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In-Vitroscreening ofHibiscus cannabinus leaves and flowerextracts for antibacterial activity against pathogenic organisms

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Research Highlights

- Hibiscus cannabinus leaves and flower extracts showes antibacterial activity on pathogenic micro-organism Shigella sp
- Shigella sp can cause Shigellosis (bacillary dysentry) , in which Hibiscus cannbinus may cure Shigellosis.

ABSTRACT
In-vitro screening of aqueous and ethanol extracts of Hibiscus cannabinus leaves and flowers for their antibacterial activity against Shigella sp,Salmonellasp, Staphylococcus aureus and Pseudomonas aeruginosa was determined by measuring zone of inhibition using disc diffusion method at different concentration range. Both extracts showed different sensitivity levels for the tested enteric pathogens and the inhibition zones ranged between 12.52±1.50 to 6.22±0.55. Among two tested extracts, ethanol extract is more susceptible to tested gram negative bacteria while compared to aqueous extract and among two extracts used aqueous extract at concentration 120000µg/10ml find to be more potent compared to ethanol exact however, not on par with that of standard employed. Among four tested organisms like Salmonella sp, Shigella sp, Staphylococcus aureus and Pseudomonas aeruginosa, Shigella spshowed more resistance. The extracts of the Hibiscus cannabinus leaves and flowers are proved to have potential antibacterial activity. Antibacterial activity was evaluated by using disc and agar diffusion methods. [Mukherjee S-2014 ,Kiran CN-2015, Asif ICM-2012]

Keywords: Hibiscus cannabinus leaves extracts, Flower extract, antibacterial activity, Shigella sp, Salmonella sp, Staphylococcus aureus and Pseudomonas aeruginosa.

I. INTRODUCTION

The use of an ethnomedicinal plants in folk medicine as a source for relief from illness can be traced back over five millennia to a written documents of the early civilization in China, India and the Near east. Among the estimated 250,000-500,000 plant species, only a small percentage has been investigated phytochemically and pharmacologically and these plants are still widely used in the form of extracts, syrups, kadha (concentrated extract), powder, mixture, tablets and asavas (fermented extracts) and that have drawn the attention from around the world.

Kenaf or Hibiscus cannabinus L. (Family-Malvaceae) is a tall annual woody herb with minute prickles on the stems and leaf stalks, native of Central Africa and indigenous to Camerron, India, Ethiopia, Zimbabwe, Mozambique, Uganda and Nigeria. Kenaf has been cultivated for over many years as an important garden crop with reddish purple, cream or scarlet throat colored flowers and as a fibre crop or as industrial crop especially in Malaysia.[Gabriel A-2005, Shivali-2010, Mohd HAB-2014]. Kenaf will grow to a height of five to six meters and produces three to five times as much fibre as southern yellow pine and rapid growth rate of Kenaf helps to alleviate global warming by absorbing CO2 gases[Morufat OB-2008] and Saba et al., reported that based on Malaysian prospective Kenaf herb will be a potential bioenergy production source in futures.[Saba N-2015] The plant is also known for its numerous synonyms viz., ambadi in Marathi or Ambashtha in Sanskrit (India)[Mukherjee S-2010].

Hibiscus cannabinus were traditionally prescribed by traditional healers and physicians to treat fever, blood and throat disorders, bruises, bilious conditions, dysentery, aches, bruises and puerperium and also Kenaf was reported to be an antidote, aphrodisiac, aperitif and anodyne, fattening, purgative, and stomachic.[N.Mahadevan-2010, Gabriel.AA] and fiber of Kenaf was used for

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the production of pulp and in paper manufacturing industries from dates back to the very time[Ahmad AM-2010, Babita G-2014]. Chemically, Kenaf contains several active phytoconstituents namely polyphenols, tannins, steroids, alkaloids, saponins, lignans such as boehmenan K, boehmenan H, threo-carolignan K and threocarolignan H, essential oils such as (E)-phytol (28.16%), (Z)-phytol (8.02%), N-nonanal (5.70%), benzene acetaldehyde (4.39%), (E)-2-hexanal (3.10%) and 5-methyl furfural (3.00%), ethyl alcohol, isobutyl alcohol, limonene, phellandrenee[Anushabala-2014, Mozaina K-2001]and glucosides such as cannabiscitrin, cannabiscetin and anthocyanin glycoside cannabinidin[Julius E-2005, Pradeep Kamboj-2010]. Kenaf seed has higher level of unsaturated fatty acid and high protein quality and it recently received attention in livestock industry as feed ingredient due to its nutritional profile [K.D.Olawepo-2014]. To one side from the presence of different chemical components a tall woody herb Hibiscus cannabinus also possess a wide spectrum of pharmacological activities viz., antioxidant [Kuvalekar AA-2010], haematinic [Chellapan KD-2012], antihyperlipidemic,[Shivali-2010, W.A. Fawole-2014] hepatoprotective activity[Julius EO-2005], anti diabetic, anti-ulcer, cytotoxic and immunomodulatory activities.[Babita G-2014]

