskeleton of control and treated cells. The screening of *A. vasica* leaves extracts results have shown significant antibacterial, antioxidant and anticancer properties due to the presence of therapeutically important constituents which can be used in the development of herbal formulations in treating respiratory diseases.

Keywords: *Adhatoda vasica*, Human lung epithelial adenocarcinoma cell line (HCC-827), MTT, FRAP,

Introduction
Medicinal components from plants play an important role in conventional as well as western medicine. Plants synthesize aromatic substances in the form of secondary metabolites as a part of their evolutionary modification and growth, most of which are phenols or their oxygen-substituted derivatives [1]. Some 12,000 secondary metabolites from various medicinal plants have been isolated and identified contributing to 10% of the total metabolites synthesized by the plants [2]. The importance, necessity and efficacy of medicinal plants in practice of medicine today are well established and cannot be overlooked [3]. Medicinal plant extracts can be used directly or indirectly for the treatment of different diseases. The use of traditional medicine and medicinal plants in most developing countries is widely observed as a basis for the maintenance of good health [4]. Scientists throughout the world are trying to explore the therapeutic values of medicinal plants to help the humans. In the world 30% and more of the pharmaceutical preparations are plant based [5]. From the ancient times it is well documented that the active ingredients from the plant origin have been used to treat various diseases and microbial infections. Medicinal values of plants are due to the presence of bioactive compounds in small quantities, which help maintaining the consistency in the human and animal body functioning [6]. These active principles have provided many effective molecules in search of new drug medicines [7]. A large number of these plants are used in the form of powder, decoction and infusion for the treatment of various diseases including the microbial infection [3].

Traditional medicinal plants are yet to be systematically investigated against various pathogens, which have developed resistance to the present day antibiotics, drugs or any other means of treatment [8]. Plants as antimicrobials should be tested against the specific pathogens to test their therapeutic values. The antimicrobial potential of plant extracts have been explored by a very large number of scientists world over [9, 10]. In spite of the tremendous advancement of medical sciences and technology, the diseases are the leading health problems particularly in underprivileged population in the remote rural areas in the developing countries.

The role of medicinal plants in herbal remedies and in healthcare preparations is well recorded in earliest literature of ayurveda. Nowadays, interest has been renewed towards herbal medicines due to the incapability of synthetic pharmaceutical products to control major diseases [11].

Adhatoda vasica nees (Acanthaceae) commonly known as vasaka distributed throughout India up to an attitude of 1300m the leaves, flowers, fruit, and roots are extensively used for treating cold cough, whooping cough, chronic bronchitis and asthma as sedative, expectorant and antispasmodic [12]. *Adhatoda vasica* has been used as a well-known drug in the unani and ayurvedic medicine [13] and the plant has been used in the indegenous system of medicine in India for more than 2000 years [14]. *A. vasica* is known for its antibacterial, antispamodiac, anti-arthritis, antiseptic, expectorant and antituberculosis properties [15]. The phytochemical studies of the various parts of *Adhatoda vasica* revealed the presence of alkaloids, phytosterols, polyphenolics and glycosides as a major class of compounds. Its principal constituents are quinazoline alkaloids with vasicine as its chief alkaloid. The leaves are rich in Vitamin C and carotene and yield an essential oil. Chemical compounds found in leaves and roots of this plant includes essential oils, fats, resins, sugar, gum, amino acids, proteins and vitamin C etc [16]. In the present work we have selected *A. vasica* as herbal medicine to screen its medicinal properties in reference. This study emphasizes upon the *in vitro* antibacterial activity, antioxidant property, anticancer potential and phytochemical anlaysis leading to the identification of phytoconstituents responsible for medicinal activities.

Materials and Methods

Plant collection: The dried leaves of *Adhatoda vasica* were obtained from local market of Dehradun, India.

Extraction of Plant material: 25g of powder was taken as a thimble charge and organic {ethyl acetate (4.4), methanol (5.1)} and water (9.0) extraction was done in succession using soxhlet extraction method [17]. All the extracts were made solvent free and concentrated using rotary evaporator and preserved at 4°C in airtight bottle until further use.

