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Evaluation of Phytochemical Constituents in the Methanolic Leaf Extracts of *Acorus calamus* L.

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ABSTRACT

The chemical constituents of *Acorus calamus* L. leaves have been evaluated using GC-MS and FT-IR. Building upon prior studies that identified antimicrobial activity of *A. calamus* leaf extracts, the objective of this study was to identify the bioactive antimicrobial and other phytochemical compounds in the leaf extract using GC-MS and FT-IR analyses. Preliminary analysis revealed the presence of tannins, terpenoids, alkaloids, phenols, glycosides, and steroids. Of all identified compounds in methanol extracts, the abundant compounds were asarone (41.09%), 9, 12, 15-octadecanoic acid (Z, Z, Z-) (4.74%), Hexadecanoic acid (4%), alpha linolenic acid (3.66%), vitamin E (1.80%), 2-methoxy-4 vinyl phenol (2.54%), phytol (0.46%) and stigmast-5-en-3-ol, (3. beta)-. (0.75%). The FTIR analysis verified the presence of alcohols, phenols, alkanes, alkenes, aldehydes, ketone, and aromatic amines. The study presented here reports the first conclusive evidence indicating the presence of important phytochemical constituents in the leaf extracts of *A. calamus,* which may suggest leaf extract as potential candidate for effective pharmaceutical formulations. The current method of using rhizome limits the plant usage to one time only. Using leaves extract of *A. calamus* avoids culmination of entire plant and hence, preserves this endangered herb.

INTRODUCTION

Natural remedies from medicinal plants for treatment of various diseases have been shown to be safe and effective. Many plant species have been used in folklore medicine for the treatment of various ailments (Babamurugan et al., 2012). Phytoconstituents from medicinal plants are important in pharmaceutical industry for preparation of therapeutic agents and drug development (Nisha et al., 2011). The development of medicines starts with the identification of active compounds, detailed biological assays and dosage formulations followed by clinical analysis to establish safety and efficacy of the new drug (Ncube et al., 2008). Plants and fruits are considered as one of the main sources of important phytocompounds. Plants contain large number of secondary metabolites such as flavonoids, phenols, steroids, alkaloids, saponins and glycosides (Shahidi, 2008).

Acorus calamus L. (Family: Acoraceae), commonly known as Sweet Flag is a perennial monocot herb and identified as an endangered medicinal plant species (Balakumbahan et al., 2010). It is an extensively branched, cylindrical rhizome up to 2.5 cm thick, purplish brown to light brown

externally and white internally. It is used as a relief to the digestion disorders, appetite, stomach cramps and colic (Balakumbahan et al., 2010). Plant leaves, rhizomes and its essential oil has various biological actions like antispasmodic, carminative, mental ailments remedy, chronic diarrhea, dysentery, tumors reliever and used for treatment of epilepsy (Devi and Ganjewala 2009). It has antifungal, antibacterial, antidiarrheal, insecticidal properties (Phongpaichit et al., 2005), tranquilizing, antidyslipidemic, antioxidant, anticholinesterase, spasmolytic, neuroprotective, vascular modulator activities (Shaha and Gilani, 2010). Various extracts of *A. calamus* are traditionally used for the antidiabetic, immunosuppressive, hypolipidemic, mitogenic, antiproliferative, and anticarcinogenic activity towards human lymphocytes (Palani et al., 2010). It has been reported that leaves extracts of *A. calamus* show antimicrobial activities (Khatri et al., 2016).

A knowledge of the chemical compounds of plants is desirable not only for the discovery of therapeutic agents, but also because such information may be of immense value in producing new phytocompounds for discovering the actual significance of folkloric remedies (Milne, 1993). Hence a thorough validation of the herbal drugs has emerged as a new branch of science emphasizing and prioritizing the standardization of natural drugs and products because several of the phytochemicals have complementary and overlapping mechanism of action. Mass spectrometry, coupled with chromatographic separations such as Gas chromatography (GC/MS) is used for direct analysis of components present in medicinal plants and traditional medicines. For the analyses of non-polar components and volatile essential oil, fatty acids, lipids and alkaloids, GC-MS approach is a valuable method (Jie and Choi, 1991; Betz et al., 1997). Gas Chromatography Mass Spectroscopy is a reliable and compatible method to identify the unknown organic compounds in a complex mixture by matching the spectra with reference spectra. FTIR (Fourier Transform Infrared Spectroscopic Analysis) and GC-MS, are powerful tools for identification and determination of bioactive constituents and have attracted great attention among researchers (Roberts and Xia, 1995).

