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# ISOLATION, STRUCTURAL CHARACTERIZATION OF BIOACTIVE COMPOUND ALPHA ASARONE AND ITS ANTIMICROBIAL POTENTIAL

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## Abstract

The detection of herbal medicine has led to the development of new therapeutic agents. The explore for natural or biological products and compounds derived from natural sources has played an crucial role in drug discovery due to their pharmacological significance.The current study focus to isolate, structurally characterize and identify the bioactive compounds from *Acorus calamus* by using thin layer chromatography, Column chromatography, Nuclear Magnetic resonance, Fourier-transform infrared spectroscopy (FTIR) and High-resolution mass spectrometry (HRMS). Identification of the compounds were based on the molecular structure, molecular mass and calculated fragments. The name, molecular weight and structure of the components of the test materials were ascertained using Nuclear Magnetic Resonance, Fourier Transform Infra-Red Spectrometry (FTIR) and High-resolution mass (HRMS). The FTIR spectrum shows the presence of major functional groups. From the results of NMR data, FTIR and HRMS the compound is found to be Alpha Asarone ($C_{12}H_{16}O_3$) which was isolated from *Acorus calamus*. The antibacterial activity also determined by using intestinal microorganisms. This study revealed that this compound has good antibacterial potential. In future the Alpha Asarone can be used as a candidate for anti-cancer drug development based on further research

***Keywords:*** Extraction, TLC, Isolation, Characterization, *Acorus calamus*, NMR, FT-IR, HRMS and antibacterial.

## 1. Introduction

Plants are healthy and natural resource of life. In particular, medicinal plants are of great importance with endless therapeutic properties useful for curing various diseases with an advantage of being natural. At present, there are un-countable products in market, with adverse side effects on once heath. Therefore, the use of secondary metabolites from plant origin could be an advantage and best solution to narrow down the use of unhealthy products. In past, the plant or microbial extracts in crude or partially-purified forms were the only sources of medication available for the treatment of human

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and animal diseases. This gave an idea that the effect of a drug in human body is due to an interaction of drug with biological molecules. This opened new doors in pharmacology, as pure, isolated chemicals, instead of extracts, as the standard for the treatment of diseases. At present, there are innumerable number of such bioactive compounds isolated form crude extracts and their chemical structure were elucidated. Moreover, plants have always been a source of a wide array of secondary metabolites with potential pharmacological properties (Vivek K. Bajpai *et al.*, 2016).

*Acoruscalamus* is a natural plant belonging to the order Acorales and family Acoraceae. The genus name is Acorus and its species is called A. calamus. This plant has a very long history of medicinal use in Chinese and Indian herbal traditions. This plant was present in Indian markets nearly two thousand years agoand it had been sold as a medicine in every Indian shop. It was used for ailments such as dyspepsia, mouth and throat diseases, fevers, epilepsy, bronchitis, hysteria, tumors, rat bites, ear worms, toothaches, pains of the chest and kidneys, insomnia, melancholia, neurosis, loss of memory depression and mental disorders, asthma, diarrhoea, dysentery, flatulence (ChinnappanSudhakar *et al.*, 2015).

The aim of the current study was to provide step-by-step visual demonstration of fractionation and isolation of biologically active plant secondary metabolites using TLC, column-chromatographic and NMR, FTIR and HRMS spectroscopy techniques and evaluation of the antimicrobial activity to check their biomedical importance.

## 2. Material and Methods

### 2.1. Preparation of ethanol/methanol extract of Vasambu (Acorus calamus)

To an oven-dried round bottom flask (250 mL) equipped with a magnetic stir-bar was added the Vasampu powder (a sum of 30 grams) and ethanol (50 mL),and the mixture was strenuouslystirred at room temperature for another 2-3 hours. Then the mother liquid (organic layer) was decanted. Subsequently,the residue in the RB flask was re-diluted with ethanol (50 mL) and refluxed for another 1 hour at 80ºC. Then the supernatant liquid was transferred and combined room temperature extracts.In a similar way, the methanol extracts were also prepared with the residue. In detail, in the beginning, we extracted Vasampu (Acorus calamus) with ethanol (2 X 50 mL) at room temperature as well as at reflux conditions.

Similarly, we have followed the identical extraction procedure with methanol (2 X 50 mL) to get the maximum yield of the desired natural product; this quantitative amount of product can be utilized for different preliminary studies of biological application.

