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Potential *in vitro* antimicrobial efficacy of *Holigarna arnottiana* (Hook F)Aseer Manilal<sup>1\*</sup>, Akbar Idhayadhulla<sup>2</sup><sup>1</sup>Department of Medical Laboratory Sciences, College of Medicine and Human Health, Arba Minch University, Arba Minch, Ethiopia<sup>2</sup>School of Chemical Engineering, Yeungnam University, Gyeongsan 712-749, South Korea

## PEER REVIEW

## Peer reviewer

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## Comments

This is a preliminary study, conducted in a distinguished way to assess the presence of potential antimicrobial activity in organic solvent extract of *H. arnottiana* leaves. The results are interesting and further study is required to understand whether the candidate may have some application in human or veterinary health care system.

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## ABSTRACT

**Objective:** To explore the *in vitro* antimicrobial potential of *Holigarna arnottiana* (*H. arnottiana*) against human and shrimp pathogenic bacteria and use GC-MS analysis to elucidate its antimicrobial principles.

**Methods:** In the present study, organic extract of *H. arnottiana* was examined for *in vitro* antimicrobial potency against five clinical human pathogens, seven species of human type culture pathogens, six pathogenic *Vibrio* strains isolated from moribund tiger shrimp (*Penaeus monodon*) and seven type cultures (Microbial Type Culture Collection, MTCC) of prominent shrimp pathogens.

**Results:** The extraction of *H. arnottiana* with ethyl acetate yielded bioactive crude extract that efficiently repressed the growth of all tested pathogens. Among the pathogens tested, shrimp pathogens were the most susceptible organisms while clinical pathogens were found to be a little resistant. The chemical constituents of the *H. arnottiana* were analysed by GC-MS which revealed the presence of major compounds such as 3,7,11,15-tetramethyl-2-hexadecen-1-ol (42.1%), 1-lodo-2-methylundecane (34.5%) and squalene (11.1%) which might have a functional role in the chemical defence against microbial invasion.

**Conclusions:** Based on the finding it could be inferred that *H. arnottiana* would be a reliable source for developing shrimp and human bio-therapeutics in future.

## KEYWORDS

Anacardiaceae, *Holigarna arnottiana*, Plant extract, Bioactive compounds, Chemical constituents

## 1. Introduction

The haphazard employment of synthetic antibiotic medicaments for the management of dreadful infectious diseases had ineluctably led to multiple drug resistance in human, animal and plant pathogenic microorganisms<sup>[1,2]</sup>. To circumvent the ruinous effects of synthetic antibiotics, an alternative control is urgently required. The development of novel antibiotics to combat emerging and existing dreadful diseases using natural products as a reliable source is found to be a current trend. In contrary to the synthetic drugs, antimicrobials from natural origin are not associated with negative effects and have an enormous therapeutic potential to heal many infectious diseases. Terrestrial and

marine vegetation are known to biosynthesize a cornucopia of bioactive secondary metabolites which stave off many potential invaders either in a constitutive or an inducible way<sup>[3,4]</sup>. Plants have been mined for novel secondary metabolites since 1800's. Nearly, 80% of the terrestrial floras are virtually untapped sources of bioactive metabolites. Hitherto, more than one hundred thousand bioactive compounds have been sourced from higher plants<sup>[5]</sup>. With the advent of modern sophisticated technology, it is now possible to explore the lead compounds and assess their potential antimicrobial activity.

Diverse plant species belonging to Anacardiaceae family from Indian subcontinent has been extensively investigated for antimicrobial potentials<sup>[6-9]</sup>. The plant of genus

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*Holigarna* occurs mostly in the evergreen forest of Western Ghats region of Indian subcontinent. Several species of genus *Holigarna* are popular for the management of arthritis, dysentery, hemorrhoids, skin diseases, cancer and also as antiseptic in folk medicine[10–12]. Retrospective researches evidenced that *Holigarna arnottiana* (*H. arnottiana*) express antioxidant[13], antifeedant[14], anticancer and allergic potentials[15,16]. These activities might be due to the presence of diverse bioactives metabolites in this species. However, literature pertained to the antimicrobial activity of *H. arnottiana* from the Indian subcontinent has been scant. In the light of this, the present study is intended to explore the *in vitro* antimicrobial potential of *H. arnottiana* against a battery of human and shrimp pathogenic bacteria and use GC–MS analysis to elucidate its antimicrobial principles.

