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An evaluation of the Antimicrobial activity of leaf extracts of Momordica Charantia against human pathogens

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ABSTRACT
Momordica Charantia (Cucurbitaceae) is commonly known as bitter gourd. The fruit has claimed to contain charantin, steroidal saponin, momordicosides, carbohydrate, mineral matters, ascorbic acid, alkaloids, glucoside, etc. The plant used as stomachic, carminative, tonic, antipyretic, antidiabetic, in rheumatoid arthritis and gout. The present investigation was carried out to isolate, purify and characterize Charantin leaves of Momordica Charantia Linn. The isolated charantia leaves was further characterized with the help of Thin Layer Chromatography, Fourier Transform Infra Red Spectroscopy confirmed the identification. The antimicrobial activity of charantia was tested by using Agar Diffusion method. The present studies confirm better antimicrobial activity of Charantia when compared with standard, against microbes.

KEYWORDS: Momordica Charantia, Antimicrobial, Staphylococcus aureus, Streptococcus pyogenes, Well diffusion, FTIR, herbal medicines.

I. INTRODUCTION

Medicinal plants are the nature's gift to human being to make disease free healthy life. In India, thousands of species are known to have medicinal values and the use of different parts of several medicinal plants to cure specific ailments has been in vogue since ancient times [1]. Momordica charantia is a medicinal plant belonging to the family cucurbitaceae is known as bitter melon, bitter gourd, balsam pear, karela, and pare [2,3]. It found in tropical and subtropical regions of the world and widely used as herbal medicine. It is a slender climbing annual vine with long-stalked leaves and yellow, solitary male and female flowers borne in the leaf ails. The Latin name Momordica means "to bite" referring to the jagged edges of the leaves, which appear as if they have bitten[4]. Momordica charantia is known to contain compounds such as momorchanins, momordolol, charantin, charine, cryptoxanthin, cucurbitns, cucuritanes, cycloartenols, diosgenin, elaeostearic acids, erythrodiol, galacturonic acid, goyaglycosides, goyasaponins, and multiflorenol[5,6] .The fruit and leaves contain alkaloids, glycoside, saponin like substances, rennin an aromatic volatile oil mucilage[7,8]. Bitter gourd shows a significant antimicrobial activity and is of great use in medicine for treatment of many diseases such as piles, leprosy, jaundice, diabetes and snake bite[9,10]. Its fruits and leaves have been shown to exhibit various biological activities including anti-diabetic, anti-rheumatic, anti-ulcer, anti-inflammatory and anti-tumor[11,12]. There are evidences that explain that increased uptake of fruits and vegetables reduce the risk of cancer [13,14]. This is attributed by antioxidants presents in fruits and vegetables[15-17].An antimicrobial is a compound that kills or inhibits the growth of microbes. The present study was carried out to evaluate the antimicrobial efficacy of the dry leaves extracts and to evaluate the antibacterial activity of Momordica charantia extracts .

II. MATERIALS AND METHODS

Collection of Plant Materials

Bitter Gourd Leaves were collected from Sengappadai, Madurai. The leaves were removed and washed thoroughly with tap water followed by sterile distilled water. The leaves were completely shadow dried and ground into fine powder. These powders were stored in air tight containers in refrigerator at 4°C until use.

Preparation of Plant samples

Aqueous and methanol are used as solvents for extraction of the plant materials. The air dried and fine powdered leaves were extracted with water and methanol using soxhlet extraction apparatus according to soxhlet method where materials are extracted by repeated percolation which lasts about 6-8 hours under reflux in a specialized glassware. The aqueous and methanolic extract obtained was used for study.

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Collection of Microorganisms
The following Microorganisms were obtained from The Department of Microbiology, The Madura College, Madurai.

Gram-positive Bacteria:

- Staphylococcus aureus
- Streptococcus pyogenes.

The mother cultures were maintained in nutrient agar plates and kept at 4ºC in refrigerator. The bacterial strains were further identified and characterized by the following biochemical tests.

Microscopic Observation of Bacteria
For the Morphological identification, the microscopic observation has been done for the 4 test samples collected

Gram staining technique
Gram staining, a differential staining technique, separates bacteria into two groups, Gram-positive and Gram-negative.

