

Original Research Article

Microwave Assisted Extraction and Evaluation of herbal plant Extract against Mastitis causing *Staphylococcus aureus*

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A B S T R A C T

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Mastitis is inflammation of the mammary gland and udder tissue. It is a key endemic disease of mammals including bovine and it stood next to Foot and Mouth Disease (FMD). It causes approximately 55% of total annual loss in dairy industries. It is occurred due to incursion of pathogenic bacteria through teat canal. Many bacteria are accountable for the disease but *Staphylococcus aureus* is consider as the focal causative agent of mastitis. Presence of many virulence gene and multi drug resistance gene makes *S. aureus* highly infectious. In the present study leaf extracts of *Withania somnifera*, *Calotropis procera*, *Tinospora cordifolia*, *Azadirachta indica*, *Datura innoxia* and bark extract of *Ficus racemosa* prepared in five different solvent was examined for its antibacterial activity against *S.aureus* isolated from mastitic samples. Maximum antimicrobial activity was observed in crude extract of *Datura innoxia* and *Ficus racemosa* which was prepared using methanol and methanol: Acetic acid mixture. Plant extracts which showed highest antimicrobial activity were further evaluated for its antioxidant activity and amongst it *D. innoxia* showed highest antioxidant activity. Partial purification of crude extract was carried out using column chromatography. Different fractions after column purification were analyzed using gas chromatography coupled mass spectrometry (GC-MS) to identify compounds. A GC-MS spectrum indicates presence of, gamalonic acid, chloropyramine, and scopolamine in fraction-1, whereas fraction-2 consists of cyclododecene, gamelonic acid, octadecenol 2-bromo, pentadecanoic, 1-heptadecene and octadecenoic. The purified product may be used as pharmaceutical agents.

Introduction

Medicinal plants have been extensively used for the treatment of different human and animal disease and have contain of high therapeutic value (Salem *et al.*, 2013). The antimicrobial assay of these plants has great

value to check its importance since the antibiotic resistance has become global concern. Infectious disease is the colonization of pathogenic microbes in the human and animal system and pathogen is

the micro-organism that has potentiality to cause the disease. India has ancient heritage of traditional medicine and have includes about 2000 species and has vast geographical area. Indian traditional medicines are based on various systems like ayurveda, sidhdha. unani and homeopathy and evaluation of this drug is primarily based on phytochemical, pharmacological and other allied approaches. The techniques involved for the evaluation of medicines are chromatography, microscopy and other (Padmaa, 2009). Human beings have used plants for the treatment of diverse ailment for thousands of year. According to world health organization, most population still rely on traditional medicines for their psychological and physiological health care, since they cannot afford the products of western pharmaceutical industries together with their side effects and lack of healthcare facilities. Rural area of many developing countries are primarily depends for their healthcare and have found place in day-to-day life.

Herbal molecules are safe and would overcome the resistance produced by the pathogens as they exist in a combined form or in a pooled form of more than one molecule in the protoplasm of the plant cell. The plant chemicals are classified as primary and secondary metabolites. Primary metabolites are widely distributed in nature, occurring in one form or another in virtually all organisms. Secondary metabolites have no apparent function in a plant's primary metabolism, but often have an ecological role, as pollinator attractants, represent chemical adaptations to environmental stresses or serve as chemical defense against micro-organisms, insects and higher predators and even other plants. In contrast to primary metabolites, they are synthesized in specialized cell types and at distinct developmental stages, making their

extraction and purification difficult. As a result, secondary metabolites that are used commercially as biologically active compounds, are generally high value-low volume products than the primary metabolites (e.g. steroids, quinines, alkaloids, terpenoids and flavonoids), which are used in drug manufacture by the pharmaceutical industries. These are generally obtained from plant materials by steam distillation or by extraction with organic or aqueous solvents and the molecular weight are generally less than 2000. Antimicrobial substances are the substances that kill or inhibit the growth of microbes. And plants have capacity to synthesize chemical that help them to defense against attack from predator. By chance some of this compound while being toxic to plant predator turn out to be beneficial to treat human disease. Antimicrobial activity of many plant extract has been reported by many authors (Chan *et al.*, 2010). The curative properties of medicinal plants are mainly due to the presence of various complex chemical substances of different composition which occur as secondary metabolites. Compounds that can scavenge free radicals, antioxidant compound have great potential in ameliorating these disease processes (Reena *et al.*, 2013). There are different plants which have got antimicrobial and antioxidant activity and their different parts have different properties.