## 2. Material and methods

### 2.1. Sample collection

Tender leaves of plant, *H. arnottiana* was sourced from the Kollam (08° 54' N and 76° 38' E) prefecture. The collected specimen was identified taxonomically with the help of an eminent taxonomist, Prof. N. Ravi, Sree Narayana College, Kollam. The specimen was transported to the laboratory in polythene bags and voucher specimen was frozen at –5 °C for future reference. Prior to extraction, the leaves were cleaned to remove dirt and other associated debris. The cleaned samples were chopped into small pieces and stored at 2 °C until analyzed.

### 2.2. Preparation of organic extracts

The crude organic extract was prepared from fresh plant material using the procedure reported in our earlier study[3] with trivial modification. Briefly, to obtain the organic extract a definite quantity of plant material was pulverized in a ceramic mortar and heavy round ended pestle using different organic solvents such as hexane, chloroform, ethyl acetate, dichloromethane, ethanol and phosphate buffer saline of increasing polarity at room temperature. These extracts were submerged in Scott Duran flasks with respective solvents and placed in a shaker (REMI) at 100 r/min for twenty hours in order to permit maximum extraction of the active compounds. After twenty hours, extracts were filtered using Whatman filter paper No. 1 to remove the tissue residue. The extracts were then centrifuged for 10 min at 10000 r/min and evaporated to dryness in a distillation system and weighed. Each organic extract filtrate was subsequently dissolved in the corresponding extraction solvent for a final concentration of 5 mg/mL and stored in the refrigerator for further studies.

### 2.3. Assay microorganisms

All the organic extracts were tested against a panel of human and shrimp pathogenic bacteria (Table 1). The human and shrimp pathogens with MTCC number were obtained from Institute of Microbial Technology, Chandigarh, India. The human clinical isolates were obtained from Medilab Speciality Laboratories (India) Pvt. Ltd, Kochi, Kerala, India. The shrimp *Vibrio* isolates used in this study are isolated from diseased tiger shrimp *Penaeus monodon*[2]. The test

organisms were maintained on nutrient agar (Hi Media, India) at 4 °C and sub-cultured before used.

**Table 1**

Panel of pathogens used for antimicrobial assay.

Group	Species
Clinical isolates	<i>Staphylococcus aureus</i>
	<i>Pseudomonas</i> sp.
	<i>Proteus vulgaris</i>
	<i>Escherichia coli</i>
	<i>Klebsiella</i> sp.
Human pathogens (MTCC)	<i>Staphylococcus aureus</i> (MTCC 96)
	<i>Streptococcus mutans</i> (MTCC 890)
	<i>Bacillus amyloliquefaciens</i> (10441)
	<i>Micrococcus luteus</i> (MTCC106)
	<i>Klebsiella pneumonia</i> (MTCC 109)
	<i>Shigella flexneri</i> (MTCC 1457)
	<i>Vibrio mimicus</i> (MTCC 4434)
	<i>Vibrio alginolyticus</i> (MTCC 4439)
Shrimp pathogens (MTCC)	<i>Vibrio alcaligenes</i> (MTCC 4442)
	<i>Vibrio vulnificus</i> (MTCC 1145)
	<i>Vibrio parahaemolyticus</i> (MTCC 451)
	<i>Vibrio fischeri</i> (MTCC 1738)
	<i>Vibrio harveyi</i> (MTCC 3438)
	<i>Aeromonas hydrophila</i> (MTCC 1739)
	<i>Photobacterium damsela</i> (Vb26)
Shrimp <i>Vibrio</i> isolates	<i>Vibrio harveyi</i> (Vb5)
	<i>Vibrio alginolyticus</i> (Vb11)
	<i>Vibrio vulnificus</i> (Vb14)
	<i>Vibrio fischeri</i> (Vb17)
	<i>Vibrio parahaemolyticus</i> (Vb21)

