MICROPROPAGATION, REGENERATION AND EVALUATION OF ANTIMICROBIAL ACTIVITY OF LIPPIA NODIFLORA

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ABSTRACT
Lippianodiflora (L) is an important medicinal plant used in the treatment of diseases such as Chronic indolent ulcer, gastrointestinal and respiratory disorders, diabetes etc. In the present study Nodes, internodes and leaves were used as explants for the initiation of root, shoot and callus and cultured on Murashige and Skoog (Ms) medium supplemented with different growth regulators like Indole-3-acetic acid(IAA), 2,4-dichlorophenoxy acetic acid(2,4-D), α-Naphthalene acetic acid(NAA) and Benzyl adenine(BAP). The callus and root were induced in medium supplemented with IAA (1mg/m) l and shoot were induced in BAP medium (1mg/ml). Antimicrobial activity were studied in different solvent extracts (acetone, chloroform, ethyl acetate, hexane and methanol) by well diffusion method against human pathogenic microorganisms Bacillus subtilis, Salmonella typhi, Staphylococcus aureus, Escherichia coli, Vibrio cholera and Candida krusei. The study also concluded that the antimicrobial activity against all the microorganism increase with increased concentration of the extract. Highest antimicrobial activity were observed in hexane stem extract (27.77%) against S. aureus, chloroform leaf extract (26.66%) against E. coli, ethyl acetate leaf extract (22.22%) against E.coli and S. typhi , acetone leaf and stem extracts were effective (23.33%)against B. subtilis and S. aureus respectively. The ethyl acetate extract of callus were effective against C. krusei and B. subtilis than the wild extract .Hence Lippia nodiflora were proved to possess antimicrobial activity and so the callus can be grown on large scale to treat vaginitis, soft tissue abscess, food poisioning and allergic reactions.

KEYWORDS: *Lippianodiflora, MS medium, Bacillus subtilis, Staphylococcus aureus, Salmonella typhi, Escherichia coli, Vibrio cholera, Candida krusei, callus*.

I. INTRODUCTION

Lippia nodiflora (Phyla nodiflora) is a small perennial herb belonging to the family verbenaceae. Its branched stems bear numerous leaves and small pink or white flowers. The plant is distributed all over the world particularly in African sub-continent and most of the tropical and subtropical regions, particularly in maritime areas close to rivers [1-5]. The presence of phytochemicals in medicinal plants has attracted a great deal of attention, concentrate on their role in preventing diseases [6]. L. nodiflora is reported to possess acrid, cooling, aphrodisiac, astringent, anthelmintic, alexiteric, emmenagogue, bactericide, diuretic, antiseptic, antitussive, antipyretic and anti-inflammatory properties and is effective against bronchitis, respiratory diseases, arthritis, fever, dyspepsia, hookworm, gonorrhea, ulcers, stomachic, wounds, burning neuralgia, sores, spasms and vertigo [7-14]. The antimicrobial activity of plant extracts has formed the basis of many applications in food preservations, pharmaceuticals, alternative medicines and natural therapies [15]. Phytochemical investigations on this plant have revealed the presence of flavone glycosides, alkaloids, essential oil, resin, stigmasterol, β-sitosterol, sugars, mono and diflavone, sulphates of neptin, jaceosidin, hispidulin and 6hydroxyluteoli[16-18] . Recent studies on this plant have also resulted in the finding of a new terpenoide known as lippiacian and another component named halleridone [1] .

Development of new antimicrobial compounds against different microorganisms is becoming critically important, as infectious diseases are still one of the leading causes of death in the world. Medicinal plants have been well-known natural sources of remedies for the treatment of various diseases since antiquity. According to a report by the World Health Organization (WHO), that infectious and parasitic diseases account for nearly 11 million among the 57 million total deaths in 2003 and medicinal plants would be the best source to obtain a variety of drugs, nearly 20,000 plant species are currently being used. The use of plant compounds for pharmaceutical purposes has gradually increased in the world. About 80% of individuals from developed countries use traditional medicine, which has compounds derived from medicinal plants. Hence, more studies pertaining to the use of plants, as therapeutic agents should be emphasized. Due to increasing population the natural ecosystems are rapidly eroded and many of the the plant species are extinct. In order to cope up with this alarming situation, the recent developments in biotechnology have come as a boon to consume and mass produce these important medicinal plants [19]. Plant tissue culture techniques offer a viable tool for mass multiplication and germplasm conservation of medicinal plants in order to meet the needs of pharmaceutical industry [20].The objective of this research is to micro propagate Lippia nodiflora, which has potential antimicrobial activity against the human pathogens and to evaluate the antimicrobial Activity (against Gram positive, Gram negative bacteria and Fungi) of wild plant with different solvent extracts and compare with the callus for its efficacy.