Motility
Most bacterial microscopic preparations result in death of the microorganisms as a result of heat-fixing and staining. Simple wet mounts and the hanging drop technique allow observation of living cells to determine motility.

Biochemical Identification of Bacteria
Followed by the morphological identification, the biochemical tests were done for the identification of bacteria

IMVIC TESTS
Indole production test
The tryptone broth was sterilized in test tubes. The indole produced during the reaction was detected by adding 0.5 ml Kovac's reagent after 48 hrs of incubation at 37°C and allowed to stand for 10 to 15 min. Formation of cherry red reagent layer indicates the production of indole from the microbial catabolism of tryptophan

Methyl-Red and Voges-Proskauer test
MRVP tests were performed using MR-VP broth simultaneously and separately. This test enabled to detect the formation of acidic (Organic acids - formic, acetic, lactic, succinic acid etc.) and neutral product acetoin (acetyl methyl carbinol) as end product during glucose fermentation by bacteria. The bacterial cultures were inoculated into 5 ml sterilized broth in respective labeled test tubes and incubated at 35ºC for 48 hrs. For M.R. test, a few drops of methyl red solution was added to the culture broths. A red colour indicated positive reaction and the pH has been reduced to 4.5 or less due to the accumulation of acidic products of glucose fermentation.

For V.P. test, 0.5 ml of 5% alcoholic α-naphthol solution and 0.5 ml of 40% KOH solution was added to another set of the culture broths. Appearance of pink or erosion red colour in 5-15 minutes indicates positive reaction due to the oxidation of acetyl methyl carbinol to diacetyl by the reagent (Baritt's method), thereby producing red colour with guanidine residue in the broth. A yellow colour in broth the test indicated negative reaction when compared to the uninoculated broth (control).

Citrate utilization test
It is performed to check the ability to bacteria to utilize citrate as the sole carbon source which depends on the presence of citrase, an enzyme produced by the organism, which breaks down the citrate to oxaloacetic acid and acetic acid, which are converted to pyruvic acid and carbon dioxide enzymatically. The bacterial cultures were inoculated into respective labeled simmon's citrate agar slants and incubated at 35°C for 48 hrs, where sodium citrate is the only source of carbon and energy. The CO_2 generated during the break down of citric acid combines with sodium and water forming sodium carbonate, an alkaline product, which changes the colour of the indicator from green to blue which indicates positive test. Bromothymol blue, the indicator is green when acidic (pH- 6.8 and below) and blue when alkaline (pH - 7.6 and above).

Starch hydrolysis test
This test is used to identify bacteria that can hydrolyze starch (amylose and amylopectin) using the enzymes a-amylase and oligo-1,6-glucosidase. These enzymes break the starch molecules into smaller glucose subunits which can then enter directly into the glycolytic pathway. In order to interpret the results of the starch hydrolysis test, iodine must be added to the agar. The iodine reacts with the starch to form a dark brown color. Thus, hydrolysis of the starch will create a clear zone around the bacterial growth. Starch agar plates were prepared in aseptic conditions and the test organisms were inoculated on to the respective labeled plates with a sterile transfer loop. An

uninoculated plate was kept as control and the plates were incubated at 35°C for 48 hrs. After incubation the plates were flooded with Gram's iodine and the Plates were observed for clear zone around the test organisms in each plate.

Urease production test

Microorganisms with the ability to produce hydrolytic enzyme urease, which breaks down urea with the liberation of ammonia. The urease test was performed by inoculating the bacterial isolates into urease agar plates and was observed after incubation as above. The colour change from yellow to pink indicated a positive result due to the accumulation of ammonia which raises the pH of the medium.

Carbohydrate fermentation test (Glucose)

It tests an organism's ability to ferment the sugar glucose as well as its ability to convert the end product of glycolysis, pyruvic acid into gaseous byproducts. This is a test commonly used when trying to identify Gram-negative enteric bacteria, all of which are glucose fermenters but only some of which produce gas. Fermentation medium was prepared with specific carbohydrate - glucose and the medium was sterilized using autoclave. Glucose fermentation broth tubes were inoculated with test organisms and one uninoculated tube was kept as comparative control. All the tubes were incubated for 24 hrs at 37°C in incubator and after incubation the tubes were observed for the change in color (due to production of acid) or change in color and appearance of bubbles (due to production of acid and gas).