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## 2.2. Analytical techniques

The filtered organic layers of ethanolic/methanolic extracts were analyzed with thin layer chromatography (*tlc*); both extracts havea similar pattern (*tlc* spots). Later, the organic layers were combined and concentrated,which afforded 18.31 gm of crude residue. Unless otherwise stated, oven-dried glassware was used, and all solvents and silica-gel (100-200 mesh) were purchased from SD-Fine/TCI Chemicals and used without further purification. Thin layerchromatography was carried out on commercially available pre-coated aluminum sheets (Merck silica-gel $F_{254}$) and visualized using UV light (254 nm) or with Iodine.

The manual gravity column chromatography was carried out using 100-200 mesh silica gel packed in glass columns using n-hexane and ethyl acetate as an eluent.The nuclear magnetic resonance (NMR) spectrawererecorded on a Bruker Avance-III 400 NMR spectrometer.Chemical shifts are reported in parts per million (ppm) concerningtetramethylsilane (TMS) as an internal standard, and thecouplingconstants (*J)* are reported in Hertz (Hz).

The multiplicity of $^{1}H$ NMR was denoted as s = singlet, d = doublet, t = triplet, q = quartet, and m = multiplet. A proton-decoupled $^{13}C$ NMR data were recorded for unambiguous understandings. High-resolution mass spectra (HRMS) were recorded using an Electro-spray Ionization (ESI)on a time of flight (TOF) technique using WATERS–XEVO G2-XS-QtoF spectrometer. FT-IR spectra were recorded on Affinity-I Shimadzu FT-IR spectrometers with a universal ATR accessory and are reported in wavenumbers ($cm^{-1}$).

## 2.3. Antibacterial activity

### 2.3.1. Agar well diffusion method

The agar well diffusion method was applied to investigate antibacterial activities of methanol, extracts [M. Rahimi-Nasrabadi, *et al*., 2013] against 4 bacteria. Besides, the concentrations of 10, 20, 30, and 40 µg/mL were employed to evaluate the antibacterial activities of Alpha Asarone. First, 0.5 McFarland standard of bacteria was prepared. Then, Mueller-Hinton agar plates were seeded with a lawn of bacteria. By using of a cup-borer, well was prepared in the plates and the tested microorganisms was pipetted in the well. The plates were placed at room temperature for 1 h in order to penetrate of compound into the agar. The Petri dishes were incubated at 37 °C for 24 h. Ciprofloxacin used as standard. Finally, the inhibition zone diameter was measured in term of mm.

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## 3. Result and Discussion

### 3.1. Preparation of ethanol/methanol extract of Vasambu (Acorus calamus)

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Similarly, we have followed the identical extraction procedure with methanol (2 X 50 mL) to get the maximum yield of the desired natural product; this quantitative amount of product can be utilized for different preliminary studies of biological application. Then the filtered organic layers of ethanolic/methanolic extracts were analyzed with thin layer chromatography (*tlc*); both extracts havea similar pattern (*tlc* spots). Later, the organic layers were combined and concentrated,which afforded 18.31 gm of crude residue.

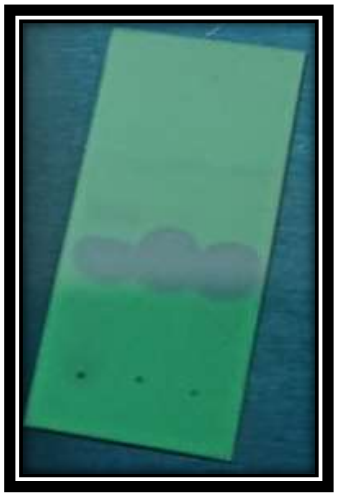

**Figure 1. Representation of crude residue TLC image under UV light (254 nm)**

### 3.2. Isolation of components using column chromatography

It is unusual and quite surprising to note that the *tlc* pattern was evident, consisting of one significant product spot along with one mild spot in the difference of 0.3 Rf. Also, it is noteworthy to mention that the aroma of the crude product itself is gratifying.