Though research has been conducted on the essential oil extracted from rhizomes of *A. calamus* (Sharma et al., 2020), limited information is still available from leaves oils. To our best knowledge, this is the first description on the phytochemical constituents of the leaves extracts of *A. calamus* which show pharmacological significance. The objective of this study was to identify the phytochemical compounds in the methanolic extract of A. calamus leaves using GC-MS and FT-IR analyses.

MATERIALS AND METHODS

Plant Material. Acorus calamus plants used in this study were collected from Ch. Devi Lal Herbal Nature Park – Chuharpur, Yamuna Nagar (Haryana) and maintained in the university nursery.

Preparation of the Extract. The fresh and healthy *A. calamus* leaves closest to the shoot meristem were collected in the month of May, washed and air dried at room temperature for 7 days until no moisture was present. Later, the dried leaves were ground and 10g of powdered plant part were subject to successive extraction with 50 mL of methanol solvent using Soxhlet apparatus. The solvents were evaporated to dryness and the residue was stored at 4°C in a refrigerator.

Phytochemical Screening. The methanolic plant extract was tested for the presence (+) or absence (-) of secondary metabolites such as flavonoids, sugars, amino acids, and proteins, terpenoids, tannins, phenols, alkaloids, saponins and phytosterols.

Shinoda's Test for Flavonoids. About 0.5 g of plant extract was dissolved in ethanol, warmed, and then filtered. One piece of magnesium chip was then added to the filtrate followed by concentrated HCl added dropwise and heated. A pink, orange, or red to purple coloration confirmed the presence of flavonoids (Sofowara, 1993, Trease and Evans, 2000).

Test for Terpenoids. About 0.5 g of plant extract was

dissolved in ethanol, then added 1 mL of acetic anhydride was added followed by the addition of concentrated H_2SO_4. A reddish-brown color at interface showed the presence of terpenoids (Sofowara, 1993, Trease and Evans, 2000).

Test for Tannins and Phenols. About 0.5 g of plant extract was dissolved in 10 mL of distilled water and then filtered. 1 mL of 1% ferric chloride solution was added to 2 ml of the filtrate. A dark green or blue-green precipitate indicated the presence of tannins and phenols (Kumar et al., 2007, Khatri et al., 2017).

Test for Alkaloids. Mayer's test. About 0.5 g of plant extract was boiled with 1 ml of 1% HCl on water bath and filtered. To this filtrate, few drops of Mayer's reagent were added, and appearance of precipitate was taken as positive test for alkaloids (Kumar et al., 2007; Khatri et al., 2017).

Test for Saponins. Approximately, 1 g of dried leaves was boiled with 5 ml of distilled water and filtered. The filtrate was diluted with 3 ml of distilled water and shaken for 15 minutes. The development of stable foam confirmed for the presence of saponins (Kumar et al., 2007; Khatri et al., 2017).

Test for Sugar. About 0.5g of plant extract was dissolved in 4 mL distilled water and then filtered. The filtrate was heated with 5 mL of Fehling's solution. The formation of a red precipitate of cuprous oxide indicated the presence of sugar (Sofowara, 1993; Trease and Evans, 2000).

Test for Glycosides and Sterols. Salkowski test. 10 mg of extract was dissolved in 2 mL of chloroform and 2mL of concentrated sulphuric acid were added from the side of the test tube. Test tube was shaken for few minutes and the formation of red color in chloroform layer confirmed the presence of glycosides and sterols (Kumar et al., 2007; Khatri et al., 2017).

Test for Amino acids and Proteins. Few drops of Millan's reagent were added to 0.5g of plant extract and followed by gentle heat. A reddish-brown coloration or precipitate indicated the presence of amino acids and proteins (Sofowara, 1993; Trease and Evans, 2000).

GC-MS Analysis. Methanolic extract of *A. calamus* leaves was investigated using GC-MS analyzer (GCMS-QP2010 Plus). Carrier gas was Helium at a regular flow of 1.2 mL/min, an injection volume of 2.0 µL, injector temperature 260°C and ion source temperature 230°C was used. The oven temperature operating mode: 100°C held for 1 min, rising at the rate of 10°C per min up to 250°C with 6 min hold, rising at the rate of 15°C per min up to 300°C with 20 min hold up. The total running time of GC-MS was about 46 min.