In our study we have checked antimicrobial and antioxidant activity of different plants in different extract like bark of *Ficus racemosa* leaves of *Calotropis procera*, *Withania somnifera*, *Tinospora cordifolia*, *Azadirachta indica* and *Datura innoxia*. *Ficus Racemosa* stem and bark contain two leucoanthocyanins: leucocyanidin-3-O- β -glucaopyranoside, leucopelarogonidin-3-O- α -L-rhanmopyranoside, β -sitosterol,

unidentified long chain ketone, ceryl behenate, lupeol, its acetate, α -amyrin acetate. From trunk bark lupeol, β -sitosterol, and stigmasterol were isolated (Hamed, 2011; Salem *et al.*, 2013). *Calotropis procera* contain several types of compounds such as Cardenolide, triterpenoids, alkaloids, resins, anthocyanins and proteolytic enzymes in latex, flavonoids, tannins, sterol, saponins, cardiac glycosides (Kaushik and Goyal, 2008; Quazi *et al.*, 2013). Roots of *Withania somnifera* contain different types of secondary metabolites and other compounds like alkaloids, steroids, reducing sugar, glycosides (Mahmood *et al.*, 2012). Stem of *tinospora cordifolia* contains berberine, palmatine, tembetarine mangoflorine, tinosporine (Garima Pandey *et al.*, 2014). Tinosporide, cordifolide, cordifol, heptacosenol, columbin (Ch *et al.*, 2012). *Azadirachta Indica* contains Azadirachtin, meliacin, gedunin, nimbidin, nimbolides, salanin, nimbin, valassin, meliacin. *Datura Innoxia* plants contain tropane alkaloids such as hyocyamine, scopolamine, and atropine. It has been used for long time in some cultures as a poison and hallucinogen because of presence of these substance And this compound plays a key role in killing or bacteria (Kaushik and Goyal, 2008).

Mastitis is an inflammation of the mammary gland characterized by physical, chemical, bacteriological, cytological changes in milk and pathological changes in the gland. Clinical mastitis recognized by abnormal milk, gland swelling and /or systemic illness whereas subclinical mastitis characterized by apparently normal milk with an increase in SCC due to influx of leukocyte with reduces in milk production. The reduction in milk production attributed to sub-clinical mastitis may account for 70%-80% of the total losses (Philpot and Nickerson, 1991). Mastitis pathogens are found either in the udder (contagious pathogens: *S. aureus*, *Str.*

agalactia and *Mycoplasma*) or the cow's surrounding (environmental pathogens: *Str. uberis*, *Str. dysgalactiae* and *E. coli*) (Andrews *et al.*, 1992). Coagulase-negative Staphylococci (CoNS) represent a diverse collection of bacterial species able to cause bovine mastitis as well as a variety of typically opportunistic infections in humans. They have further significance as a potential source of antimicrobial resistance and virulence genes which can be transferred by horizontal gene transfer to *S. aureus* thus promoting its ability to cause disease. Some Staphylococcal isolates produce methicillin-resistant transpeptidase, an enzyme that inactivates many penicillinase resistant penicillins and other beta-lactam antibiotics such as methicillin.

Organisms exhibiting this type of resistance are referred to as Methicillin Resistant Staphylococci. *Staphylococcus aureus* strains that showed resistance to methicillin were reported (Jevons, 1961). The specific genetic mechanism of its resistance has been identified as a mobile genetic element (staphylococcal cassette chromosome SSCmec) integrated into the *S. aureus* chromosome, within which the *mecA* gene encodes the enzyme.

This protein has a low affinity for β -lactam antimicrobial drugs. Three different types described *mecA*, *mecB*, and *mecC*. SSC mec elements have been subdivided into type I to XI (Ito *et al.*, 1999; Katayama *et al.*, 2000). Multilocus sequence typing (MLST) are genotyping methods used to characterize and distinguish specific clones among the isolates. To control mastitis and to avoid potential problems associated with bacterial resistance and treatment failure, it is important to be aware of antimicrobial resistance characteristics of mastitis causing bacteria. Herbal medicine represents a great advance for the design of new strategies for

the prevention/ treatment of the mastitis disease.