Chemical and reagents: 2, 4, 6-tripyidyl-s-triazine (TPTZ), Mueller Hinton Agar were purchased from Hi-Media (Mumbai, India), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT, 98%) reagent, Fetal bovine serum (FBS) and streptomycin-penicillin antibiotic solutions were purchased from Sigma Aldrich, South Korea. The chemicals and reagents used for the study are of pure grade. The human lung epithelial adenocarcinoma cell line (HCC- 827) was procured from Korean cell bank, South Korea.

Antimicrobial susceptibly testing

Bacterial strains: Pathogens responsible for causing respiartory diseases in humans i.e., *Klebsiella pneumoniae Staphylococcus aureus, Streptococcus mutans, Streptococcus pyogenes, Streptococcus pneumoniae* were procured from IMTECH Chandigarh (Punjab), for the study.

Agar well diffusion method: The antimicrobial activity of all the extracts (ethyl acetate, methanol, aqueous) of *Ahatoda vasica* leaves was determined by agar well diffusion method [18]. The extracts were dissolved in DMSO (dimethylsulphoxide) to obtain the concentration of 0.5mg/100µl and 1mg/100µl.The antibiotic Gentamicin (0.5mg/100µl, 1mg/ 100µl) was used as positive control and DMSO as negative control. The tests were performed in triplicates and the final results were presented as the mean zone of inhibition and standard deviation were calculated.

Broth Dilution MIC tests: The Minimal Inhibitory Concentration (MIC) of the plant extracts was determined by macro broth dilution assay [19]. On the basis of the results obtained from Agar well diffusion method (ZOI) two-fold serial dilutions of all the extracts were prepared in well plates with Mueller-Hinton Broth (Hi-media, Mumbai, India) as diluents. 20µl of test microorganisms of the standard concentration (5×10^5 cfu/ml) was inoculated in the each dilution. Two-fold serial dilution of DMSO and Gentamicin was used as experimental negative and positive control respectively. The plates were incubated at 37ºC for 24hours. The lowest concentration at which the extract or standard drug showed no visible growth (turbidity) was taken as MIC.

Determination of Minimum Bactericidal Concentration: 20µl of the MIC test broth tube solutions were spread over MHA plates and incubated for 18-24h at 37ºC. The plates showing no single bacterial growth, the dilution was considered as MBC (Minimum Bactericidal Count) concentration of the extract that is bactericidal in nature. The MIC index (MIC/MBC) was performed to determine whether an extract is bactericidal (MIC/MBC <4) in nature. MIC index values of greater than 4 and less than 32 are considered as bacteriostatic [20]. The test was performed in triplicates and its mean MIC and MBC values were calculated. The results were expressed in terms of standard deviation.

Antioxidant Power (Ferric reducing ability of plants): The FRAP assay was performed as described by **[21].** The stock solution of various extracts of 2.5mg/ml concentration was prepared in DMSO. 10µl -100µl of extract was mixed with 1.5ml of FRAP reagent and the volume was adjusted to 5ml with distilled water. The tubes were incubated at 37ºC for 15minutes and absorbance was noted at 593nm.

Anticancer activity: The human lung epithelial adenocarcinoma HCC-827 cell line were cultured and maintained in 90% DMEM media substituted with 10% Foetal Bovine serum (FBS) and 1% antibiotic for 24h (22). The media was then removed and the cell layer was washed with phosphate buffer saline (PBS) (0.1M pH7.0) to remove the traces of media. Later, 500 □l of trypsin-EDTA was added to the culture flask to remove the adherent cell layer from the flask. After 5min, 2ml of the media was added and single cells were collected. The cells were counted on the haemocytometer to get the exact viability and cell count for the experiments. 1×10^5 cells of the human lung epithelial adenocarcinoma-HCC-827 were used for the anticancer study of the extracts in reference.