Gelatin hydrolysis test

The gelatin hydrolysis test is used to determine the ability of a microbe to produce gelatinase, an enzyme which hydrolyses the protein gelatin as shown below. If the gelatin is liquid, gelatinase is present and if gelatin is solid, no gelatinase is present (i.e., the control is solid). Gelatin medium was prepared in test tubes at aseptic conditions and the bacterial cultures were stab inoculated using inoculating needle and labeled appropriately. The tubes were incubated at 37ºC for 4 to 7 days. After incubation, the tubes were placed in refrigerator at 4°C for 15 minutes. The tubes were observed for liquification.

Catalase test

The enzyme catalase present in some microorganisms breaks down hydrogen peroxide into water and oxygen as shown below which helps them in their survival.The bacterial isolates were inoculated into nutrient broth in the test tubes. After proper growth, catalase production was determined by introducing 3-4 drops of H2O2 (20%) onto a drop of cultures which were placed on the centre of a clean glass slides. The cultures were observed for the appearance or absence of gas bubbles.

Oxidase test

Oxidase enzyme plays a vital role in the operation of the electron transport system during aerobic respiration. Cytochrome oxidase catalyses the oxidation of reduced cytochrome by molecular oxygen, resulting in the fermentation of H2O or H_2O_2. The cultures were grown on nutrient agar slopes for 24-48 hrs at optimum temperature. A loop-full of each culture were rubbed on the moistened oxidase discs using a sterile loop. The color of the smear was checked exactly 15-30 seconds after rubbing the cells on the reagent moistened discs. Appearance of deep blue colour indicated positive reaction while no colour change was a negative reaction.

Coagulase test

Coagulase is an enzyme that clots blood plasma. This test is performed on Gram positive, catalase positive species to identify the coagulase positive Staphylococcus aureus. Coagulase is a virulence factor of S. aureus. The slide was labeled with the name of the organism to be inoculated. Aseptically, added the 0.5 ml of a 1:4 dilution of citrated human plasma and 0.1 ml of test culture on to the slide. The bacterial plasma suspension was examined for clot formation at 5 minutes and 20 minutes.

Blood haemolysis test

Blood Agar Plate tests the ability of an organism to produce hemolysins, enzymes that damage / lyse red blood cells (erythrocytes). The degree of hemolysis by these hemolysins are helpful in differentiating members of the genera Staphylococcus, Streptococcus and Enterococcus. β-hemolysis is complete hemolysis. Partial hemolysis is termed α-hemolysis. If no hemolysis occurs, this is termed γ-hemolysis. The blood agar plates were prepared in aseptic conditions and inoculate the test organisms with quadrant streak technique on to the blood agar plates. An uninoculated plate was kept as control. Then the plates were incubated at 37ºC for 24 hrs and after

incubation, the plates were observed for the zone of haemolysis around the growth of the test organism.

Triple Sugar Iron agar test

It tests for organisms' ability to ferment glucose and lactose to acid and acid plus gas end products. It also helps in identification of sulfur reducers. Triple sugar iron agar slants were prepared in aseptic conditions and using aseptic loop, inoculate each experimental organisms into its appropriately labeled tubes by means of a stab-and-streak inoculation. An uninoculated tube was kept as a control. The tubes were incubated in ambient air for 18 to 24 hours at 37°C. After Incubation, the tubes were examined for the color of both the butt and slant. The tubes were also examined for the presence or absence of blackening within the medium.

PHYTOCHEMICAL SCREENING

Small quantity of aqueous and methanolic extracts of Momordica charantia was dissolved and used for detection of phytochemicals such as glycosides, phytosterols, proteins, alkaloids, flavonoids, tannins, saponins, fats & fixed oils, gums and mucilages.

Test for glycosides

The extract was hydrolyzed with HCL for few hours on hot water bath and the hydrolysate was subjected to Fehling's, Benedict's, Barfoed's tests and the result was recorded.