Materials and Methods

Sample collection

Dhatura innoxia, *Calotropis procera*, *Tinospora cordifolia*, *Ficus racemosa*, *Azadirachta indica* and *Withania somnifera* collected from botanical garden of Vagahai, Dang district.

Preparation of plant extract

For the preparation of plant extract, bark of *F. racemosa* and leaves of remaining all plant were collected because of bark of consist of *F. racemosa* consist of maximum amount of secondary metabolites which shows higher antimicrobial activity. After the collection leaves and bark of plants were

dry for 4-5 days in hot air oven at 40°C.

Followed by drying, powders of all plants were prepared using mixer grinder and then extraction was carried out by microwave assisted extraction technique. Then extracts were centrifuged at 7000rpm for 10 min. Supernatant was collected in petriplates and solvent was allowed to evaporate room temperature. Powder was scrapped from the petriplates from which the solvent has been evaporated and samples were prepared by dissolving it in DMSO solvent.

Anti-microbial activity

For the preparation of bacterial suspension, strain of *S. aureus* were transferred to sterile nutrient broth medium and incubated at 37°C for 24 hrs. And this suspension was used as a test organism in antibacterial activity. This bacterial suspension was

mixed with warmed, melted, sterile autoclaved nutrient agar and poured into plates under aseptic condition. As soon as the agar gets solidify wells were prepared by using 6 mm cork borer that was sterilized with alcohol and flame. Then different plant extract was poured in to wells. The plates were labeled and placed into refrigerator for 2hrs for diffusion of extract. Plates were incubated at 37°C for 24 hrs. After incubation diameter of zone of inhibition was measured antibacterial activity against *S. aureus* were further used to check its antioxidant activity.

Antioxidant activity

Sample which showed good zone of inhibition against test organism in antibacterial activity were considered for checking its antioxidant activity. Preparation of DPPH: 0.004 gm of DPPH dissolved in 2 ml of methanol. Preparation of standard: 0.001gm ascorbic acid was dissolved in 10 ml of methanol. For standard assay, aliquot of 0.2-1.0 ml of standard were taken into tubes and make up with the 1 ml of

methanol and to it 39µl of DPPH was added

in each tubes and allow it to incubate in dark for 30 min and O.D. was taken at 516 nm. For sample assay, aliquot of 0.2-1.0 of sample were taken into and make up with 1

ml of methanol and to it 39µl of DPPH was

added in each tubes and incubated it in dark for 30 min and absorbance (A) was measures at 516 nm. For blank 3ml of methanol was used and for control 3ml

methanol and 39 μ l of DPPH was used and

% scavenging activity was calculated by following formula (Reena *et al.*, 2012).

$$\% \text{ scavenging activity} = \frac{A \text{ control} - A \text{ test}}{A \text{ control}} \times 100$$

Column chromatography

The methanol extract of *D. inoxia* was loaded on a silica gel column (packed with hexane), then eluted with hexane and chloroform mixture (ratio from 9:1 to 1:9) and 100% chloroform afterward ethyl acetate and methanol and all 20 fractions were separately collected and tested. Subsequently the purity of eluted fraction was checked using thin layer chromatography. The fraction which shows maximum activity was subjected to GC-MS analysis to identify the active principle of extracts.

Results and Discussion

In this study different secondary metabolites were extracted from leaves of *Dhatura inoxia*, *Calotropis procera*, *Withania somnifera*, *Azadirachta indica*, *Tinospora cordifolia* and bark of *Ficus racemosus* using microwave assisted method. Till date extraction was done by using Soxhlet Method, but in 1996, Ganzler *et al.* were able to extract vicine, gossypol and alkaloids using Microwave Assisted Extraction method. During 1995, Young *et al.* was used this method for extraction of ergosterol and withanoloides (Young *et al.*, 1995). The advantage of this over the Soxhlet method is its easy, rapid and the solvent content required is less. Table 1 shows that by using various solvents the plants extract could be

prepared which can be further utilized for antimicrobial activity. In this study, alkaloids using methanol: acetic acid solvent, essential oils using hexane, taxenes using 95% ethanol can be extracted from the dry plant powder (Ganzler *et al.*, 1996).