Cell viability assay: The viability of the cells was assessed by MTT (3, 4, 5-dimethylthiazol-2yl)-2-5-diphenyltetrazolium bromide) assay. 1×10^5 cells of the human lung epithelial adenocarcinoma (HCC-827) cell line were incubated in DMEM (Dulbecco's modified Eagle medium) containing extracts of various concentrations (10µg/ml, 50µg/ml, and 100µg/ml) in 5% CO_2 incubator at 37ºC. The metabolic activity of each concentration was assessed using MTT assay at 570nm after 24h and 48 h.

Image analysis: Phalloidin staining of the control and treated cells was performed to check the changes in the structure of cytoskeleton of the cancerous cells. Cells were fixed in 4% paraformaldheyde for 10 min, and were then permeabilized with 0.1% Triton-X 100 for 5min and after each step thorough rinsing with 0.01M PBS at room temperature (in LAF) was performed. The working solution of FITC labeled Phalloidin stain was made up in 1:200 dilutions with 1% BSA and cells were incubated for 15 min before imaging. The morphology of the cell cytoskeleton was observed under microscope (NIKON, TE – 2000 U) [22].

Phytochemical Analysis of the extracts

Gas Chromatography and Mass Spectroscopy (GC-MS): The GC-MS analysis of ethyl acetate, methanol and aqueous extracts were carried out using REX column. 2µl of samples were introduced *via* an all-glass injector working in the split mode, with helium as the carrier gas. Temperature programme: 70□C -300□C at 6min, with 10min hold at 300□C. The identification of components was accomplished using computer searches in commercial library (Wiley 8 and NIST).

Results

Antibacterial activity: For finding potential new substances of therapeutic uses, it is important to screen medicinal plants for their antibacterial activities and phytochemicals. The pathogens selected for the study play an important role in causing pathogenecity in humans especially various types of respiratory disorders. The *in vitro* antibacterial screening of ethyl acetate, methanol, water extracts of *A .vasica* were performed against *K. pneumoniae*, *S. aureus*, *S. mutans*, *S. pyogenes* and *S. pneumoniae* by the Agar well diffusion method. The antimicrobial activity of all the extracts of *A. vasica* was compared with reference antibiotic Gentamicin at the same concentration. The Genatmicin being chemically synthesized and pure form of aminoglycosides showed strong bactericidal nature against respiratory diseases causing pathogens. In our study aqueous extract of the leaves of *A. vasica* was found to be most active against all the Pathogens responsible for causing respiratory afflictions followed by methanolic and ethyl acetate extract (Table 5.25, 5.27, 5.29).

The bactericidal effects of plant extracts became prominent with the increasing concentration. *S. pneumoniae* (19mm), *S. pyogenes* (22mm) and *S. mutans* (13mm) were effectively inhibited by aqueous extract whereas methanol extract showed moderate inhibition against *S. pyogenes* (19mm) and *K. pneumoniae* (16mm) and significant inhibition against *S. aureus* (24mm) (Table 5.27, 5.29). The data also revealed the specific pathogenecity suppressing behaviour of plant extracts. [23] Grange and Snell, 1996 have studied the antimycobacterial activity of semi-synthetic derivatives of compounds of *A. vasica* against *M. tuberculosis* by conventional method. The antimicrobial activity of ethanol and petroleum ether extracts of *A. vasica* leaf against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillussubtilis*, *Proteus vulgaris* and *Candida albicans* had been established by [24] Karthikeyan, *et al.*, 2009. The results obtained showed the ethanolic extract to be more effective than the petroleum ether extract by exhibiting 19mm ZOI against *Staphylococcus aureus* and could not inhibit *Klebsiella pneumoniae*. In our results the methanolic extract showed strong inhibitory activity against *S. aureus* by exhibiting 24mm ZOI and even 16mm ZOI against *K. pneumoniae* (Table4.26). So data obtained suggested that the methanolic extract is more effective in inhibiting the pathogens in reference and found better than the previously reported studies [24]. We could expect that methanolic (S5) extract of *A. vasica* could provide generic antimicrobial activity for different applications.