*Identification of Biomolecules***.** The identity of the component in the plant extract was based on their peak retention time, peak area (%), height (%) and mass spectral fragmentation patterns. Interpretation of mass spectrum GC-MS was done using the database of National Institute Standard and Technique (NIST). The spectrum of the unknown component was studied by the comparison of their retention indices and mass spectra fragmentation patterns with those stored in the WILEY library (Khatri et al., 2017).

FTIR Spectroscopic Analysis. FTIR analysis was achieved using Perkin Elmer Frontier spectrophotometer system to detect the individual peaks and their functional groups using ATR (Attenuated Total Reflectance) accessory. The IR scan was attained in the wave number region of 4000-550 cm^{-1} (mid- infrared range).

RESULTS AND DISCUSSION

Preliminary Screening. For preliminary screening of secondary metabolites, chemical tests were performed on the plant extracts (methanol) to confirm the presence of tannins and phenols, proteins, sugars, glycosides, and sterols, terpenoids and alkaloids (Table 1).

The GC-MS analysis of methanolic extract of *A. calamus* showed the presence of 15 compounds that could contribute towards medicinal value of the

plant. The detection of the phytochemical compounds was based on the peak area, area percentage and retention time. The active principles with their retention time (RT), peak area and area in percentage are provided in (Figure 1 and Table 2). The compound identified with less retention time (19.033) was pentadecanoic acid whereas stigmast-5-en-3-ol, (3. beta.)- was the last compound with longest retention time (40.455) in the methanolic extract of *A. calamus*. Asarone was identified as the major component with highest area percentage (41.09%), follow by 9, 12, 15- octadecatrienoic acid, (Z, Z, Z)- (4.74%), hexadecanoic acid (4.02%), alpha linolenic acid (3.66%), 2-methoxy-4 vinyl phenol (2.56%), 2-ketohexanoic acid (2.28%), phytol (0.46%) and octadecenoic acids was detected as the minor compound (0.27%) (Table 2).

Similar phyto-compounds were also detected in methanol fruit extract of *Terminalia bellirica* - 9,12,15-octadecatrienoic acid, (Z, Z, Z)- possesses anti-inflammatory, insecticidal, hypocholesterolemia, cancer preventive, nematicide, antiarthritic and anticoronary properties. n-hexadecanoic acid - palmitic acid can be an antioxidant, hypocholesterolemic, nematicide, pesticide, lubricant activities and hemolytic 5-alpha is a reductase inhibitor (Amala and Jeyaraj, 2014). In the leaf oils of *A. calamus*, phenolic compounds analyzed using GC and GC-MS, showed strong inhibitory effect against *Mycobacterium sp., Bacillus subtilis, Fusarium avenacium* and *Rhizomucor pusillus* (Radusiene et al., 2006). In addition, methanolic extract of *A. calamus* has shown antimicrobial activity against gram positive and gram-negative bacteria (Khatri et al., 2016). Phytol is an antimicrobial, anticancer, anti-inflammatory, and diuretic agent (Kumar et al., 2010 a). A total of 15 compounds have been identified which show biological activity in other plant species (Table 3). Likewise, Rani et al. (2011) observed the presence of phytol in the leaves of *Lantana camara* and Sridharan et al. (2011) in *Mimosa pudica* leaves. Phytol was observed to have antibacterial activities against *Staphylococcus aureus* causing damage to cell membranes as a result there is a leakage of potassium ions from bacterial cells (Inoue et al., 2005).

Asarone was isolated from the rhizome of *A. calamus* and has shown to possess antibacterial, anthelmintic, antipyretic, antispasmodic activity and used as cardio depressant. Asarone has also reported to have fungicidal action against phytopathogenic fungus (Sharma et al.,1961; Sharma et al., 1969; Nigam et al., 1990; Devi and Ganjewala, 2009; Kumar et al., 2015). Kumar et al. (2010a) has reported the presence of benzene, 1,2-dimethoxy-4-(2-propenyl)-, linolenic acid, n-hexadecanoic acid, 9,12-octadecadienoicacid in the ethanolic rhizome extract. *A. calamus* ethanol rhizome extracts has revealed the presence of 7 compounds such as phytol (major compound), 4,5,7trihydroxy isoflavone, picrotoxinin, 9,11-octadecadienoic acid, Piperazine,1- [5-methoxy-3,4-methylenedioxyphenyl]-4[4-methylbenzyl], Pregn-4ene- 3.20-dione, 16-methyl-6 methylene, (16a), Corynan-17- ol,18,19-didehydro-10-methoxy, acetate(ester) (Shanmuga Priya et al., 2017).