Test for alkaloids

Presence of alkaloids was tested with four reagents: Mayer's reagent (potassium mercuric iodide solution), Dragendorff's reagent (potassium bismuth iodide solution), Hager's reagent (saturated solution of picric acid), and Wagner's reagent (iodine and potassium iodide solution).

Test for phytosterols

Lieberman-Burchard test and Salkowski test was performed to identify the presence of phytosterols. The residue was dissolved in few drops of acetic acid and three drops of acetic anhydride was added followed by few drops of concentrated sulfuric acid. Bluish green colour was formed shows the presence of phytosterol.

Test of saponins

Foam test was conducted by diluting the extract with 20 ml of distilled and agitated in graduated cylinder 0.1cm layer of foam was formed and the result was recorded.

Test for phenolic compounds

A small quantity of extract was taken in water and FeCl3 test was performed to identify the presence of phenolic compound.

Antibacterial assay of Crude leaf extracts
Kirby-Bauer disc diffusion method

Briefly, 100 µl of the crude extracts obtained from Bitter gourd were loaded onto the sterile Whatmann No. 1 filter paper discs (5 mm) and allowed to absorb well and then it was dried till the solvents get evaporated. Nutrient agar plates were prepared in aseptic conditions and 24 hrs old cultures from nutrient broth were swabbed onto the respective labeled petriplates using sterile cotton swabs. The discs were then transferred using sterile forceps to the inoculated plates and left for incubation for 24 hrs at 37ºC. 100 µl of the pure chloroform and ethanol were used as negative control and the standard antibiotics mentioned below were used as positive control for comparison. After incubation, all the plates were observed for the zone of inhibition and the diameter of the zones were measured.

Selection of Solvent system for Thin Layer Chromatography

Glass slides (8 x 2.5 cm) were cleaned with ethanol and dried in an oven at 50ºC for 15 minutes. Slurry of silica gel was prepared and spread over the glass slides. The glass slides were then dried at 100ºC in the hot air oven for 2 – 3 hours before use. 100 µl of the different crude extracts were loaded onto the silica gel coated glass slides and developed in the solvent mixtures Chloroform : Methanol (9 : 1), n-Butanol : Acetic acid : Distilled Water (4 : 1 : 5). After the separation of the compounds the slides were observed under UV light. Among these 2 different solvent system used, Chloroform : Methanol (9 : 1) was found to be the most suitable for the separation of the bioactive constituents in both the plant samples. These solvents were further used for the Preparative TLC.

Preparative TLC

Glass slides of 16.5 x 14 cm were washed with distilled water followed by ethanol rinsed and dried. The slurry of silica gel in distilled water (4 g in 20 ml) was prepared and poured into the applicator. The coated glass slides were activated at

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80ºC for 3 hours. After cooling to room temperature, the plates were stored in slide chamber until further study (Shahverdi, 2007).The Plant extracts were loaded on the TLC slides just 2 cm from the bottom of the slides using capillary tubes. The slides were left for 30 minutes at room temperature for allowing the solvent system mentioned earlier. After the solvent front reached 5 – 11 cm height, the slides were removed and the separated bands were observed under visible light and marked. After recording the Rf values, the separated bands were scrapped off and redissolved in the respective solvents and then filtered through Whatman No. 1 filter paper using sintered glass funnel. The bioactive compounds were characterized using an FTIR spectrophotometer (Make: Schimadzu, Japan, Model: IR-Affinity 1) in order to identify the nature of the compounds. The TLC separated fractions were also used for the antibacterial assays

Antibacterial assay of TLC fractions of Plant extracts

The TLC fractions from three extracts such as BEE, PCE, PEE were selected for the antibacterial assay, based on the antibacterial potential of the crude extracts on the selected microorganisms. The disc diffusion method of Kirby-Bauer (Ukaegbu-Obi et al., 2018) was employed for determining the antibacterial activity of the TLC separated active fractions. 100 µl of the filtered TLC fractions obtained from the Thin Layer Chromatography of Carica papaya (PCE, PEE) and Musa paradisiaca L. (BEE) were loaded onto the sterile Whatmann No. 1 filter paper discs (5 mm) and allowed to absorb well and then it was dried till the solvents get evaporated. Nutrient agar plates were prepared in aseptic conditions and 24 hrs old cultures prepared in nutrient broth were swabbed onto the respective labeled petriplates using sterile cotton swabs. The discs were then transferred using sterile forceps to the inoculated plates and left for incubation for 24 hrs at 37ºC. 100 µl of the pure chloroform and ethanol were used as negative control. After incubation, all the plates were observed for the zone of inhibition and the diameter of the zones were measured and expressed in centimeters (cm).