Antimicrobial assay by agar well diffusion method

0.1% DMSO was used to dissolve 30 plant extracts in such a way that the final concentration obtained was 1mg/ml. This was further used to check the antimicrobial activity against the *S. aureus* using agar well diffusion method. The zone of inhibition obtained for different plant extract against *S. aureus* is as shown in table 2. It was found that among all extract, methanolic extract of *D. inoxia* and methanol: acetic acid of *F. racemosa* showed maximum zones of inhibition i.e. 14mm and 19mm respectively. 0.1 % DMSO which was added in control plate did not give any zone of inhibition which shows that it doesn't have any effect on the growth of *S. aureus*.

Antioxidant activity

Antioxidant activity was performed by 1,1-diphenyl-2-picryl-hydrazil method (Reena *et al.*, 2012) and significance difference was observed in the antioxidant activity of different plants that were used Graph was plotted against percentage inhibition v/s. concentration (Figure 1). The free radical scavenging activity of different plant extract mentioned above was studied to find out its ability to reduce the DPPH, a stable free radical and any molecule that can donate an electron or hydrogen to DPPH, can react with it and thereby bleach the DPPH. DPPH is a purple color dye having absorbance maxima of 517 nm and upon reaction with hydrogen donor the purple color fades or disappears due to conversion of it to 2, 2-

diphenyl-1-picryl hydrazine resulting in decrease absorbance. It was observed that methanolic extract exhibited considerable free radical scavenging activity as indicated by their IC₅₀ values. Comparison of antioxidant activity of plant extract was done using standard curve of ascorbic acid (Figure 1a). Highest percentage inhibition was observed by *D. inoxia* plant. It may be due to alkaloids, which show high antimicrobial activity. Alkaloids are secondary metabolites which generally occur in plants. It is also shows antioxidant activity and antimicrobial activity in plants. Generally antioxidant compound reacts with free radicals which are form in the body and causes cell damage so antioxidant compound donate their charge and neutralize the free radicals in the body and prevent cell damage and some disorders like cancer (Reena *et al.*, 2013).

Quality analysis and antimicrobial activity of fraction eluted

Thin Layer Chromatography was performed for crude extract and its eluted 26 fraction. In crude extract, smear observed in UV chamber because of mixture of compound while in eluted fraction only single band was observed (Figure 3). Antimicrobial activity of all the extracts of *D. inoxia* and *F. racemosa* obtained after column extraction was performed using agar well diffusion method (Table 3). Ethyl acetate: methanol (1:9) fraction of *D. inoxia* gave maximum zone of inhibition i.e. 53mm (Figure 2). Ethyl acetate: methanol (2:8) fraction of methanolic extract of *F. racemosa* was showed good zone of inhibition (42mm). Alkaloids have maximum antimicrobial activity than the others.

GC-MS analysis

Column fraction were further analyze for the identification of compound, for this the

partially purified product was used. The most potent fraction against *S. aureus* which was founded out in the study was ethyl acetate fraction of *D. inoxia* showing highest zone of inhibition against *S. aureus*. Gas chromatography–mass spectrometry (GC-MS) is an analytical technique which merge the features of gas-liquid chromatography and mass spectrometry for identify various substances within a test sample. So efforts were made to find out which compounds was present in the fraction by doing GC-MS analysis. Results of GC-MS are as shown in figure 5. Further 21.80, 23.882 and 24.527 peak was identified which showed the presence of gamolenic acid, scopolamine and chloropyramine. Scopolamine is a tropane alkaloid drug with muscarinic antagonist effects, amongst the secondary metabolites of plants from Solanaceae (nightshade) family of plants, such as *Datura*, *Brugmansia*, and *Duboisia*. (Muranaka *et al.*, 1993). Scopolamine, as a medicine is used for various treatment like Postoperative nausea and vomiting and sea sickness, leading to its use by scuba divers. Motion sickness (where it is often applied as a transdermal patch behind the ear), Gastrointestinal spasms, renal or biliary spasms, aid in GI radiology and endoscopy, Irritable bowel syndrome (IBS), Clozapine-induced hypersalivation (drooling), Bowel colic and also has anti bacterial activity against board range of bacteria (Rossi *et al.*, 2013 and Joint Formulary Committee, 2013). Chloropyramine is a classical ("old" or first generation) antihistamine drug approved in some Eastern European countries for the treatment of allergic conjunctivitis, allergic rhinitis, bronchial asthma, and other atopic (allergic) conditions. Related indications for clinical use include Quincke's edema, allergic reactions to insect bites, food and drug allergies, and anaphylactic shock. Gamolenic acid) is a fatty acid found

primarily in vegetable oils. It is sold as a dietary supplement for a variety of human health problems, although there is little or no evidence of its effectiveness. GLA has been promoted as medication for a variety of ailments including breast pain and eczema. GLA is also sometimes promoted as an anti-cancer agent. According to the American

Cancer Society there is very little evidence for its effectiveness, and "neither GLA nor other GLA-rich supplements (such as evening primrose oil) have been convincingly shown to be useful in preventing or treating any other health conditions (Williams, 2003).

