

***IN-VITRO* SCREENING OF *HIBISCUS CANNABINUS L.* LEAVES EXTRACTS FOR ANTIBACTERIAL ACTIVITY**

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Article Received on
14 April 2016,

Revised on 04 May 2016,
Accepted on 24 May 2016

DOI: 10.20959/wjpr20166-6144

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ABSTRACT

In-vitro screening of aqueous and ethanol extracts of *Hibiscus cannabinus L.* leaves for their antibacterial activity against *Salmonella typhimurium* 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 pathogen and the inhibition zones ranged between 12.67 ± 1.52 to 6.33 ± 0.58 . 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 $120000 \mu\text{g}/10\text{ml}$ find to be more potent compared to ethanol exact however, not on par with that of standard employed.

KEYWORDS: *Hibiscus cannabinus L.*, leaves extracts, antibacterial activity, *Salmonella typhimurium*.

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.^[1-3]

Kenaf or *Hibiscus cannabinus* L. (Family-Malvoceae) 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.^[4,5,6] Kenaf will grow to a height of five to six meters and produces three to five times as much fibre as sothern yellow pine and rapid growth rate of Kenaf helps to alleviate global warming by absorbing CO₂ gases^[7] and Saba et al., reported that based on Malaysian prospective Kenaf herb will be a potential bioenergy production source in future.^[8] The plant is also known for its numerous synonyms *viz.*, ambadi in Marathi or Ambashtha in Sanskrit (India).^[1]

H. 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, stomachic^{[5][9]} and fiber of Kenaf was used for the production of pulp and in paper manufacturing industries from dates back to the very time.^[10-11] 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, phellandrene^{[11][12]} and glucosides such as cannabiscitrin, cannabiscetin and anthocyanin glycoside cannabinidin.^[4-5] 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.^[13] To one side from the presence of different chemical components a tall woody herb *H. cannabinus* also possess a wide spectrum of pharmacological activities *viz.*, antioxidant^[1], haematinic^[3], antihyperlipidemic^{[5][14]}, hepatoprotective activity^[9], anti diabetic, anti-ulcer, cytotoxic and immunomodulatory activities.^[11]

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. Typhoid is an example of such infectious disease and a serious public health problem in developing countries caused

by pathogen *Salmonella typhimurium* (*S.typhi*). *S.typhi* is Gram-negative bacteria predominantly found in intestinal lumen and an enteric pathogen believes to be resistance to most antibiotics. It causes enteric fever with the clinical features of infection include fever, headache, anorexia maligns and insidious onset malaise.^[15-16] 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 of leaves extracts of *Hibiscus cannabinus L.* against *Salmonella typhimurium*.

MATERIALS AND METHODS

Plant Material and Solvent Extraction

The herb *Hibiscus cannabinus L* or Kenaf was collected from Cameron Highland Pahang. The authentication was established with the help of the institute of Bioscience, University Putra Malaysia (UPM) (Voucher specimen number: SK 2228/13). 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.

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 50°C to the solid form of crude extracts.^[2-3]

Bacterial Strain and Growth Condition

Bacteria culture of *S. typhi* obtained from the Department of Medical Lab Technology, Masterskill global college were used in the present study. Stock culture were maintained aseptically under optimal conditions for *S. typhi* and subcultured from Hektoen enteric agar onto Mueller hinton agar plates followed by incubation at 37°C for 24 hours. The type of bacteria present on each agar plate was confirmed through gram staining (Figure 1), Triple sugar iron test (Figure 2) and color changes in Hektoen enteric agar (Figure 3).^[15-16]

Minimum Inhibitory Concentration (MIC)

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.

$$\begin{aligned}\text{Concentration} &= \text{Weight of coarse powder (g)} / \text{Volume of solvent (L)} \\ &= 90\text{g} / 0.75\text{L} \\ &= 120\text{g/L} \\ &= 120\text{g}/1000\text{ml} \\ &= 0.12\text{g/ml}\end{aligned}$$

$$\text{For } 10\text{ml} = 1.2\text{g}/10\text{ml}$$

MIC values were determined for distilled water and ethanol extracts to evaluate the anti *Salmonella typhimurium* 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 $\mu\text{g ml}^{-1}$ to 12 $\mu\text{g ml}^{-1}$ against 24 hours broth culture of *Salmonella typhimurium*. A grade AA discs with 6 mm diameter (Sterile blank discs, Whatman International Ltd. England) soaked in each concentrations of extracts were placed on the plates of Mueller Hinton agar followed by incubation at 37°C for 24 hours. Plates were observed after 24 hours and inhibition zones were measured (Figure 4-5).^{[2][15-16]} The experiments were replicated three times with duplicate samples per replicate and data was collected, analyzed and were summarized in Table 1.

Minimum bactericidal concentration (MBC)

The MBC 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.^{[2,49-52] [2][17-20]}

Antimicrobial susceptibility testing (AST)

The AST 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 and

to assure susceptibility to drug of choice (Figure 6).^{[2][21]} AST results were summarized in Table 2.

RESULTS AND DISCUSSION

Confirmatory Test for *S. typhi*

Gram stain: Gram stain was done to identify the morphology of *S. typhi* and to 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 1).

Triple sugar iron test: The test revealed blackening of butt due to the production of hydrogen sulfide with red surface of slant which indicates that *Salmonella typhimurium* does not ferment lactose and sucrose (Figure 2).

The color changes in Hektoen enteric agar to green colonies with black back centers conform the growth is of *S.typhi* (Figure 3).