III.RESULTS

Sample collection

Leaves from bitter gourd plant were collected from Sengappadai, Madurai. The leaves were removed and washed thoroughly with tap water followed by sterile distilled water. The leaves were completely shadow dried and ground into fine powder. These powders were stored in air tight containers in refrigerator at 4ºC until use.

Fig 1-Leaves of Bitter gourd

Collection of Microorganisms
The following Microorganisms were obtained from The Department of Microbiology, The Madura College Madurai.

Gram-positive Bacteria:
- Staphylococcus aureus
- Streptococcus pyogenes

Fig 2 -Collection of Microorganisms

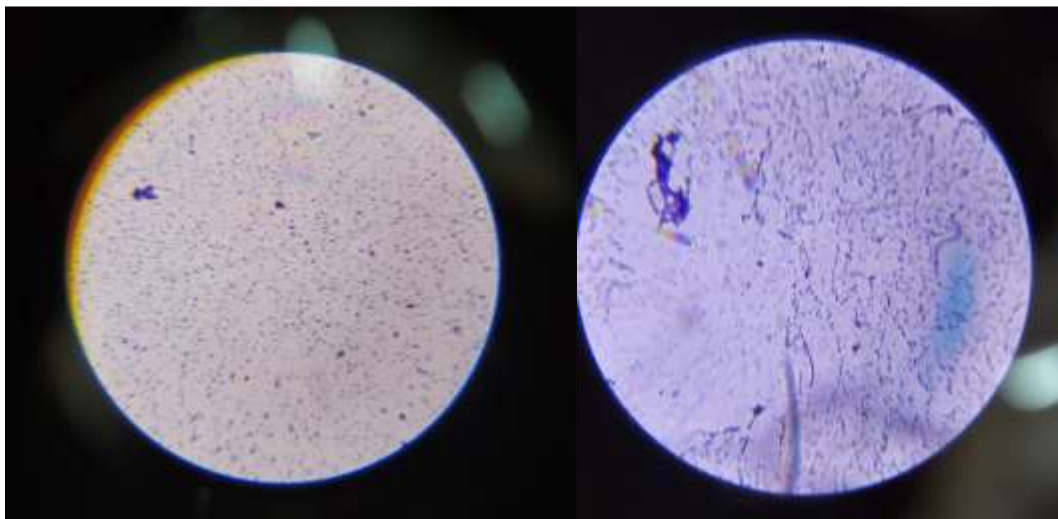

Fig 3- Microscopy of Staphylococcus aureus **Fig 4- Streptococcus pyogenes**

Table 1- Morphological and Biochemical Identification of Bacterial pathogens

Characteristics & Tests	Staphylococcus aureus	Streptococcus pyogenes
Gram Staining	+	+
Shape	Cocci	Cocci

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Motility	Non-Motile	Non-Motile
Capsule	Non-Capsulated	Capsulated
Spore	Non-Sporing	Non-Sporing
Flagella	-	Swimming or Swarming
Indole	-	-
Methyl Red	+	+
Vogues Proskauer	+	-
Citrate Utilization	+	+
Starch Hydrolysis	+	+
Urease Production	+	+
Carbohyrate fermentation (Glucose)	+	+
Gelatin Hydrolysis	+	+
Catalase	+	-
Oxidase	-	+
Coagulase	+	NA
CAMP	+	NA
Blood Haemolysis	β – Haemolysis	NA
Triple Sugar Iron Agar (TSIA)	Acid + H_2S -	Acid + ,H_2S - , Gas +

Fig 5 -Anti-Bacterial Activity of Crude Extract (Methanol) of Bitter Gourd Leaves Against Staphylococcus aureus

Fig 6 -Anti-Bacterial Activity of Crude Extract (Methanol) of Bitter Gourd Leaves Against Streptococcus pyogenes

Separation of Bioactive fraction by TLC and its Antibacterial efficacy

In Thin Layer Chromatography (TLC), solvent system involving Chloroform: Methanol (C : M) in the ratio 9:1 was found to be efficient for the separation of active principles present in the crude extracts The Retention factor (Rf) were noted and tabulated .