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The crude residue from the combined ethanolic/methanolic extraction was re-dissolved in chloroform to get a transparent solution and made into a slurry using silicagel (100-200 mesh). Subsequently, it was loaded into a freshly packed glass column (Silica gel, 100-200 mesh) and was used for this purification.We began with pure hexane as an eluent, and after that, we slowly increased the polarity with ethyl acetate (3-5%) and maintained the same ratio throughout column completion. This mixture ratio furnished the desired α-Asarone as a pure and significant product along with a minor product in traces. The isolated major product is a yellow oily liquid (8.36 gm), and the aroma is little resemblance to the odor of deep-fried pepper. Moreover, the unidentified minor product (off-white solid, 18 mg) is not enough for complete characterization/ bio-application, so we are not interested in exploring it.

**Figure 2. Traditional column chromatography image of crude residue.**

**Figure 3. Image of crude slurry (left-A) and pure compound 1 after isolation (right-B)**

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### 3.3. Characterization of compound 1

Considering the importance of unexplored natural products with the embedded fascinating bio-profile of this current analogue has been explored. Although we have isolated the desired product in highly pure form, it was thoroughly characterized using analytical techniques. The isolated scaffold seems interesting, and mainly the compound has been reported meagerly in the scientific literature with limited biological application.

### 3.4. NMR analysis of isolates

The nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance-III 400 NMR spectrometer. Proton (1H) and carbon (13C) NMR analysis were carried out for the purely isolated compound 1 in liquid state samples.The structure of compound **1**was unambiguously elucidated using 1D NMR ($^{1}$H and$^{13}$C).

### 3.5 FTIR Analysis of isolates

FT-IR spectra were recorded on Affinity-I Shimadzu FT-IR spectrometers with a universal ATR accessory and are reported in wavenumbers ($cm^{-1}$).Similarly, the structure was further evidently confirmed and validated by FTIR, high-resolution mass spectral data. For instance, the olefinic CH and phenyl ring CH and $cm^{-1}$and two hydroxyl groups were identified at 2933, 1506, 1207, 1026, 858, and 756 $cm^{-1,}$ respectively.

### 3.6. HRMS Analysis of isolates

The isolated compound α-Asarone (1)mass was confirmed on High-resolution mass spectra (HRMS) were recorded using Electro-spray Ionization (ESI) on a time of flight (TOF) techniques using WATERS–XEVO G2-XS-QtoF spectrometer.Similarly, in high-resolution mass spectra (HRMS), the calculated mass of the isolated scaffold is209.1172(for $C_{12}H_{17}O_3$ $[M+H]^+$) and found massof 209.2646matches the calculated one.

### 3.7. Spectral dataof α-Asarone (1)

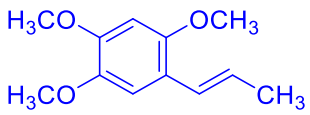


**Figure 4: (*E*)-1,2,4-trimethoxy-5-(prop-1-en-1-yl) benzene**

The crudeproduct was extracted from the Vasampu powder (*Acorus calamus*) using the typical method. The combined organic layer was concentrated under a vacuum. The resulting crude mixture was purified by manual gravity-column chromatography on 100-200 mesh silica gel using n-hexane in EtOAc (3-5%) as an eluent to affordα-Asarone**1**as apale yellow oily-liquid (8.36 g).

**$^{1}$H NMR** ($CDCl_3$, 400 MHz): δ 6.85 (s, 1H), 6.54 (s, 1H), 6.50-6.47 (m, 1H), 5.80-5.75 (m, 1H), 3.90 (s, 3H), 3.84 (s, 3H), 3.82 (s, 3H), 1.85-1.83 (m, 3H).

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**$^{13}C\{^{1}H\}$ NMR** ($CDCl_3$, 100.6 MHz): δ 151.6, 148.6, 142.4, 125.9, 124.8, 118.1, 114.2, 97.6, 56.7, 56.5, 56.1, 14.7.