The different extracts of *A. vasica* exhibited significant antimicrobial activity against chosen pathogens (Table: 2, 3, 4). *K. pneumoniae* was effectively inhibited by aqueous extract (18mm at 0.5mg/100µl) followed by methanol extract (16mm) and ethyl acetate extract (11mm) respectively. *S. aureus* showed maximum susceptibility against extracts in the order: methanol, aqueous and ethyl acetate extracts. Out of the three extracts subjected to determine the potency of plant against the diseases caused by the pathogens, the aqueous extract responded well on the basis of the data obtained. *S. mutans* was found to be the least susceptible pathogen. The aqueous extract exhibited potential antibacterial activity against *S. pneumoniae* and *S. pyogenes*. On comparing the data it was found that *S. pyogenes* was effectively inhibited by aqueous extract whereas moderately inhibited by methanol and least inhibition against ethyl acetate extract. *K. pneumoniae* and *S. pneumoniae* were highly susceptible against aqueous extract. *S. aureus* was most sensitive against methanol extract moderate sensitive against aqueous extract and least inhibition against ethyl acetate extract. *S. mutans* as effectively inhibited by aqueous extract followed by methanol and ethyl acetate extract. Activities of the various extracts of *A. vasica* were comparable to those of standard antibacterial agent Gentamicin as control (Table 1). The extracts affected the growth of bacteria effectively and significantly but less than Gentamicin (Chemically synthesized).

The MIC values for the ethyl acetate extract against *S. pneumoniae* and *S. pyogenes* was found to be 0.0625mg/ml. Minimal requirement of the extract to inhibit the growth of *K. pneumoniae* was found to be 0.125mg/ml for all the three extracts. *S. aureus* was moderately inhibited by the extracts methanol and aqueous at a concentration of 0.0156mg/ml and at 0.125mg/ml of ethyl acetate extract. The MBC values for all the extracts were determined and found to be higher than the MIC values respectively. The MIC index obtained was 2 for all the extracts and control, showing the significant bactericidal property of *A. vasica* (Table 3).

Table 1: Represents the ZOI of control (Genatamicin) against the pathogenic bacteria.

S. No.	Micro-Organisms	Zone of Inhibition (mm) Concentration of the drug in mg/100µl	
		0.5mg/100µl	1.0mg/100µl
1.	*K. pneumoniae*	28 ± 0.816	29 ± 0.816
2.	*S. aureus*	31 ± 0.816	32 ± 0.816
3.	*S. mutans*	27 ± 0.816	28 ± 0.816
4.	*S. pyogenes*	31 ± 0.816	32 ± 0.816
5.	*S. pneumoniae*	29 ± 0.816	30 ± 0.816

Table 2: Represents the ZOI of ethyl acetate extract against the pathogenic bacteria.

S. No.	Micro-Organisms	Zone of Inhibition (mm) Concentration of the ethyl acetate in mg/100µl	
		0.5mg/100µl	1.0mg/100µl
1.	*K. pneumoniae*	11 ± 0.00	12 ± 0.816
2.	*S. aureus*	16 ± 0.816	20 ± 0.816
3.	*S. mutans*	5 ± 0.00	7 ± 0.816
4.	*S. pyogenes*	14 ± 0.816	18 ± 0.816
5.	*S. pneumoniae*	13 ± 0.816	20 ± 0.816

Table 3: Represents the ZOI of methanol extract against the pathogenic bacteria.

S. No.	Micro-Organisms	Zone of Inhibition (mm) Concentration of the drug in mg/100µl	
		0.5mg/100µl	1.0mg/100µl
1.	*K. pneumoniae*	16 ± 0.816	19 ± 0.816
2.	*S. aureus*	24 ± 1.63	27 ± 0.816
3.	*S. mutans*	9 ± 0.816	17 ± 0.816
4.	*S. pyogenes*	19 ± 1.24	23 ± 0.816
5.	*S. pneumoniae*	18 ± 0.816	22 ± 1.63

Table 4: Represents the ZOI of aqueous extract against the pathogenic bacteria.