Fig 7 - TLC separation of bioactive fractions from crude extracts

Spectral characterization of bioactive components by Fourier-Transform Infrared Spectroscopy (FTIR)

In the present investigation, the FTIR analysis of crude sample of bitter guard leaf ,revealed the presence of functions groups such as N – H, O – H, C – H, C = C, C - O, C – F which are associated with the existence of strong primary amines, alcohols, phenols, alkyenes, alkenes, carboxylic acids, ammonium ions, alcohol, ether, alkyl halide, vinyl compounds in the crude leaf sample. From the FTIR analysis of bioactive fraction, it could be concluded that the antibacterial effect of bitter gourd leaf might be due to the presence of alkanes and alcohols.

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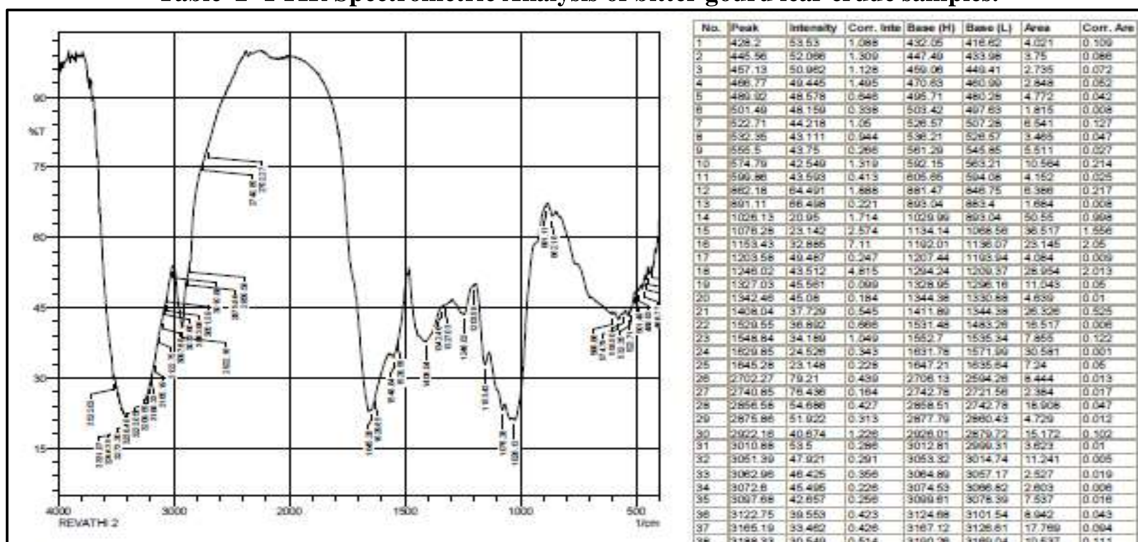
Table 2- FTIR Spectrometric Analysis of bitter gourd leaf-crude samples.