Stigmasterol is a steroidal compound used as the precursor of vitamin D3 and it may be employed as antimicrobial, anticancer, anti-arthritic, antiasthma, diuretic and anti-inflammatory and useful in prevention of certain cancers, including ovarian, prostate, breast and colon cancers (Gabay et al., 2010). Present study has also reported the same and more phytochemical compounds as asarone, phytol, stigmasterols, n-hexadecanoic acid, benzene acetaldehyde, 2-ketohexanoic acid, 5- hydroxy methyl furfural, pentadecanoic acid, beta-D-Glucopyranose, 1, 6- anhydro, linolenic acid, octadecanoic acid that are present in the rhizome of *A. calamus* which shows that the leaf extract can also be used for pharmaceutical purposes. Chemical components including hexadecanoic acid, 9, 12-octadecanoic acid, octadecanoic acid, 9-octadecenoic acid, eicosanoic acid, benzoic acid, linolenic acid and stigmasterol were reported in Gmelina *arborea* and the medicinal properties of *G. arborea* was attributed to the presence of those compounds (Ukkinen, 1982; Vijay et al., 2011; Mahadkar et al., 2013). As per best of our knowledge very few or no report is present on the GC-MS study of methanolic leaf extracts of *A. calamus*.

Phytochemical Constituents and Their Functional Groups Identification using FT-IR Spectrum Analysis. The results of FT-IR spectroscopic analysis of *A. calamus* revealed the presence of alcohols, phenols, alkanes, alkynes, alpha and beta- unsaturated aldehydes, ketones, aromatic ring, aromatic amines, alkyl halides, aliphatic amines, ether linkage and alkenes (Figure 2 and Table 4). The absorption at 3352.4 cm^{-1} is due to the H-bond responsible for alcohols and phenols present in the extract. The band at 2975.6 cm^{-1}, 1382 cm^{-1} and 2901cm^{-1} is due to C-H stretching showing alkanes. The band in 1919.8cm^{-1} showed alkynes (C=C stretch), band at 1649cm^{-1} showing alpha and beta-unsaturated aldehydes and ketones (C=O stretch), the band at 1452.7 cm-1 showed aromatic ring (C=C stretch). The band at 1323.2 cm-1 showed aromatic amines and the band at 1276.1 cm^{-1} showed alkyl halides C-H wag (-CH_2 X). The band at 1083.8 cm^{-1} showed aliphatic amines compounds (C-N stretch) and the band at 1040.6 cm^{-1} showed alkenes (C-H stretch).

FT-IR spectral analysis has been especially useful for compound identification, when run under IR region in the range of 400-4000 cm-1 there was a variation in the peaks of plant samples (Kalaiselvi et al., 2012; Khatri et al., 2019). In the present analysis, the methanolic extract of *A. calamus* (leaves) was subjected to FT-IR analysis, the functional groups of the components were separated based on its peak ratio and hence chemical compounds were identified. The peak at 2923.95 - 2975.6 cm^{-1} assigned to the C-H stretching which means that alkane compounds existed in rare medicinal plants (Starlin et al., 2012). The bands between 3000 and 3352.4 cm^{-1} represent C-H stretching vibrations that are generated by alcohols and phenols (Mohani et al., 2014).

CONCLUSIONS

This report shows the presence of important phytochemical compounds in methanol leaf extract such as asarone (41.09%), 9, 12, 15-Octadecatrienoic acid, (Z, Z, Z)- (4.74%), hexadecenoic acid (4.02%), alpha linolenic acid (3.66%), 2-methoxy-4 vinyl phenol (2.56%), 2-ketohexanoic acid (2.28%), vitamin E (1.80%) and stigmast-5-en-3-ol, (3. beta)- (0.75%) which may help for effective pharmaceutical formulations. Usage of leaf extract leads to an efficient way of extracting the compounds from same plant multiple times. Current method of using rhizome and essential oil from rhizome limits the plant usage to one time only. Thus, using leaves for compound extraction provides significant advantages over rhizome harvesting including 1) overall time reduction (leaves provide a consistent and readily available source) and 2) the use of one plantation multiple times. This proposal will lead to shorter drug formulation times and will help protect this endangered plant.