S. No.	Micro-Organisms	Zone of Inhibition (mm) Concentration of the drug in mg/100µl	
		0.5mg/100µl	1.0mg/100µl
1.	*K. pneumoniae*	18 ± 0.816	20 ± 0.816
2.	*S. aureus*	19 ± 0.816	23 ± 0.816
3.	*S. mutans*	13 ± 0.816	18 ± 0.816
4.	*S. pyogenes*	22 ± 0.816	24 ± 0.816
5.	*S. pneumoniae*	19 ± 0.816	22 ± 1.63

Table 5: Represents the MIC, MBC and MIC Index values of the control and ethyl acetate extract.

Organism	Range (mg/ml)	MIC (control) (mg/ml)	MBC (control) (mg/ml)	MIC (extract) (mg/ml)	MBC (extract) (mg/ml)	MIC Index (control)	MIC Index (extract)
P1	0.5- 0.0156	0.0156	0.0312	0.125	0.25	2	2
P2	0.5- 0.0156	0.0156	0.0312	0.125	0.25	2	2
P3	0.5- 0.0156	0.0156	0.0312	0.0625	0.125	2	2
P4	0.5- 0.0156	0.0156	0.0312	0.0625	0.125	2	2
P5	0.5- 0.0156	0.0156	0.0312	0.0625	0.125	2	2

Table 6: Represents the MIC, MBC and MIC Index values of the control and methanol extract.

Organism	Range (mg/ml)	MIC (control) (mg/ml)	MBC (control) (mg/ml)	MIC (extract) (mg/ml)	MBC (extract) (mg/ml)	MIC Index (control)	MIC Index (extract)
P1	0.5- 0.0156	0.0156	0.0312	0.125	0.25	2	2
P2	0.5- 0.0156	0.0156	0.0312	0.0156	0.0312	2	2
P3	0.5- 0.0156	0.0156	0.0312	0.125	0.25	2	2
P4	0.5- 0.0156	0.0156	0.0312	0.125	0.25	2	2
P5	0.5- 0.0156	0.0156	0.0312	0.125	0.25	2	2

Table 7: Represents the MIC, MBC and MIC Index values of the control and aqueous extract.

Organism	Range (mg/ml)	MIC (control) (mg/ml)	MBC (control) (mg/ml)	MIC (extract) (mg/ml)	MBC (extract) (mg/ml)	MIC Index (control)	MIC Index (extract)
P1	0.5- 0.0156	0.0156	0.0312	0.125	0.25	2	2
P2	0.5- 0.0156	0.0156	0.0312	0.0156	0.0312	2	2
P3	0.5- 0.0156	0.0156	0.0312	0.0625	0.125	2	2
P4	0.5- 0.0156	0.0156	0.0312	0.0156	0.0312	2	2
P5	0.5- 0.0156	0.0156	0.0312	0.0156	0.0312	2	2

Antioxidant Power: Oxidative stress is the result of overproduction of free radicals, which gets stabilised through electron pairing with biological macromolecules in healthy human cells and cause protein and DNA damage along with lipid peroxidation [25]. The interest has been renewed towards the antioxidants based on natural resources because they exhibit beneficial protective effects, including antibacterial, antiviral, anti-allergic, and antithrombotic and because they are associated with lower occurrence of cardiovascular diseases and certain types of cancer diseases [26]. Radical scavenging activity was estimated by FRAP assay and the results were compared with that of Gallic acid. Gallic acid (Merck) being in the pure form had better antioxidant power than the extracts, which has chemicals either in combined or conjugated form affecting the antioxidant power of the plants. FRAP assay is based upon ferric to ferrous ion reduction at low pH causing a colored ferrous-tripyridyltriazine complex to form.