**IR (neat):**2933, 1506, 1207, 1026, 858, 756 $cm^{-1}$.

**HRMS** (ESI) m/z calcd. for $C_{12}H_{17}O_3$ $[M+H]^+$: 209.1172, found: 209.2646.

**Figure 5: $^{1}H$ NMR (with expansion) spectra of α-Asarone**

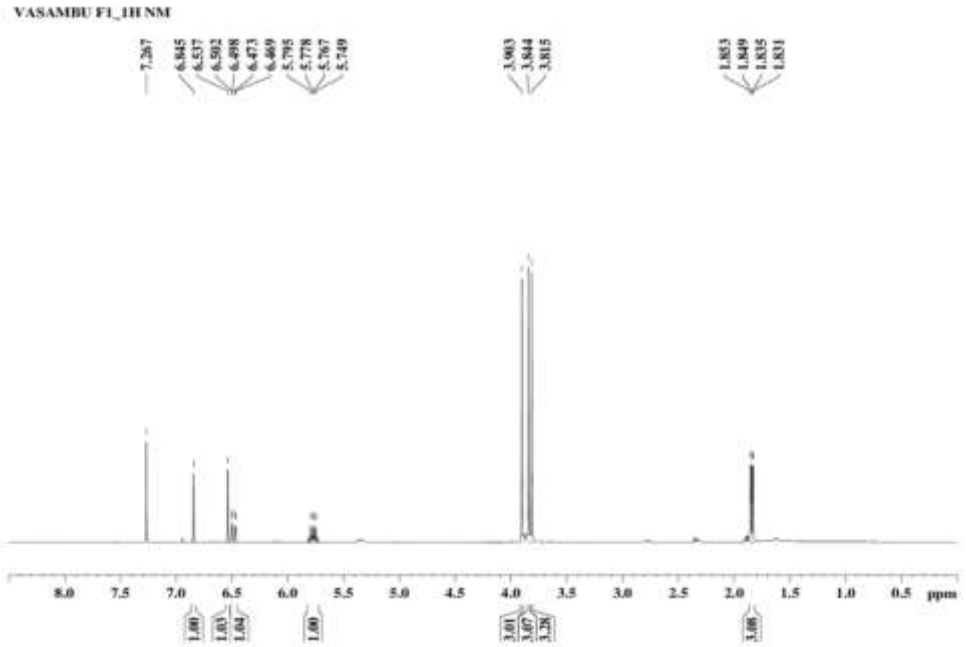