Table.1 Preparation of plant extracts

Plants	Concentration of dry plant extract (gm)				
	95% ethanol	Hexane	Methanol	Methanol: acetic acid (7:1)	Methanol:water (7:7)
<i>A. indica</i>	1.96	0.21	0.22	0.15	0.09
<i>T. cordifolia</i>	0.50	0.01	0.13	0.09	0.03
<i>W. somnifera</i>	0.23	0.37	0.11	0.01	0.15
<i>C. procera</i>	0.12	0.11	0.05	0.08	0.05
<i>D. innoxia</i>	0.04	0.02	0.04	0.15	0.15
<i>F. racemosa</i>	0.04	0.01	0.04	0.03	0.03

Table.2 Antimicrobial activity of different plant extracts

Plant	Zone of inhibition (mm)	Plant	Zone of inhibition (mm)
	<i>S. aureus</i>		<i>S. aureus</i>
<i>Withania somnifera</i>		<i>Calotropis procera</i>	
EE	4	EE	-
ME	2	ME	-
MAE	6	MAE	-
HE	-	HE	12
MWE	-	MWE	-
<i>Tinospora cordifolia</i>		<i>Dhaturoa innoxia</i>	
EE	-	EE	-
ME	2	ME	14
MAE	-	MAE	6
HE	-	HE	-
MWE	-	MWE	12
<i>Azadirachta indica</i>		<i>Ficus racemosa</i>	
EE	-	EE	15
ME	11	ME	18
MAE	2	MAE	19
HE	-	HE	17

MWE	-	MWE	-
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EE- Ethanolic extract, ME- Methanolic extract, MAE- Methanol:acetic acid extract, HE- Hexane, MWE- Methanol: water extract

Figure.1 A) Standard curve for DPPH activity B) Antioxidant activity of crude plant extract

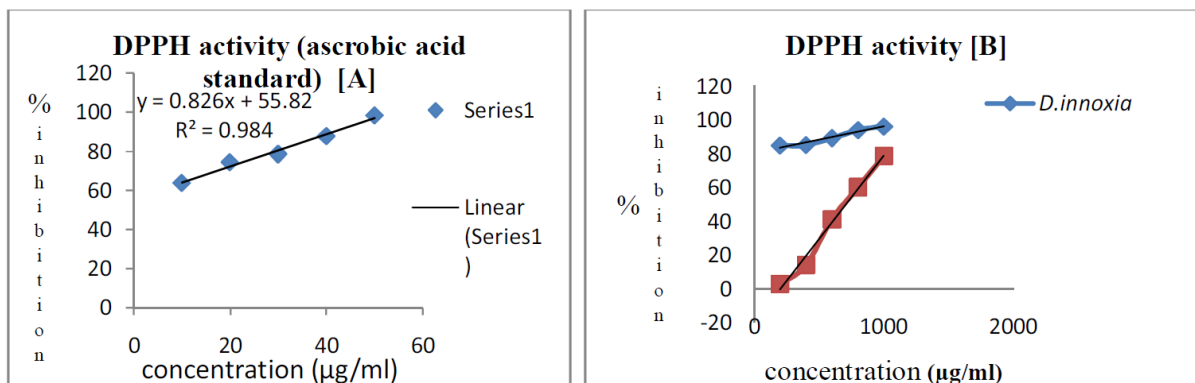
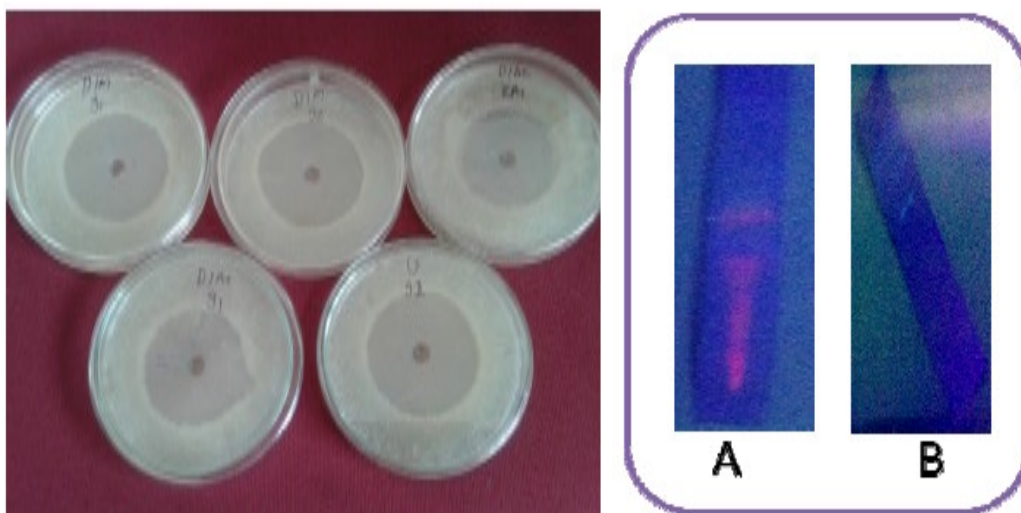