Table 1. Preliminary phytochemical screening methods used for the methanolic leaf extract of *A. calamus*

Phytochemicals	Tests	Test result*
Flavonoids	Shinoda's test	-
Saponins	Foam test	-
Tannins and Phenols	Ferric chloride test	+
Amino acids and Proteins	Millions test	+
Sugars	Fehling's Test	+
Glycosides and Sterols	Salkowski test	+
Terpenoids	Salkowski test	+
Alkaloids	Shinoda test	+

(+) = Positive (present); (-) = Negative (absent)

Table 2. Phytocompounds identified in methanolic extract *of A. calamus* leaves as per NIST library

Biomolecules	*A. calamus*		
	Retention time	Total Area	Area%
Alpha Linolenic acid	21.458	17459925	3.66
Vitamin E	35.384	8566587	1.80
Benzene acetaldehyde	7.062	2467712	0.52
2- Ketohexanoic acid	14.117	10888247	2.28
2-methoxy-4 vinyl phenol	11.500	12237182	2.56
Pentadecanoic acid	19.051	14276093	2.99
Hexadecanoic acid, Ethyl ester	19.815	19166191	4.02
Phytol	20.497	2178513	0.46
9, 12, 15- Octadecatrienoic acid, (Z, Z, Z)-	20.802	22618804	4.74
Octadecanoic acid	18.882	1136533	0.24
9- Octadecenoic acid (z)-	22.234	1104473	0.23
Stigmast-5-en-3-ol, (3. beta)-	40.455	3555070	0.75
Asarone	15.498	196028044	41.09