In-vitro antibacterial activity of aqueous and ethanol extracts of *H. cannabinus L.* leaves at concentration range between 120000 $\mu\text{g}/10\text{ml}$ to 12 $\mu\text{g}/10\text{ml}$ were determined by measuring zone of inhibition and the results are presented in Table1. Both extracts showed different sensitivity levels for the tested enteric pathogen *Salmonella typhimurium* 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) 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 (Figure 5-8) and among two extracts used aqueous extract at concentration 120000 $\mu\text{g}/10\text{ml}$ find to be more potent compared to ethanol exact (120000 $\mu\text{g}/10\text{ml}$). It can be inferred from the results that leaves extracts of *H. cannabinus L.* has potential antibacterial property against the pathogen which is believe to be resistance to most antibiotic *Salmonella typhimurium*. 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 to

microbial infection or against predation of insects and herbivores or to serve as plant defense mechanisms.^{[2-3] [22-23]}

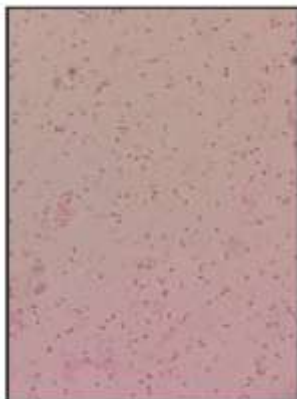


Figure 1: Gram stain



Figure 2: Triple sugar iron test



Figure 3: Color changes in Hektoen Enteric Agar

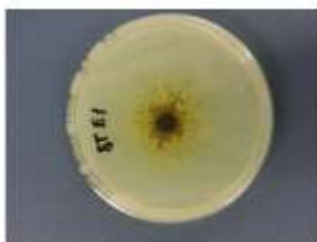


Zone of inhibition: Aqueous extract (120000µg/10ml)

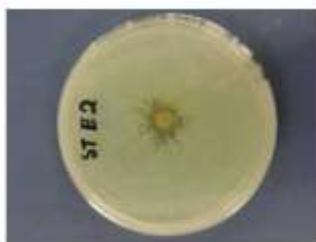


Zone of inhibition: Aqueous extract (12000µg/10ml)

Figure 4



120000µg/10ml



12000µg/10ml



1200µg/10ml



120µg/10ml



12µg/10ml

Figure 5: Zone of inhibition of ethanol extract

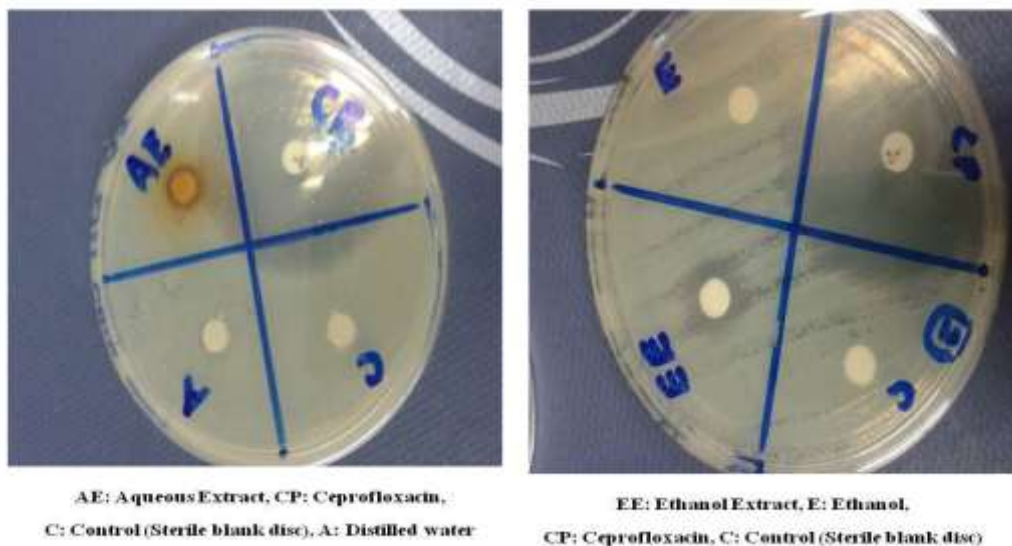


Figure 6: Antimicrobial Susceptibility Test (AST)

Table 1: Minimum inhibitory concentration (MIC) of leaves extracts of *Hibiscus cannabinus L.* (Kenaf)

Extract concentration	Zone of inhibition (in mm) <i>Salmonella typhimurium</i>
Aqueous ($\mu\text{g}/10\text{ml}$)	
120000	12.67 \pm 1.52
12000	6.67 \pm 1.15
1200	0.00 \pm 0.00
120	0.00 \pm 0.00
12	0.00 \pm 0.00
Ethanol ($\mu\text{g}/10\text{ml}$)	
120000	7.33 \pm 0.58
12000	7.33 \pm 0.58
1200	12.33 \pm 2.08
120	10.67 \pm 1.53
12	6.33 \pm 0.58

All values are in mean \pm SD. N=3 (The experiment was performed in three replicates)

Table 2: Antimicrobial Susceptibility Test (AST) against *Salmonella typhimurium*

Extract and Disc	Zone of inhibition (in mm) <i>Salmonella typhimurium</i>
Aqueous Extract	
Control	0
Distilled Water	0
Aqueous Extract	9.33 \pm 1,15
Cephalosporin	41.67 \pm 0.58
Ethanol Extract	
Control	0

Ethanol	0
Ethanol Extract	11.33±2.08
Ceprofloxacin	44.33±4.04

All values are in mean \pm SD. N=3 (The experiment was performed in three replicates)

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 *H. cannabinus L.* 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 *Salmonella typhimurium* and other enteric pathogens.

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