**Figure 6: $^{13}C$NMR spectra of α-Asarone**

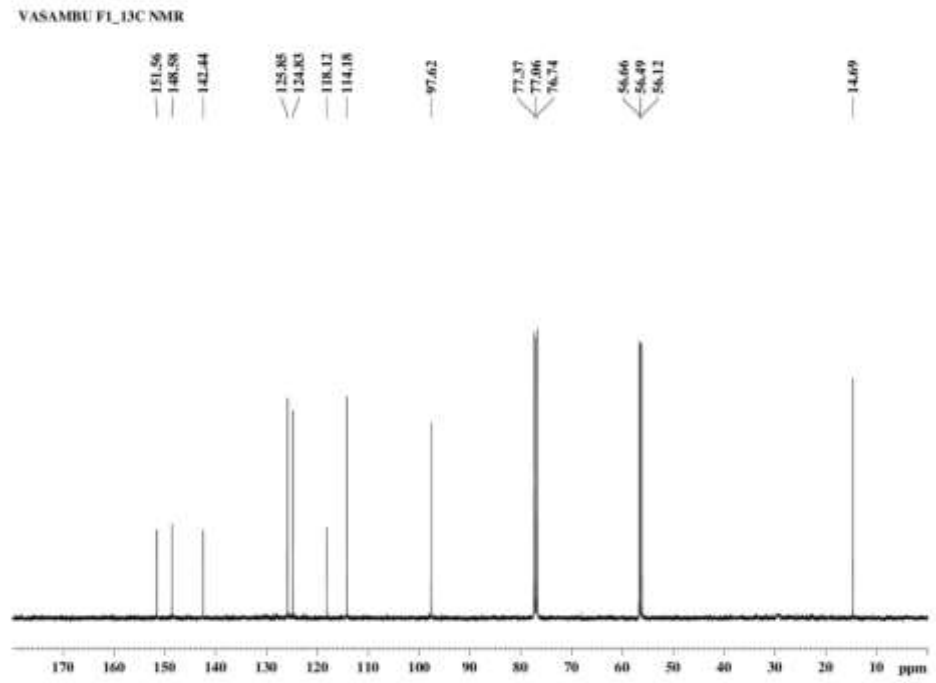

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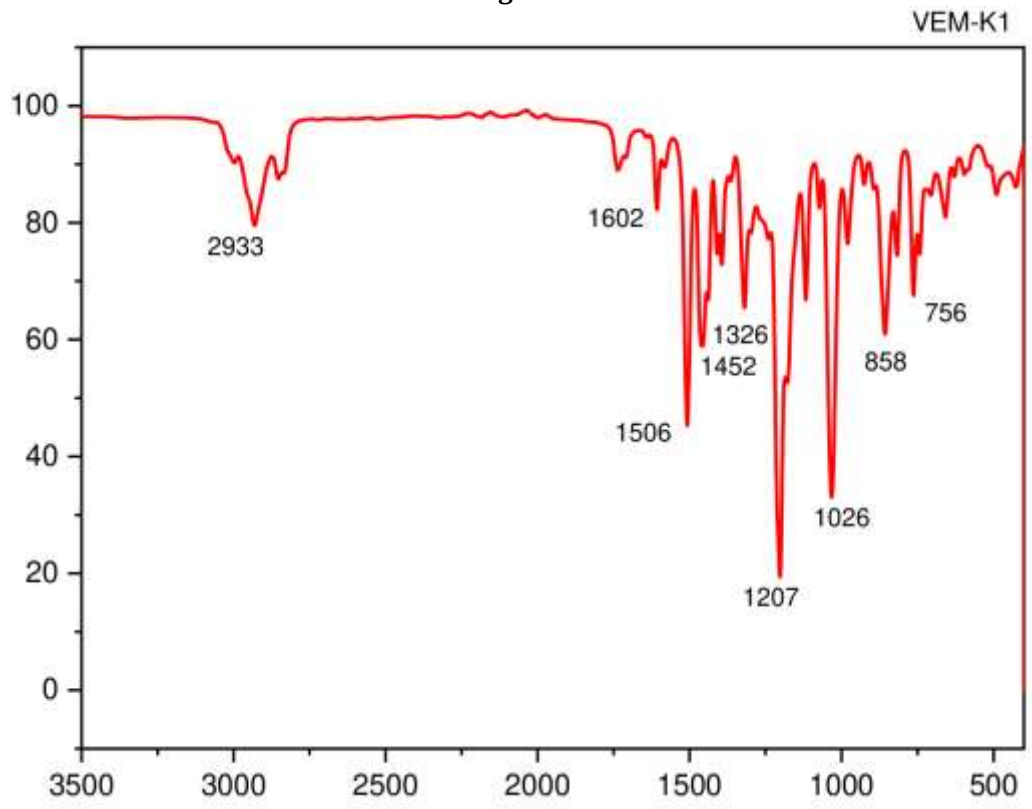
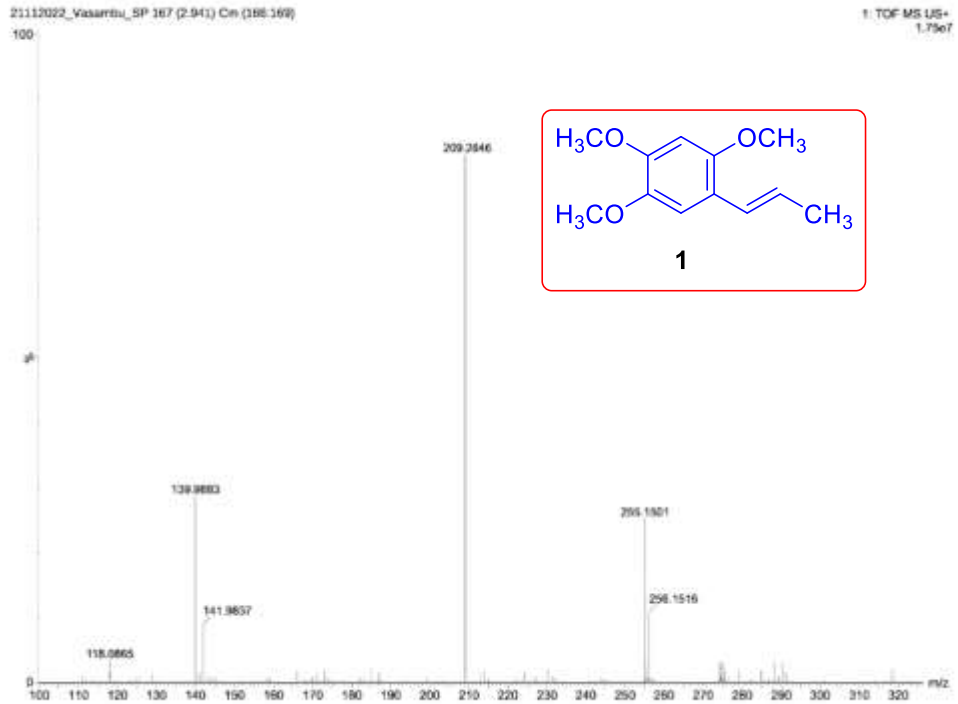
**Figure 7: FT-IR**