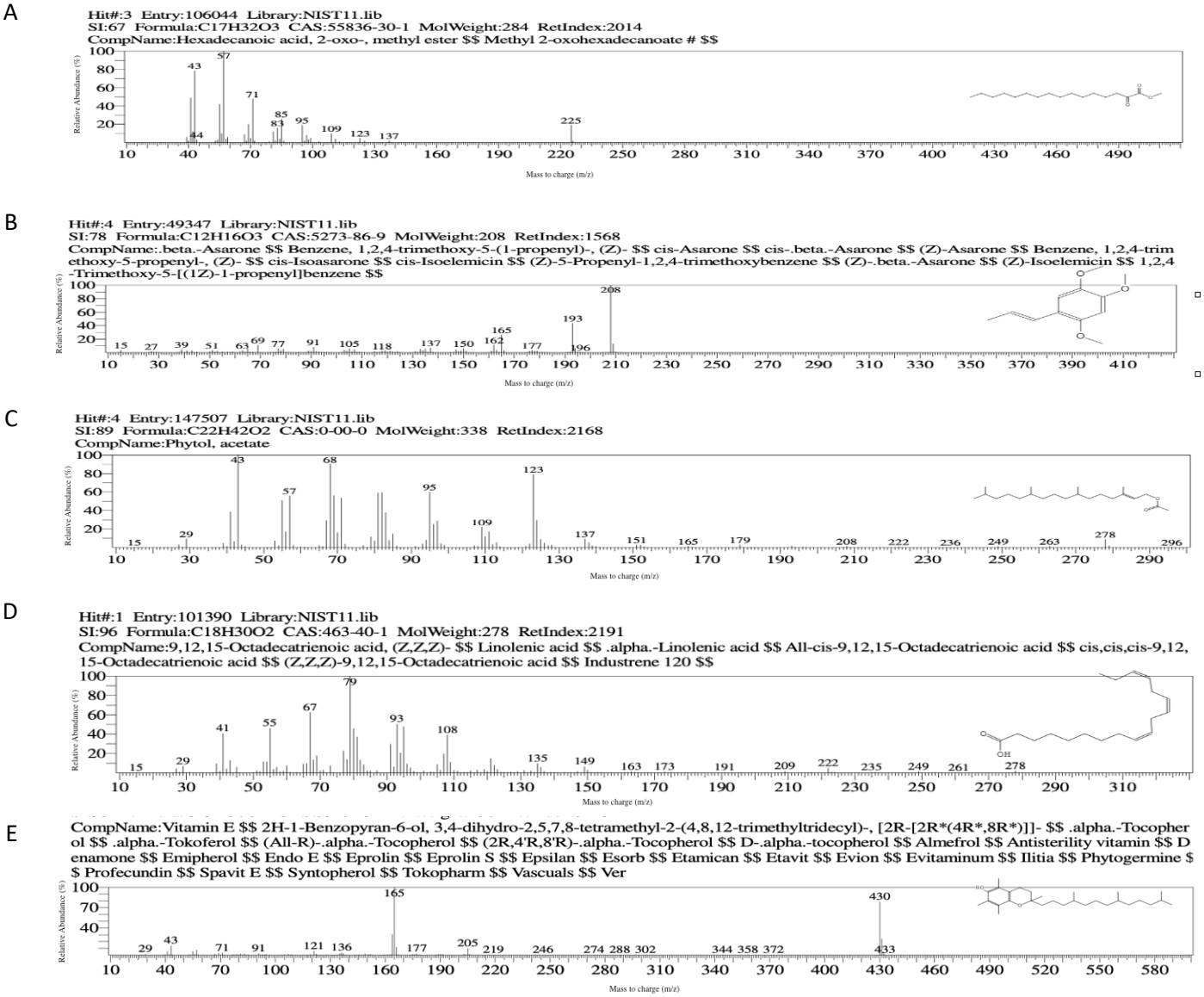

Figure 1. Mass spectra of identified compound from methanolic leaf extract of *A. calamus*. A) Hexadecanoic acid, B) beta-asarone, C) Phytol, D) 9, 12, 15- Octadecatrienoic acid, (Z, Z, Z), E) Vitamin E.

Table 3. Phytochemical analysis of major components in the methanolic extract of *A. calamus* (per NIST library)

Compound	Nature	Activity/ Role	References
Vitamin E	Vitamin	Anticancer, Anti-aging, Analgesic, Antidiabetic, Anti-inflammatory, Antioxidant, Antitumor Hepatoprotective, Hypocholesterolemic, Antirachitics, Anticoronary Antidermatitic, Antileukemic, Antiulcerogenic, Vasodilator, Antispasmodic	Kumar et al., 2010 a; Aparna et al., 2012
Benzene acetaldehyde	Aldehyde compound	Antimicrobial, Preservative	Sathish *et al*., 2012
2- Ketohexanoic acid	Fatty acid ester	Antioxidant, Hypercholesterolemia, Anticoronary, Androgenic, Hemolytic, Cancer preventive Hepatoprotective	Kumar et al., 2010 a; Aparna et al., 2012
5- Hydroxy methyl furfural	Aldehyde	Antiseptic, Flavor, Fungicide, Pesticide, Insecticides	Kumar et al., 2010 a
Benzene dicarboxylic acid	Plasticizer compound	Colorless crystalline acid used in synthesis of dyes and perfumes; Neurodegenerative disorders, Antimicrobial	Sathish *et al*., 2012
Alpha- Linolenic acid	Linolenic acid	Anti-inflammatory, Hypocholesterolemic, Cancer preventive, Hepatoprotective Nematicide, Insectifuge Antihistaminic, Antiacne, 5-Alpha reductase inhibitor Antiandrogenic, Antiarthritic, Anticoronary, Insectifuge	Ukkinen, 1982; Kumar et al., 2010 a
2-methoxy-4 Vinyl phenol	Phenolic compound	Antiviral, Fungicide, Antibacterial, Antioxidant, Antiseptic, Cancer preventive,	Kumar et al., 2010 a
Beta-D-Glucopyranose, 1, 6- Anhydro	Sugar moiety	Preservative	Khatri et al., 2017
Pentadecanoic acid	Fatty acid	Unique Fatty acid in nature, Used as Flavoring agent	Vijay et al., 2011; Mahadkar et al., 2013
Hexadecanoic acid, Ethyl ester	Palmitic acid ester	Antiandrogenic, Hemolytic 5-Alpha reductase Inhibitor, Antioxidant, Hypocholesterolemic, Nematicide, Pesticide, Flavor, Lubricant,	Sharafzadeh *et al*., 2011
Phytol	Diterpene	Antimicrobial, Anti-inflammatory, Diuretic, Anticancer	Rani et al., 2011; Khatri et al; 2017
9, 12, 15-Octadecatrienoic acid, (Z, Z, Z)-	Linolenic acid	Anti-inflammatory, 5-Alpha Reductase Inhibitor, Antiandrogenic, Antiarthritic, Anticoronary, Hypocholesterolemic, Cancer preventive, Hepatoprotective, Nematicide, Insectifuge, Antihistaminic, Antieczemic, Antiacne	Vijay et al., 2011; Mahadkar et al., 2013
9- Octadecenoic acid (z)-	Fatty acid	Anti-inflammatory, Anti arthritic, Hepatoprotective Hypercholesterolemic, Nematicide, Antimicrobial	Vijay et al., 2011; Mahadkar et al., 2013
Stigmast-5-en-3-ol, (3. beta)-	Steroidal compound	Precursor of vitamin D3, employed as antimicrobial, Anticancer, Anti-arthritic, Antiasthma, Diuretic and anti-inflammatory and useful in prevention of certain cancers including ovarian, Prostate, breast and colon cancers	Gabay et al., 2010
Asarone	Aromatic compound	Fungicidal action against phytopathogenic fungus, Antipyretic, Antispasmodic, Emetic Fungicide, Mutagenic, Sedative Myorelaxant, Tranquilizer, Pesticide, Cardio depressant, Psychoactive,	Sharma et al.,1961; 1969; Nigam et al., 1990; Devi and Ganjewala, 2009; Kumar et al., 2015