The results obtained in our study have shown relevance to the earlier reported studies where ethyl acetate, methanol and aqueous extracts exhibited strong antioxidant power to prevent the oxidative stress responsible for causing variety of diseases. At a concentration of 5×10^{-3}g/l, the extracts showed varied antioxidant power. In all cases the appreciation of

FRAP value was noticed with the increase of extract concentration. The comparative analysis of antioxidant power shown by all the extracts of *A. vasica* used in the study falls in the order: S5> S4> S6.

Free radical scavenging properties of the extracts of *A. vasica* were determined by FRAP assay and tabulated in Table 8. The antioxidant power of each of the extract registered a rise with increase in concentration of the extracts. The results were compared to the known antioxidant Gallic acid used as control, exhibited strong antioxidant power than plant extracts. Out of the three extracts subjected, methanol extract was found to be the best free radical scavenger as it exhibited the highest antioxidant power (54.91µM/l) followed by ethyl acetate extract (40.00µM/l) and aqueous extract (25. 00µM/l) at a concentration of 100µl (50×10^{-3}g/l).

Table 8: Represents the antioxidant power of Gallic acid, ethyl acetate extract, methanol extract and aqueous extract.

Concentration of Gallic acid (g/l)	Antioxidant power (Gallic acid) (µM)	Antioxidant power (Ethyl acetate extract) (µM)	Antioxidant power (Methanol extract) (µM)	Antioxidant power (Aqueous extract) (µM)
5 X 10^{-3}	49.08	12.25	6.91	6
10 X 10^{-3}	49.25	16.41	13.5	9.58
15 X 10^{-3}	51.25	20	15.9	10.58
20 X 10^{-3}	58.16	21.08	22.08	12.33
25 X 10^{-3}	60.25	26.33	27.5	15.58
30 X 10^{-3}	72.25	28.75	33.66	16
35 X 10^{-3}	75	30	41.83	16.33
40 X 10^{-3}	79.33	33.66	48	21.41
45 X 10^{-3}	79.75	37.50	51.91	23.58
50 X 10^{-3}	80	40	54.91	25

Anticancer activity: The significant antimicrobial and antioxidant activity of the *A. vasica* various solvent extracts have formed the basis for evaluation of its cytotoxic activity against Human lung epithelial adenocarcinoma cell line (HCC-827). In our study we have explored *A. vasica* as an alternative means of treatment to cure respiratory afflictions caused by the microbial activity of the pathogens used in the study, overproduction of free radicals and leading to severe diseases like cancer. The various solvent extracts of the plant was tested for its antitumor activity on HCC-827 cell line using 3- (4, 5-dimethyl thiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT assay), which is based on the reduction of MTT by the mitochondrial dehydrogenase of viable cells to a purple formazon product. The effect of different concentrations of ethyl acetate, methanol and aqueous extracts on cell viability of cancer cells was assessed by MTT assay. Results clearly showed that different solvent extracts of the plant exhibited effective to moderate inhibition on lung cancer cells. Inhibitions of the growth of carcinoma cells were found to be dose dependent and time dependent. The potency of *A. vasica* extract to inhibit the cancerous growth was recorded in terms of decrease in viable cell count as compared to the control value using MTT assay (Table 9, 10, 11). The data obtained showed that the methanol extract had pronounced cytotoxic effect on the cancerous cells after 24h and 48h of treatment, which increased with the increase in dose of the extract. Methanol extract completely inactivated the metabolic activity of HCC-827 at the concentration of 100µg/ml after 24h and 48h of treatment followed by ethyl acetate extract and aqueous extract.

Table 9: Represents the effect of ethyl acetate extract on HCC-827 cells after 24h and 48h.