### 2.4. Antimicrobial assay

The antimicrobial activity of *H. arnottiana* was investigated *in vitro* according to the method described in our previous study[17]. Mueller Hinton agar plates were prepared and swabbed with respective pathogens. The wells were made on agar plates by using a sterile cork borer. The resultant wells were filled with 120 µL of the appropriate organic extract. Subsequently, the plates were incubated at (30±2) °C for 24 h. The well with solvent used for dissolution was considered as negative control while chloramphenicol (1 mg/mL) and nalidixic acid (1 mg/mL) were used as the positive controls for shrimp and human pathogens. The assay was performed in triplicates of individual Petri-dishes. Clear zone of inhibition formed around wells were considered indicative of antimicrobial activity. The inhibitory activity was measured by calculating the area of inhibition zone. The antibiogram was statistically analyzed for the determination of Skewness among the tested bacterial strains.

### 2.5. Gas chromatographic mass spectroscopic analysis

The organic extract of *H. arnottiana* with highest antimicrobial activity was subjected to GC–MS analysis. The GC–MS analysis was carried out using a Clarus 500 Perkin–Elmer Gas Chromatograph equipped with mass detector Turbo mass gold– Perkin Elmer Turbomass 5.2 spectrometer and an Elite–5 MS (5% Diphenyl/ 95% Dimethyl poly siloxane), 30×0.25 mm×0.25 µm of capillary column was used with helium at a 1 mL min<sup>-1</sup> as a carrier gas. The GC oven temperature was kept at 110 °C for two minutes, programmed

to 280 °C at the rate of 5 °C/min and kept constant at 280 °C for 10 min. The split ratio was adjusted to 1:20 and the injection volume was 2 µL. The injection and detector temperature was 250 °C. The GC–MS electron ionization mode was 70 eV. Mass scan range was from  $m/z$  45–450 amu. The peaks of the gas chromatography were subjected to mass–spectral analysis. Peak identification was carried out using NIST Version 2.0 (2005).

## 2.6. Data analysis

All the data are presented as mean±standard deviation (SD). Mean values were assessed using one way analysis of variance (ANOVA) using SPSS for Windows version 11.2 (Statistical Package for Social Services, Chicago, IL, USA).

## 3. Results

The overall results of antimicrobial activity of *H. arnottiana* extracted using different organic solvents against human and shrimp pathogens are tabulated in Table 2. Of the six solvents used, ethyl acetate was found to be the best solvent for extracting antimicrobial principles from fresh material following dichloromethane and ethanol. Ethyl acetate extract of *H. arnottiana* exhibited broad spectrum of activity and it repressed the growth of all the tested pathogens. However, activity was absent in phosphate buffer saline, chloroform and hexane extract. On the basis of preliminary findings, the ethyl acetate extract of *H. arnottiana* was used for further studies.

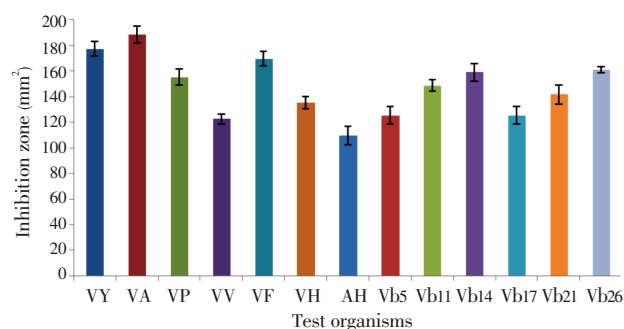
**Table 2**

Overall activity of different solvent extracts of *H. arnottiana* against different panel of test pathogens.

Solvents	% of antimicrobial activity			
	Human pathogens (MTCC)	Clinical isolates	Shrimp pathogens (MTCC)	Shrimp isolates
Hexane	0	0	0	0
Chloroform	0	0	0	0
Ethyl acetate	100	100	100	100
Dichloromethane	40	20	80	80
Ethanol	60	40	100	80
PBS	0	0	0	0

Zone of inhibition  $\geq 20$  mm<sup>2</sup> was considered as active.