At this very day; development of resistance to antimicrobial agents is very much common in a wide variety of pathogens and in a diverse organisms which were direct or indirect cause of a serious public health problem and leading cause of premature death. Shigellosis is an example of such infectious disease and a serious public health problem in developing countries caused by pathogen Shigella sp.Shigella is Gram-negative bacteria predominantly found in intestinal lumen and an enteric pathogen believes to be resistance to most antibiotics. It passes through your stomach and then multiplies in your small intestines. They then spread into spread into your large intestines (also known as colon), causing cramping in that part of your body, along with diarrhea.[Yogesh M-2007, Anna M. Witkowska-2013]. The search for a broad spectrum antimicrobial agent of both natural and synthetic origin is still on and highly focused area by researcher around the globe. In view of above observation, we thought it will be worthwhile to carry out in-vitro study on antibacterial activity ofleaves and flower extracts of Hibiscus cannabinus againstShigella sp.

II. MATERIALS AND METHODS

2.1 Collection of samples

The leaves and flowers were initially separated from the main plants body and rinsed with distilled water and shade dried and then homogenized into fine powder and stored in air tight bottles.

2.2 Plant Material and Solvent Extraction

The herb Hibiscus cannabinus L or Kenafwas collected. Any type of adulteration was strictly avoided during collection, after collection and during storage until next use. The leaves were washed thoroughly under running water, to remove any mud, clay and adhering particles and leaves were rinsed in distilled water, drained and air dried under shades until there is no differences in the weight. Dried leaves were then grounded into coarse powder form by using a grinder and stored in a well closed air tight container until further used.

A total of 10g of leaves air dried powder was weighed and was placed in 100ml of organic solvents (methanol and ethanol) in a conical flask and then kept in a rotary shaker at 190-220 rpm for 24 hours and then it was filtered with the help of muslin cloth and centrifuged at 10000 rpm for 5 minutes. The supernatant was collected and the solvent was evaporated by solvent distillation apparatus to make the final volume of one-fourth of the original volume, giving a concentration of 40 mg/ml. It was stored at 40˚C in air tight bottles for further studies. [Saba N-2015, Gabriel AA-2005]

Two types of extracts (distilled water and ethanol) were used to screen for their antibacterial activity. Extraction was carried out for each solvent (750 mL) by adaptation of versatile cold maceration method. Maceration was done by stirring the coarse powder for seven days at room temperature. The soaking waste residues were filtered off by using muslin cloth to obtain the crude extract filtrates. The collected filtrates were then evaporated in a water bath at 50ºC to the solid form of crude extracts.[Kiran CN-2015, Gunjan M-2012]

2.3 Bacterial stain and growth condition

Bacteria culture of Shigella sp, Salmonella sp, Staphylococcus aureus and Pseudomonas aeruginosa obtained from the Department of Medical Lab Technology. Stock culture were maintained aseptically under optimal conditions for Shigella sp, Salmonella sp, Staphylococcus aureus and Pseudomonas aeruginosa and subculturedonto Muller hinton agar

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plates followed by incubation at 37ºC for 24 hours. The type of bacteria present on each agar plate was confirmed through gram staining.[Mohan JSS-2007, Salma NM-2010]

2.4 Antibacterial Assay

2.4.1 TestmicroorganismforAntibacterial Assay

For the in vitro antibacterial assay the following human bacterial pathogens were studied such as Salmonella sp, Shigella sp, Staphylococcus aureus and Pseudomonas aeruginosa.