Table 4. FTIR spectra of the methanolic extract of *A. calamus* leaves.

Characteristic Absorption (s) cm ^{1}	Bond	Functional Group
3352.4	H- bonded	Alcohols, Phenols
2975.6	C-H stretch	Alkanes
2928.5	C-H stretch	Alkanes
2901.0	CH2-CH3 stretching	Alkanes
1919.8	CC stretch	Alkynes
1649.0	C=O stretching	σ, β- unsaturated aldehydes, Ketone
1452.7	C=C stretch	Aromatic ring
1382.0	C-H stretch	Alkanes
1323.2	C-N stretch	Aromatic amines
1276.1	C-H wag (-CH2 X)	Alkyl halides
1083.8	C-N stretch	Aliphatic amines
1040.6	C-O-C stretch	Ether Linkage
879.70	=C-H stretch	Alkenes

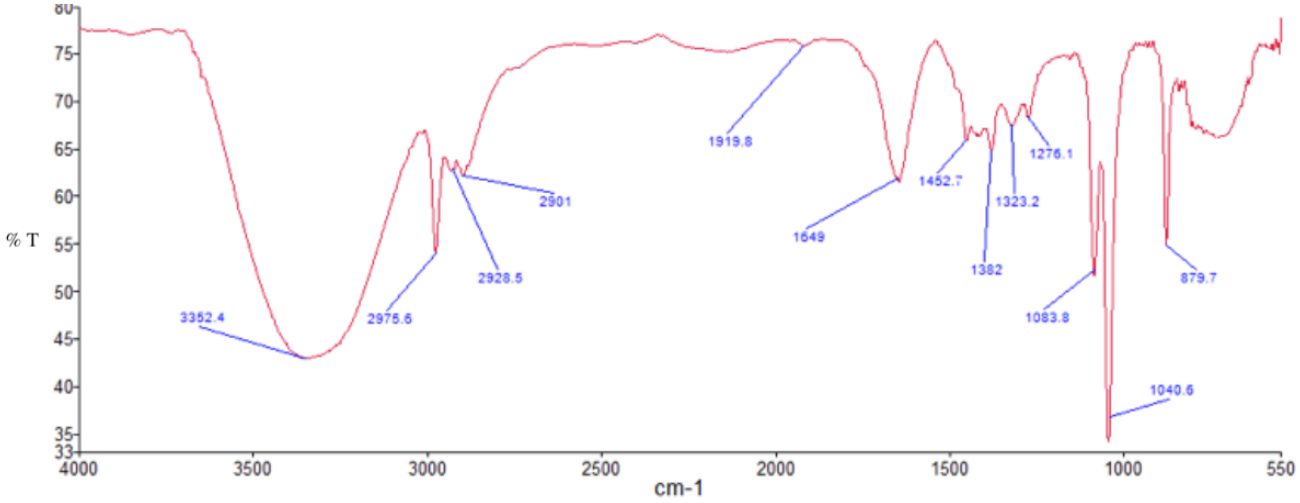

Figure 2. FTIR spectra of methanolic leaf extract of *A. calamus*.

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