S. No.	Concentration (µg/ml)	Control (24h)	Treated (24h)	Control (48h)	Treated (48h)
1.	10	$1.57x10^5$	$0.09 x10^5$	$2.17x10^5$	$0.05 x10^5$
2.	50	$1.57x10^5$	$0.05 x10^5$	$2.17x10^5$	$0.04 x10^5$
3.	100	$1.57x10^5$	$0.03 x10^5$	$2.17x10^5$	$0.01 x10^5$

Table 10: Represents the effect of methanol extract on HCC-827 cells after 24h and 48h.

S. No.	Concentration (µg/ml)	Control (24h)	Treated (24h)	Control (48h)	Treated (48h)
1.	10	$1.57x10^5$	$0.06 x10^5$	$2.17x10^5$	$0.02 x10^5$
2.	50	$1.57x10^5$	$0.03 x10^5$	$2.17x10^5$	$0.03 x10^5$
3.	100	$1.57x10^5$	0.00	$2.17x10^5$	0.00

Table 11: Represents the effect of aqueous extract on HCC-827 cells after 24h and 48h.

S. No.	Concentration (µg/ml)	Control (24h)	Treated (24h)	Control (48h)	Treated (48h)
1.	10	$1.57x10^5$	$0.07 x10^5$	$2.17x10^5$	$0.025x10^5$
2.	50	$1.57x10^5$	$0.06 x10^5$	$2.17x10^5$	$0.07 x10^5$
3.	100	$1.57x10^5$	$0.05 x10^5$	$2.17x10^5$	$0.03 x10^5$

Phytochemical analysis

Gas Chromatography and Mass Spectroscopy

Analysis of various solvent extracts of *A. vasica* detected the individual compounds by GC-MS analysis carried out at the range of 50ºC -300ºC for 75min, showed the number of compounds, ethyl acetate (82), methanol extract (33), aqueous extract(28). Some of the compounds present in all the extracts are Exo-2-(bistrifluoromethylamino-oxy) Norbornane, carbonic acid, N-(3,4,4-trimethyl-1,2-dioxoethane-3-yl-methoxy carbonyl), glycine, hydrazoic acid, Repandin A, hexanamide, Bis [1,2,3-tri(T-butyl)-2-cyclopropen-1-yl] 1,2,4,5-Tetrazine,1,2 ethaneamine,naphthoquinone,2-fluoroformyl-3,3,4,4-tetrafluoro-1,2-oxazetidine,hydroxyl amine), (2-propanone,phosphine,hydroxylamine,vinyl furan,2-pyridine propanoic acid, hydrazine carboxylic acid, 1-chloro ethyl acetate,2-pyridine ethaneamine,2,4,6-

trithiaheptane 2-oxide, erythro-1,2-dimethyl 1-methyl thio-2-hydroxyl ethane, acetic acid, 2,2,3,3-tetramethyl-1-oxa spiro(3,5)non-5-en) in aqueous extracts (Table 5.56). The mentioned compounds may be responsible for the properties exhibited by the plants in reference against the chosen infection. Derivatives of naphthoquinone show numerous biological activities such as antibacterial, antiviral, antitumour, cytotoxic, insect repellent, anti-inflammatory, antipyretic properties. Plants with such constituents are used for the treatment of malignant and parasitic diseases in China and countries of South America [27]. Benzene sulfonamide and its derivatives acts as vasodilator and reduces the risk of heart attack which is caused by various physiological stress and disorders [28]. Glycine is an essential amino acid used as a metabolic product for the growth of bacteria. In excess inhibits the growth of bacteria and used as a non-specific antiseptic agent. Therefore presence of these compounds in the extracts proves the medicinal efficacy of the extracts and its properties in reference.

Table 12: The identified phytochemicals in ethyl acetate extract were detected using GC-MS technique.

Peak No.	Phytochemicals	Molecular formula	Retention time (min)
1	Hydrazine carboxyamide	CH_5N_3O	3.307
4	2-Propanone	C_3H_6O	3.613
10	Sclerosol	C_2H_6OS	7.110
22	Phoshine	CH_5P	7.903
23	Carbamic acid	CH_3NO_2	8.070
25	Methane-D3,Nitro-	CD_3NO_2	8.883
32	2H-Benzopyran-4-Carbonitrile,6-Fluoro-3,4-Dihydro-4-[(Methylthio)Methyl]	$C_{12}H_{12}FNOS$	16.767
34	2-Pyridinepropanoic acid	$C_{11}H_{13}NO_3$	46.097
38	Amino urea	CH_5N_3O	46.413

Table 13: The identified phytochemicals in the methanol extract were detected using GC-MS technique.