2.4.2 Culturepreparationfor Antibacterial Assay

The cultures were grown on nutrient agar at 37˚C for 18 hours and the colonies were suspended in saline (0.85% Nacl) and its turbidity was adjusted to 0.5 Mac Farland standards (108 CFU/ml). This saline culture preparation was used to inoculate the plates[Rashmi P-2014]

2.4.3 Disc diffusion Method

In the agar disc diffusion method the test compounds, i.e. the flower and leaves aqueous and organic extract were introduced into a disc 0.5 mm (hi-media) and then allowed to dry. Thus the disc was completely saturated with the test compound at concentration of 40 mg/mL. Then these discs were placed directly on the surface of Muller Hinton agar plates, swabbed with the test organism and the plates were incubated at 37 ℃ for 24 h.

2.4.4 Agar well diffusion method

Muller Hinton agar plates were prepared and wells of 5 mm were cut and swabbed with different cultures. The cut wells were then filled with 50 µL of both aqueous and solvent extracts of flowers and leaves separately and the plates were kept for incubation at 37 °C for 24 hours.

2.5 Minimum Inhibitory Concentration

The initial concentration of test solution to conduct minimum inhibitory concentration (MIC) was calculated in g/L by using initial amount of leaves powder macerated in solvent followed by calculating the concentration to obtain the extract per 10 ml. Concentration = Weight of coarse powder (g) / Volume of solvent (L) = 90g / 0.75L = 120g/L = 120g/1000ml = 0.12g/ml For 10ml = 1.2g/10ml

MIC values were determined for distilled water and ethanol extracts to evaluate the anti Shigellasp potentiality by measuring the zone of inhibition using a disc diffusion method on direct inoculated plates. The extracts were tested over a range of concentrations from 120000 µg ml to 12µg ml-1 against 24 hours broth culture of Shigella sp. A grade discs with 6 mm diameter, soaked in each concentrations of extracts were placed on the plates of Muller Hinton agar followed by incubation at 37ºC for 24 hours. Plates were observed after 24 hours and inhibition zones were measured[Kiran CN-2015, Yogesh M-2007, Huda JM-2011] . The experiments were replicated three times with duplicate samples per replicate and data was collected and analyzed. There is no MIC values were determined for Salmonella sp, Staphylococcus aureus and Pseudomonas aeruginosa.

2.5.1 Minimum Bacterial Concentration

The Minimum Bacterial Concentration was defined as the lowest concentration of antimicrobial agent or the extract, which inhibits or killed a particular microorganism. To determine MBC, samples were swiped by using a cotton swab from plates with no visible growth or clear zone in MIC assay and subcultured on freshly prepared Mueller Hinton agar plate followed by incubation at appropriate temperature for 24 hours. Plates were observed after 24 hours for any visible growth and as defined formerly, the MBC was taken as the concentration of the extract that did not shown any growth on new set of agar plates.[Selvamohan T-2012, Nilugal CK-2014, Komathi P]

2.5.2 Antimicrobial Susceptibility Test

The Antibacterial Susceptibility Test was carried out by measuring zone of inhibition using disc diffusion method against standard drug (ciprofloxacin) used and control discs (discs soaked in distilled water and ethanol) which were used for comparison to detect the drug resistance in study pathogen andto assure susceptibility to drug of choice. [Kiran CN-2015, Gunjan M-2012]

2.5.3 Procedure

Mueller Hinton Agar mediumwas prepared by taking 50ml of distilled water and 1.9g of Mueller Hinton agar in a conical flask. Sterilize the medium in autoclave at 121˚C for 20 minutes. Then the medium was poured into petriplatesaseptically and allow it to solidify.Inoculate the medium with a culture of test micro-organismsand cut wells by using micro tips which has a capacity of 20µl for pouring plant extract. Incubate it for 37˚C for 24 hours.

III. RESULT AND DISCUSSION

3.1 Confirmatory Test for Shigella sp

Gram staining: Gram stain was done to identify the morphology of Shigella sp and to

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determine the bacteria is of gram positive or gram negative. The microscopic examination showed the organism as gram negative. It appeared in red and rod shaped. (Figure-1and 2)

3.2 Biochemical test

Biochemical tests are done for the confirmation of tested micro-organisms. Biochemical tests include Indole, MR, VP, Citrate, TSI, Urease, Oxidase and Catalase.(Table – 1)