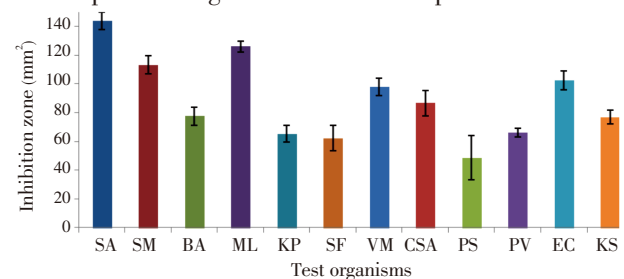
Based on the activity spectrum, inhibitory potentials of ethyl acetate extract of *H. arnottiana* against different bacteria were ranked as highly active ( $>100$  mm<sup>2</sup>), moderate active (50–100 mm<sup>2</sup>), and less active ( $<50$  mm<sup>2</sup>). The antibiogram of *H. arnottiana* revealed that the ethyl acetate extract has broad spectrum antibacterial principle. Notably the antibiogram was positively skewed towards the shrimp pathogens when compared to human type culture and clinical isolates (Figure 1). The inhibitory spectrum ranged between (109.4±8.8) to (188.6±6.4) mm<sup>2</sup> was displayed against the shrimp type culture pathogens. The highest activity rank of (188.6±6.4) mm<sup>2</sup> was extended against *Vibrio alcaligenes* followed by (177.4±5.9) mm<sup>2</sup> against *Vibrio alginolyticus*. The extract of *H. arnottiana* impeded the growth of all shrimp *Vibrio* isolates in the range of (125.34±6.9) to (161.2±2.5) mm<sup>2</sup>. The highest inhibition rank of (125.34±6.9) mm<sup>2</sup> was recorded against *Photobacterium damsela*.



**Figure 1.** Antibacterial potential of ethyl acetate extract of *H. arnottiana* against shrimp pathogens.

VY: *Vibrio alginolyticus*; VA: *Vibrio alcaligenes*; VP: *Vibrio parahaemolyticus*; VV: *Vibrio vulnificus*; VF: *Vibrio fischeri*; VH: *Vibrio harveyi*; AH: *Aeromonas hydrophila*; Vb5: *Vibrio harveyi*; Vb11: *Vibrio alginolyticus*; Vb14: *Vibrio vulnificus*; Vb17: *Vibrio fischeri*; Vb21: *Vibrio parahaemolyticus*; Vb26: *Photobacterium damsela*.

In the case of human type culture pathogens the activity rank was in the range of (62.1±4.6) to (143.8±6.1) mm<sup>2</sup> (Figure 2). The antimicrobial activity was remarkable against *Staphylococcus aureus*, *Micrococcus luteus* and *Streptococcus mutants* to the extent of (143.8±6.1) mm<sup>2</sup>, (125.9±3.1) mm<sup>2</sup> and (113.1±8.4) mm<sup>2</sup> respectively. The moderate activity rank of (97.9±3.7) mm<sup>2</sup>, (77.4±4.9) mm<sup>2</sup>, (65.11±6.5) mm<sup>2</sup> and (62.1±4.6) mm<sup>2</sup> was exhibited against the Gram negative bacteria, *Vibrio mimicus*, *Bacillus amyloliquefaciens*, *Klebsiella pneumonia* and *Shigella flexneri* respectively. On the other hand the activity of plant extract against human clinical isolates was in the range of (48.61±3.1) mm<sup>2</sup> to (102.6±6.7) mm<sup>2</sup>. The high activity rank of (102.6±6.7) mm<sup>2</sup> was displayed against *Escherichia coli* whereas the meager activity rank of (48.61±3.1) mm<sup>2</sup> was produced against *Pseudomonas* sp.