Fig.2 (a) Zone of inhibition by purified extract against *S. aureus* (b) TLC plates in UV chamber: A- Crude extract, B- purified extract



From GC-MS analysis of Ethyl acetate: methanol (2:8) fraction of methanolic extract of *F. racemosa* was consisted bioactive compounds like cyclododecene, gamelonic acid, octadecenol 2-bromo, pentadecanoic, 1-heptadecene and

octadecenoic were found. Its reported that 60% of the total amount of plant volatiles constituents studied are cyclododecane (Seong *et al.*, 2010) which may have varied antioxidant and antimicrobial properties (Jang *et al.*, 2008) which is very similar with

the constituents found in the *G. boninense* fruiting bodies based on the GC-MS profile (Russel *et al.*, 1991). Most compounds are fatty acids which are known to have antibacterial and antifungal properties (Russel *et al.*, 1991). Lipids can kill microorganisms by disrupting the cellular membrane of bacteria, fungi and yeasts (Lempe *et al.*, 1998). Pentadecenoic and octadecenoic also possess antimicrobial properties. Moreover, it has also been proposed that these fatty acids have potential antibacterial and antifungal principle for clinical application (Altieri *et al.*, 2008). Octadecenol 2- bromo have been reported for its antifungal activity. 1-heptadecene also has been reported for its antifungal and antioxidant activity. Cyclododecene have been reported for its antimicrobial and antiprotozoal properties.

Mastitis is an inflammation of udder tissue. It is very severe disease in bovine animals and it is second most infectious disease after Foot and Mouth disease (FMD). It is caused due to invasion of pathogenic bacteria from the teat canal. Many bacteria are responsible for the cause of this disease but *Staphylococcus aureus* is consider as the main causative organism. In the present study leaf extracts of *Withania somnifera*, *Calotropis procera*, *Tinospora cordifolia*, *Azadirachta indica*, *Datura inoxia* and bark extract of *Ficus racemosa* were examined for its antibacterial activity using five different solvent against *S. aureus*. Maximum antimicrobial activity was observed in crude extract of methanol and methanol: Acetic acid mixture. Plant extracts which showed highest antimicrobial activity were further evaluated for its antioxidant activity and amongst it *D. inoxia* showed highest antioxidant activity. Identification and partial purification of crude extract was carried out by column chromatography. Different fractions after

column purification were analyzed using Gas Chromatography-Mass Spectrometry (GC-MS) to identify compounds. The GC-MS activity of the selected plant extract showed production of few beneficial compounds that are helpful in herbal medicines. GC-MS of fraction-1 which included ethyl acetate and methanol of *d. inoxia* showed presence of compounds like gamalonic acid, chloropyramine, and scopolamine, whereas fraction-2 with ethyl acetate and methanol of *F. racemosa* showed presence of compound like cyclododecene, gamelonic acid, octadecenol 2-bromo, pentadecanoic, 1-heptadecene and octadecenoic. In both plant extract Gamalonic acid is common secondary metabolite, which is one of the beneficial compounds used in herbal medicines.

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