In-vitro antibacterial activity of aqueous and ethanol extracts of Hibiscus cannabinus L. leaves and flowers(Figure 3 and 4) at concentration range between 120000 µg/10ml to 12 µg/10ml were determined by measuring zone of inhibition. Both extracts showed different sensitivity levels for the tested enteric pathogen Shigella sp and the inhibition zones ranged between 12.67±1.52 to 6.67±1.15 (aqueous extract, in mm) and 12.33±2.08 to 6.33±0.58 (ethanol extract, in mm) (Table- 2) however, not on par with that of standard employed. Among two tested extracts, ethanol extract is more susceptible to tested gram negative bacteria while compared to aqueous extract and among two extracts used aqueous extract at concentration find to be more potent compared to ethanol exact . It can be inferred from the results that leaves and flower extracts of Hibiscus cannabinus L. has potential antibacterial property against the pathogen which is believe to be resistance to most antibiotic Shigella sp. At this very point we can conclude that the type of solvent used to extract appeared to have impact on their activity. However, the extracts used were in crude form and it is well documented and known fact through the literatures that crude extracts might contain wide varieties of active phytoconstituents or secondary metabolites viz., tannins and quinine which are responsible for plant pigment, terpenoids which give plants their odours and flavour, alkaloids, glycosides, saponins, flavonoids, lectin, to some extent aromatic substances, most of which are phenols or their oxygen substituted derivatives which are known to be synthesized by plants in response tomicrobial infection or against predation of insects and herbivores or to serve as plant defense mechanisms.Salmonella sp, Staphylococcus aureus and Pseudomonas aeruginosa didn't show any antibacterial activity on these leaves and flo Hibiscus cannabinus[Kiran CN-2015, Morufat OB- 2008]

IV. CONCLUSION

It is probable to conclude that the observed antibacterial activity could be attributed to the presence of various chemical constituents and secondary metabolites of tall woody herb Hibiscus cannabinus and we believe that the preliminary results of this study appear to indicate that the purified forms of isolated compounds from these two extracts would show more potential antibacterial activity against Shigella sp and other enteric pathogens.(Figure-5 and 6)

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ANNEXURE

(Table – 1)

S.No	Test micro-organism	Indole	MR test	VP test	Citrate test	TSI (Gas/H2S)	Urease test	Oxidase test	Catalase test
1	Shigella sp	+	+	-	-	+/-	-	-	+
2	Salmonella sp	-	+	-	+	+/+	-	-	+
3	Staphylococcus aureus	-	+	+	+	-/-	+	-	+
4	Pseudomonas aeruginosa	-	-	-	+	+/-	-	+	+

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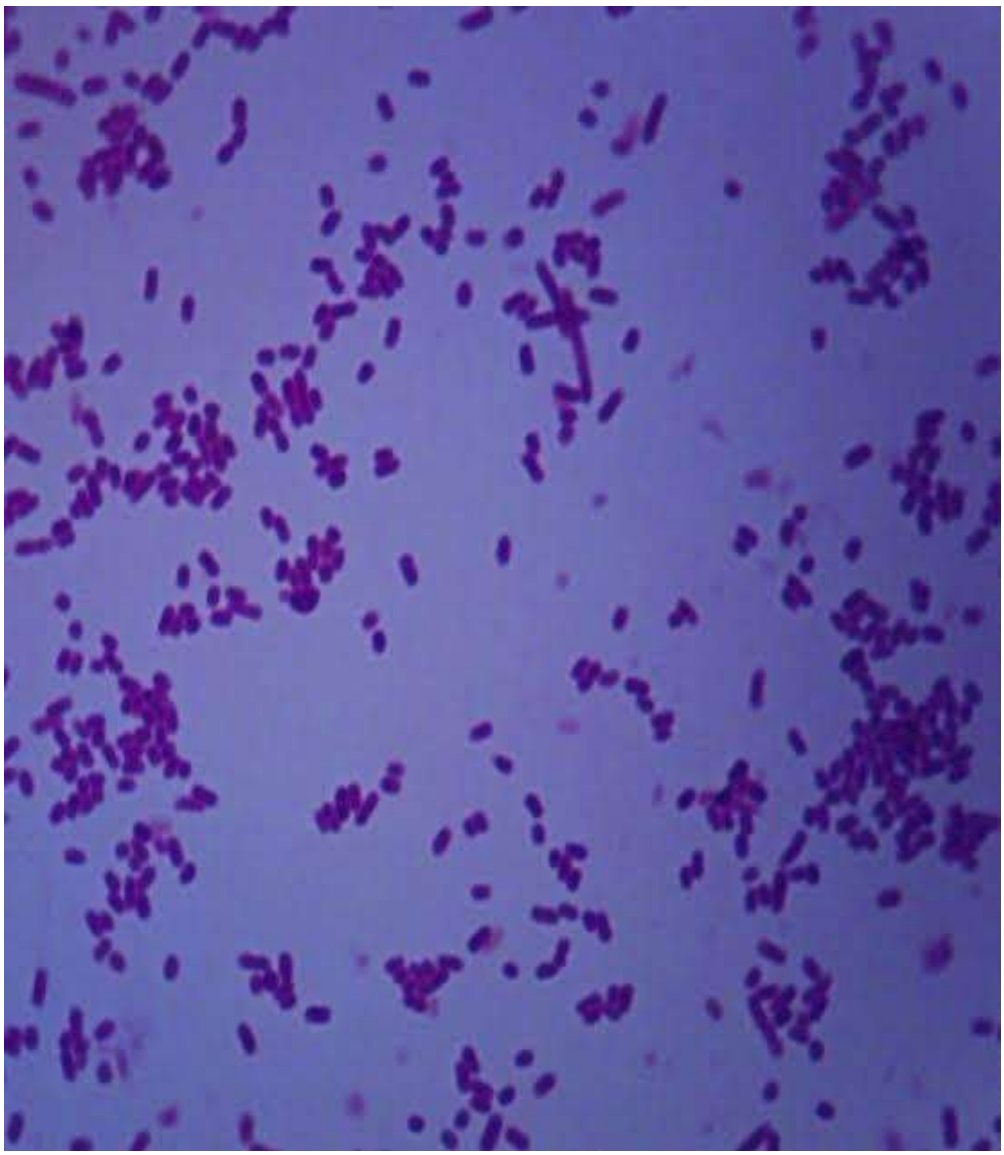
(Table - 2)