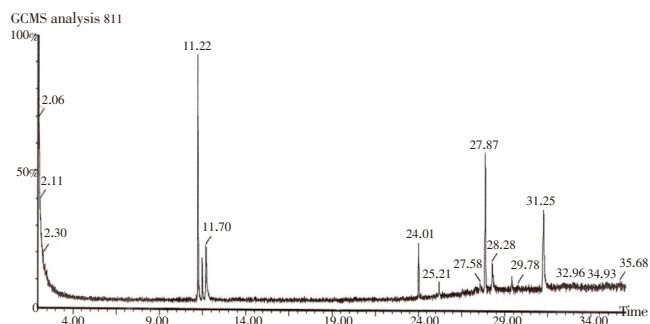


**Figure 2.** Antibacterial potential of ethyl acetate extract of *H. arnottiana* against human pathogens.

SA: *Staphylococcus aureus*; SM: *Streptococcus mutants*; BA: *Bacillus amyloliquefaciens*; ML: *Micrococcus luteus*; KP: *Klebsiella pneumonia*; SF: *Shigella flexneri*; VM: *Vibrio mimicus*; Clinical Isolates CSA: *Staphylococcus aureus*; PS: *Pseudomonas* sp.; PV: *Proteus vulgaris*; EC: *Escherichia coli*; KP: *Klebsiella* sp.

### 3.1. Spectrum of the *H. arnottiana* extract

The GC–MS analysis was carried out to determine the possible chemical constituents from *H. arnottiana*. The GC–MS chromatogram of ethyl acetate extract of *H. arnottiana* is shown in Figure 3. The relative percentage of compounds present in the *H. arnottiana* is summarized in Table 3. Analysis revealed that the main phyto–constituents present in the ethyl acetate extract of *H. arnottiana* are 3,7,11,15–tetramethyl–2–hexadecen–1–ol (42.1%) and 1–lodo–2–methylundecane (34.5%) followed by squalene (11.1%), vitamin E (8.5%) and heptadecane, 2,6,10,14–tetramethyl– (3.7%).



**Figure 3.** GC–MS chromatogram profile of the ethyl acetate extract of *H. arnottiana*.

**Table 3**

Characteristics of the peaks obtained from the ethyl acetate extract of *H. arnottiana* by GC–MS analysis.

RT	Name of the compound	Molecular formula	MW	Peak area%
11.22	3,7,11,15-tetramethyl-2-hexadecen-1-ol	C <sub>20</sub> H <sub>40</sub> O	296	42.1
24.01	Squalene	C <sub>30</sub> H <sub>50</sub>	410	11.1
27.87	1-Iodo-2-methylundecane	C <sub>12</sub> H <sub>25</sub> I	296	34.5
28.27	Vitamin E	C <sub>29</sub> H <sub>25</sub> O <sub>2</sub>	430	8.5
29.42	Heptadecane, 2,6,10,14-tetramethyl-	C <sub>21</sub> H <sub>44</sub>	296	3.7

RT: Retention time; MW: Molecular weight.

#### 4. Discussion

Terrestrial vegetation is recognized as a good candidate for development of drugs and drug leads for various diseases<sup>[18]</sup>. In the present study, *H. arnottiana* sourced from Kollam prefecture is investigated for their antimicrobial potency against human and shrimp pathogens. Findings of the present study indicated that of the six solvents used, the extract of *H. arnottiana* obtained using ethyl acetate efficiently impeded the growth of all the tested pathogens. These results envisaged that the extraction method had profound effect on the isolation of antimicrobial principles. A high rank of antimicrobial activity was revealed among shrimp type culture and *Vibrio* isolates. Thus, *H. arnottiana* can be considered to be a potential producer of vibriocidal agents that could be utilized for the development of shrimp bio-therapeutics in future. Among the human type culture pathogens, ethyl acetate extract exhibited significant activity against *Staphylococcus aureus*. On the other hand, human clinical isolates were found to be moderately susceptible to crude extracts except *Escherichia coli*. The resistance exhibited by the enteric strains might be due to the permeability barrier provided by the cell wall or to the membrane accumulation mechanism<sup>[19]</sup>. The present findings will provide new insight into the development of antibacterial agent from *H. arnottiana* for the control of clinical and type culture human pathogens. Recent studies have shown that other species of *Holigarna* were effective in inhibiting the growth of human pathogens<sup>[20]</sup>. However, limited literature is available on the aspect on the biological potential of *H. arnottiana* from India. In comparison to our study, Pradeep and Saj have shown the antimicrobial activity of *H. arnottiana* from Kerala<sup>[21]</sup>. The same authors also reported that antibacterial activity of *H. arnottiana* might be due to the presence of alkaloids, steroids, tannins, phenolic compounds, flavonoids, steroids, resins, fatty acids, and gums. Antimicrobial activity against shrimp pathogens has

not been previously reported for *H. arnottiana*.