No.	Peak	Intensity	Corr. Inte	Base (H)	Base (L)	Area	Corr. Are
1	428.2	53.53	1.088	432.05	416.62	4.021	0.109
2	445.56	52.086	1.309	447.49	433.98	3.75	0.088
3	457.13	50.982	1.128	459.06	449.41	2.735	0.072
4	468.77	49.445	1.495	470.63	460.99	2.848	0.052
5	489.32	48.578	0.848	495.71	480.28	4.772	0.042
6	501.49	48.159	0.338	503.42	497.63	1.815	0.008
7	522.71	44.218	1.05	526.57	507.28	6.541	0.127
8	532.36	43.111	0.944	538.21	528.57	3.465	0.047
9	555.5	43.75	0.288	561.29	545.85	5.511	0.027
10	574.79	42.549	1.319	592.15	563.21	10.584	0.214
11	599.88	43.593	0.413	605.66	594.08	4.152	0.025
12	862.18	64.491	1.888	881.47	848.75	6.388	0.217
13	891.11	66.498	0.221	893.04	883.4	1.684	0.008
14	1026.13	20.95	1.714	1029.99	893.04	50.55	0.998
15	1076.28	23.142	2.574	1134.14	1068.56	36.517	1.556
16	1153.43	32.885	7.11	1192.01	1136.07	23.145	2.05
17	1203.58	49.487	0.247	1207.44	1193.94	4.084	0.009
18	1246.02	43.512	4.815	1294.24	1209.37	28.954	2.013
19	1327.03	45.561	0.099	1328.95	1296.16	11.043	0.05
20	1342.46	45.08	0.184	1344.38	1330.88	4.639	0.01
21	1408.04	37.729	0.545	1411.89	1344.38	26.326	0.525
22	1529.55	36.892	0.666	1531.48	1483.26	16.517	0.006
23	1548.84	34.189	1.049	1552.7	1535.34	7.855	0.122
24	1629.85	24.526	0.343	1631.78	1571.99	30.581	0.001
25	1645.28	23.148	0.228	1647.21	1635.64	7.24	0.06
26	2702.27	79.21	0.439	2706.13	2594.26	8.444	0.013
27	2740.85	76.436	0.164	2742.78	2721.56	2.384	0.017
28	2856.58	54.686	0.427	2858.51	2742.78	16.908	0.047
29	2875.86	51.922	0.313	2877.79	2860.43	4.729	0.012
30	2922.16	40.674	1.226	2926.01	2879.72	15.172	0.102
31	3010.88	53.5	0.286	3012.81	2999.31	3.623	0.01
32	3051.39	47.921	0.291	3053.32	3014.74	11.241	0.005
33	3062.96	46.425	0.356	3064.89	3057.17	2.527	0.019
34	3072.6	45.495	0.226	3074.53	3066.82	2.603	0.006
35	3097.68	42.657	0.256	3099.61	3076.39	7.537	0.016
36	3122.75	39.553	0.423	3124.68	3101.54	8.942	0.043
37	3165.19	33.462	0.426	3167.12	3126.61	17.769	0.094
38	3188.33	30.549	0.514	3190.26	3169.04	10.537	0.111

Table 3-FTIR Spectrometric Analysis of Organic bitter gourd leaf-crude sample.

S. No	Peak (cm^{-1})	%T	Type of bond	Functional group
1	3188.33	30.549	C-H stretch	Aromatics
2	574.79	42.549	C-Br stretch	Alkyl hydes
3	1327.03	45.561	C-N stretch	Aromatic amines
4	3051.39	47.921	=C-H stretch	Alkenes
5	3209.55	28.407	-c	Alkynes(terminal)
6	2922.16	40.674	C-H stretch	Alkanes
7	1529.55	36.892	N-O asymmetric stretch	Nitro compounds
8	3165.19	33.462	C-H stretch	Aromatics
9	2856.58	54.686	=C-H stretch	Alkenes
10	1153.43	32.885	C-O stretch	Alcohols,carboxylic acids,esters,ethers
11	1408.04	37.729	C-C stretch(in-ring)	Aromatics
12	1629.85	24.526	N-H bend	1 amines
13	1076.28	23.142	C-N stretch	Aliphatic amines
14	1026.13	20.95	C-N stretch	Aliphatic amines

IV. DISCUSSION

The increased rate of resistance to the prevailing antibiotics has led to the search for newer pharmacologically active agents, which are more effective, affordable and readily available from natural sources, such as local medicinal plants and plant products. This has led to the discovery of many clinically useful therapeutic agents that play a vital role in the treatment of human diseases. The natural products are of important concern for human health and welfare, as they are endowed with bioactive compounds which are economically beneficial, safe and had promising antimicrobials effect in the treatment of infectious diseases, while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials. The medicinal plants are used worldwide for several human ailments including bacterial infections. The present work was

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designed to assess the in-vitro antibacterial efficacy of medicinal plants such as Bitter gourd against selected microbes.

V.CONCLUSION

Extracts of Momordica charantia demonstrated antimicrobial activity on tested microorganisms .Leaf extracts showed good antimicrobial activity. Further studies are recommended that will involve various parts of the plant, select different fractions of extracts and purify the active antimicrobial components.

VI.ACKNOWLEDGEMENT

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