S.no	Test micro-organisms	Zone of inhibition(in mm)			
		Ethanol extract	Aqueous extract	Control	Ceprofloxacin
1	*Shigella sp*	11.21	8.54	0	45.34±2.05
2	*Salmonella sp*	0	0	0	30.65
3	*Staphylococcus aureus*	0	0	0	41.45
4	*Pseudomonas aeruginosa*	0	0	0	-



(Figure-1)

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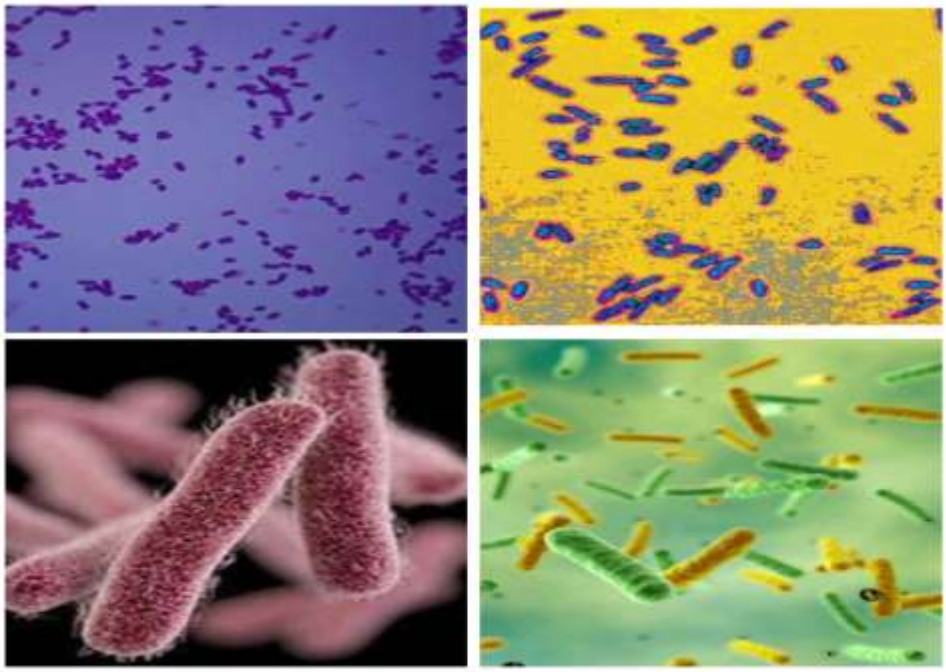

(Figure-2)

(Figure-3)

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(Figure-4)

(Figure-5)

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(Figure-6)