Peak no.	Phytochemicals	Molecular formula	Retention time (min)
3	N-(3,4,4-Trimethyl-1,2-Dioxethane-3-yl-MethoxyCarbonyl)Glycine	$C_9H_{15}NO_6$	3.620
6	Silane	$C_8H_{18}C_{12}OSi$	3.933
17	Acetamide	$C_2H_3C_{12}NO$	7.723
25	1H-Pyrimido[4,5,6-IJ][2,7]Naphthyridine-6-Carbonitrile,2-Ethyl-5,8-Dimethoxy-	$C_{14}H_{13}N_5O_2$	50.753
26	Acetonitrile-D3	C_2D_3N	50.860
28	3-Methoxy-5-(Methoxymethoxy)-7-Methyl-6-(3-(Trimethylsilyl)Propargyl)-1,4-Naphthoquinone	$C_{20}H_{24}O_5Si$	51.007
30	L-Alanine, Ethylester-	$C_5H_{11}NO_2$	52.513
32	Formamide, N-[(dibutylamino)methyl]-N-methyl-	$C_{11}H_{24}N_{20}$	53.240
34	Trans-2-((phenylthio)methyl)-1-(2-propenyl)-1,2,3,4-tetrahydronaphthalene	$C_{20}H_{22}S$	53.747
37	2-Acetonyl-3-cyano-2,3-dimethylcyclobutane-1-carboxylic acid	$C_{11}H_{15}NO_3$	54.043
45	5,5'-dicarboxy-3'-(2-chloroethyl)-4-(2-acetoxyethyl)-3,4'-dimethylpyrromethane	$C_{19}H_{23}ClN_2O_6$	56.893

Table 14: The identified phytochemicals in the aqueous extract were detected using GC-MS technique.

Peak no.	Phytochemicals	Molecular formula	Retention time (min)
10	3,3'-[1,2-hydrazindiyl-bis(Carbonyloxymethylene) [Bis(3,4,4-trimethyl-1,2-dioxethane	$C_{14}H_{24}N_2O_8$	3.620
23	D5-Ethylnitrate	$C_2D_5NO_3$	7.740
25	Methane- D3	CD_3NO_2	8.850
20	Bis (Fluoromethyl)(Dimethyl)Silane	$C_4H_{10}F_2Si$	7.343
27	Erythro-1,2-Dimethyl-1-Methylthio-2-Hydroxyethane	$C_5H_{12}OS$	10.080

Discussion

An increased antibiotic resistance has posed a problem worldwide due to the frequent use of antibiotics. The therapeutic values of plants have always been used in healing various diseases because of the wide safety profile they provide. The purpose of testing plants for its antimicrobial activity is because the plants have been used in various treatments. In recent years, there has been so much increase in the occurrence of infections due to the development of antibiotic resistance, which has become an ever-increasing therapeutic problem (Austin, D.J. *et al*., 1999) [29].

Various medicinal plants have been in use since many years for the treatment of diseases in humans and animals. Some plants have the ability to treat diseases like cancer without any adverse side effects and their products are used in more than 50% of clinical preparations. (Rosangkima, G. and Prasad, S. B., 2004) [30]. Plants have many bioactive compounds, which exhibits strong antioxidant activities. The antioxidants scavenge free radicals, protect the cell from highly reactive species and cure cancer. Many substances present in the human diet naturally act as potential chemo preventive agents so vegetables and fruits can provide prevention against cancer. (www.cancervax.com/info/index.htm; Vecchia, C. L. and Tavani A., 1998) [31].

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