Studies on chemical examination of the constituents from *H. arnottiana* date back to 1950s<sup>[22]</sup>. In the present study, the secondary metabolites of *H. arnottiana* were analyzed by GC–MS. The principle compounds present are 3,7,11,15-tetramethyl-2-hexadecen-1-ol (42.1%), 1-Iodo-2-methylundecane (34.5%) and squalene (11.1%). Evidence supporting the bioactivity of certain plants possessing these compounds has been formerly reported<sup>[23–25]</sup>. GC–MS analysis of a Malaysian medicinal plant, *Labisia pumila* evidenced the presence of 3,7,11,15-tetramethyl-2-hexadecen-1-ol<sup>[24]</sup>. Hashmi *et al.*<sup>[26]</sup> reported the presence of 3,7,11,15-tetramethyl-2-hexadecen-1-ol, 1-Iodo-2-methylundecane and squalene from a medicinal plant, *Thymus vulgaris* through GC–MS analysis. In addition, Noro *et al.* identified a tetrahydroxy squalene compound with antimycobacterial activity from *Rhus taitensis* (Anacardiaceae)<sup>[27]</sup>. The findings of the present study envisaged that the antimicrobial activity exhibited by the *H. arnottiana* might be due to the synergistic activity of these compounds. This report would be the first to explore the possible antimicrobial potential of *H. arnottiana* against human and shrimp pathogens from India. Thus, the future use of this plant for the development of human and veterinary grade antibiotics is highly promising.

#### Conflict of interest statement

We declare that we have no conflict of interest.

#### Acknowledgements

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#### Comments

##### Background

Before the advent of chemotherapy diseases were treated with topical agents or mixed unknown. The discovery of antibiotics revolutionized the treatment of many diseases that affect the human kind. Unfortunately, these magic bullets are often used improperly. This allowed appearance of drug-resistant bacteria. In attempt to develop new chemotherapys, attention is paid to compounds with antibiotic activity of plant origin.

##### Research frontiers

The study starts from the assumption that several species of the genus *Holigarna* have got therapeutic potential. The species *H. arnottiana* has attracted interest for its properties (antioxidant, anticancer potentials, *etc.*) and now the interest is to investigate whether there are also some unknown antibiotic properties.

##### Related reports

The information provided in this work are in agreement with the studies of several authors, for example Kalase *et al* (2013).

The potential offered by the knowledge of popular medicine



that uses raw drugs is a springboard to standardize the concentration of active ingredients as antibiotic or immunostimulant. Publication of Pradeep *et al.* is interesting (2010).

Kener *et al.* (Antibiotics: where did we go wrong?—Drug delivery 2004) underlined the way in order to find new antibiotics from natural sources and however, unfortunately, research, using genomic techniques, did not yield the expected results.

### Innovations and breakthroughs

The interest to find bioactive compounds against bacteria urged to investigate whether in *H. arnottiana* there is some compounds that can be used even against shrimp pathogenic bacteria. The data presented seem to be encouraging, but only further studies will determine whether the antimicrobials found will be suitable for the human or veterinary application.

### Applications

The applications are certainly focused on expanding the repertoire of antimicrobial drugs. The data seem to suggest the idea that the effect is due to the synergy of various components found in plant extracts. This opens several scenarios ranging from a search of natural chemotherapeutic agents to assess how they act, up to creation of modified molecules to extend the spectrum of action.

### Peer review

This is a preliminary study, conducted in a distinguished way to assess the presence of potential antimicrobial activity in organic solvent extract of *H. arnottiana* leaves. The results are interesting and further study is required to understand whether the candidate may have some application in human or veterinary health care system.

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