HRMS **Figure 8: Spectra of α-Asarone**



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### 3.8. Antibacterial activity of alpha asarone

After incubation the plates were removed and the results obtained were observed and noted. The method is based upon the diffusion of compound and standard from a well into solidified nutrient agar layer in a petriplate to such an extent that growth of the added microorganism is prevented entirely in a circular zone. Four different concentrations (10, 20, 30 and 40 µl) of alpha asarone were tested for antibacterial activity by using well diffusion method. In the present study, gram positive *S. aureus* and gram-negative organisms *E. coli, pseudomonas aeuriginosa, Plesiomonas shiselloide* and *Aeromonas salmonicida* were selected. Ciprofloxacin, the standard antibacterial drug used in the present study. The data are summarized and represented in Table 1 and Figure 9.

**Table: 1: Antimicrobial activity of different Bacterial species against α – Asarone**

| S. No | Organism Name (Bacterial Species) | Concentration (µl) | Zone of inhibition diameter (mm) | Activity |
|---|---|---|---|---|
| | | | | |
| 1. | *Pseudomonas aeroginosa* | 10 | 7 | Lower activity |
| | | 20 | 8 | Lower activity |
| | | 30 | 9 | Lower activity |
| | | 40 | 10 | Moderate activity |
| 2. | *Plesiomonas shiselloides* | 10 | - | Lower activity |
| | | 20 | 8 | Lower activity |
| | | 30 | 9 | Lower activity |
| | | 40 | 10 | Moderate activity |
| 3. | *Staphylococus aureus* | 10 | - | No activity |
| | | 20 | - | No activity |
| | | 30 | 7 | Lower activity |
| | | 40 | 8 | Lower activity |
| 4. | *Aeromonas salmonicida* | 10 | - | No activity |
| | | 20 | - | No activity |
| | | 30 | 7 | Lower activity |
| | | 40 | 8 | Lower activity |
| 5. | *Escherichia coli* | 10 | - | No activity |
| | | 20 | 8 | Lower activity |
| | | 30 | 9 | Lower activity |

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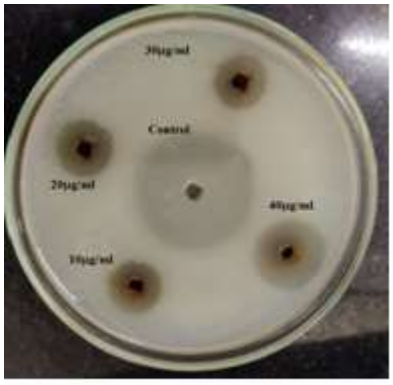
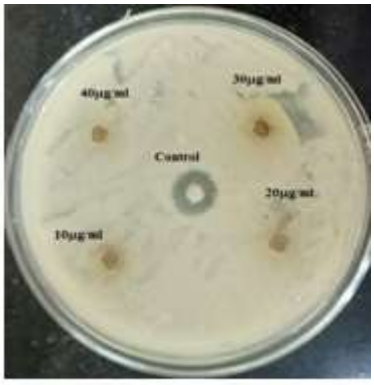
| | | | | |
|---|---|---|---|---|
| | | 40 | 10 | Moderate activity |
| 6. | *Standard* **(Ciprofloxacin)** | *Pseudomonas aeroginosa* | 14 | Moderate activity |
| | | *Plesiomonas shiselloides* | 13 | Moderate activity |
| | | *Staphylococus aureus* | 10 | Moderate activity |
| | | *Aeromonas salmonicida* | 13 | Moderate activity |
| | | *Escherichia coli* | 16 | Moderate activity |

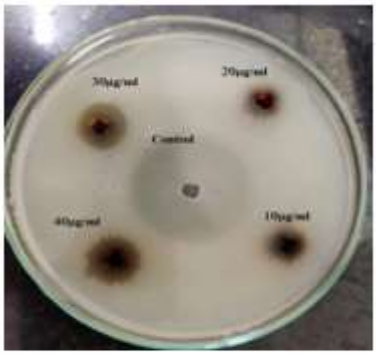
**Figure 9: Antibacterial activity of α – Asarone**



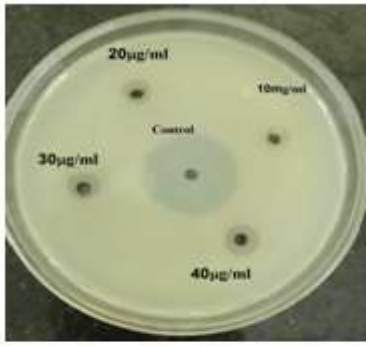
*Pseudomonas aeruginosa*



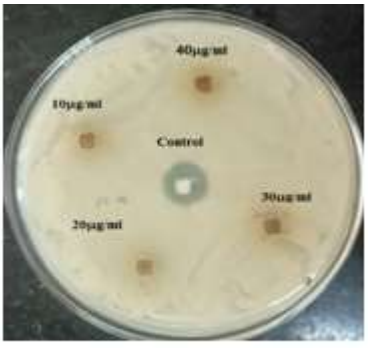
*Plesimonas shiselloides*



*Staphylococus aureus*




*Aeromonas salmonicida*



Escherichia coli

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α – Asarone showed a moderate activity against, *pseudomonas aeuriginosa (10mm at 40* µl), *Plesiomonas shiselloide (10mm at 40* µl) and *E. coli (10mm at 40* µl) respectively. Regarding other concentrations c showed a lower activity against *pseudomonas aeuriginosa* (7mm, 8mm, 9mm at 10, 20, 30 µl, respectively), *Plesiomonas shiselloide* (8mm, 9mm at 20, 30 µl, respectively), *S. aureus* (7mm, 8mm at 30, 40 µl, respectively) *Aeromonas salmonicida* (7mm, 8mm at 30, 40 µl, respectively) *E. coli* (8mm, 9mm at 20, 30 µl, respectively).

No activity against *Plesiomonas shiselloides* at 10µl, *Staphylococus aureus at* 10µl and 20µl, *Aeromonas salmonicida at* 10µl and 20µl and *Escherichia coli at 10*µl respectively. Similarly, standard Ciprofloxacin showed a moderated activity against *S. aureus (10), Aeromonas salmonicida* (13) E. coli (16), *pseudomonas aeuriginosa (14) and Plesiomonas shiselloide (13), respectively and at Escherichia coli*. From the report it was confirmed that all the organisms except *S. aureus and Aeromonas salmonicida* exhibited a moderate growth against the α – Asarone at 40µl as similar to the standard drug Ciprofloxacin.

From the results it is shown that the α – Asarone seems to give appreciable antibacterial against intestinal bacteria, this indicates that the compound acts specifically against the gram – positive cell wall, because gram – positive bacteria are surrounded by a thicker peptidoglycan cell wall than gram-negative bacteria. This outer membrane is composed of lipopolysaccharide that offer little resistance than gram – negative bacteria extra resistance against extract that cannot penetrate easier. (Nikolaidis*et al.*, 2014).

It was previously reported that Gram – Negative bacteria are more resistant to the plant extracts because of the presence of hydrophilic cell wall essentially constituted by lipo-polysaccharide (LPS) that prevents the penetration of organic extracts to the target cell membrane Reynoids 1996; Bezic*et al.*, 2003. This is the reason that Gram – positive bacteria were prone to be more sensitive to extract than Gram – negative bacteria. This is well matched with the present study that the ethanolic extract of henna inhibited the growth of all Gram-positive bacteria to some extent than Gram – negative bacteria.

**4. Conclusion**

In summary, the current work deals with crude product extraction from the commercial sample (*Acorus calamus*), isolation through column chromatography and structural elucidation through analytical data like NMR, FT-IR and HRMS and agar antibacterial activity through well diffusion method. Further, the biological

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evaluation of isolated products has been performed the anticancer study. The structurally attractive natural product α-Asarone (1) was isolated through simple extraction/column purification techniques and proved that it has antibacterial activity. Although, we firmly believe that the current study will have a beneficial place in molecular biology and will get medicinal chemist attention for evaluating against several rare diseases.

**Acknowledgement:**

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