1.1. Organization of the Manuscript

Methodology; Source of plant, Sterilization of plant materials, Preparation of media, Preparation of plant extracts, Test human pathogens, Preparation of Inoculum,, Tissue culture of plant, Antimicrobial Activity assay , Results, Discussion, Reference

II. METHODOLOGY

2.1 Source of plant

Fresh and aerial parts of *Lippia nodiflora* were collected from University of Madras, Maraimalai Campus (Guindy), Chennai, India

2.2 Sterilization of plant materials

Well grown leves, nodes and internodes were washed with running water and sterilized in Sodium hypochlorite(0.5%) solution for 15-30 minutes and Mercuric chloride(0.05%) solution for 2-5 minutes and finally washed with the sterile distilled water for three times at 5 minutes interval.

2.3 Preparation of media

MS media (21) for tissue culture contain macro, micro, minor, iron and vitamins. Iron stock was stored in a black bottle to prevent photolysis of chemicals. Sucrose 3% (30 g/L), 0.1% meso-inositol (100 mg/L) and 1mg/mL amount of plant growth hormones were added to the medium and buffered by 1N HCl or 1N NaOH to adjust the medium pH to 5.6 before autoclaving.
Muller Hinton Agar (MHA) (3.8g/ 100ml) was prepared for antimicrobial screening and sterilized in the autoclave.

2.4 Preparation of plant extracts

25g of dried and grinded stem and leaves were added to five different solvents (acetone, chloroform, ethyl acetate, hexane and methanol) separately. The extracts were filtered twice using Whatmann filter paper after every 24 hours. The extract obtained is evaporated on hot plate to concentrate.

2.5 Test human pathogens

Bacillus subtilis, Salmonella typhi, Staphylococcus aureus, Vibrio cholera, Escherichia coli and Candida krusei.

2.6 Preparation of Inoculum

Fresh cultures were prepared by inoculating the organisms in Nutrient Broth (NB) for bacteria and fungi in at 37°C for 24 hours. Each organism was suspended in sterile broths and diluted.

2.7 Tissue culture of plant

The sterilized explants (leaves, nodes and internodes) were inoculated in the MS media supplemented with various hormones namely IAA, 2,4-D, NAA and BAP. Only the initiated shoots were sub cultured to the fresh media supplemented with same combination for further proliferation and elongation.

2.8 Antimicrobial Activity assay

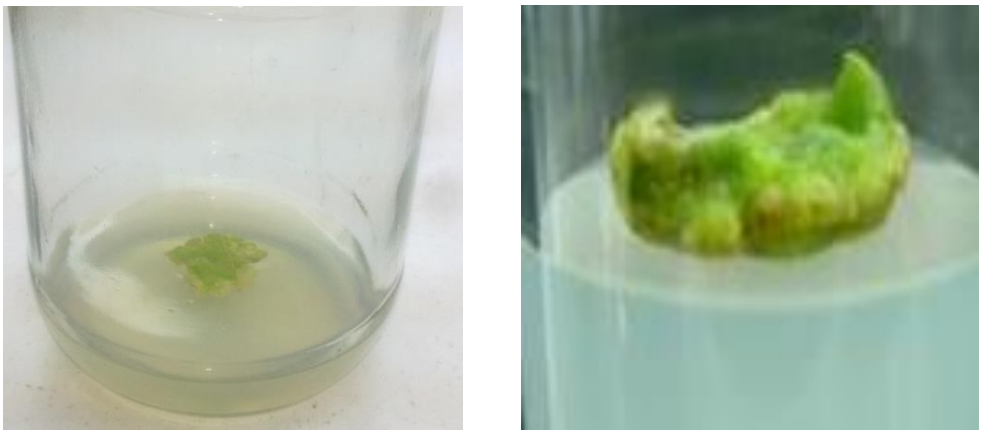
The *in vitro* antimicrobial activities of test compounds were determined by the well-diffusion method described by Nagarajan *et.al*., 2010 [22]. The prepared MHA media were poured half-filled to the Petri plates and allowed for solidify. The bacterial and fungal test cultures were evenly spread over the media by sterile cotton swabs. Then, wells were made by sterile cork borer, 25µl, 50µl and 70µl of the plant extracts were transferred into separate wells. Then these plates were incubated at 37°C for 24 hours. After incubation period the result were observed and measured the zone of inhibition around the each well.

III. RESULTS

The explants (leaves, nodes and internodes) of *Lippia nodiflora* inoculated in MS medium supplemented with different hormones (IAA, 2, 4-D, NAA and BAP) were micro propagated and produced various plant parts. The stored Sodium alginate beads inoculated in MS medium amended with different hormones, showed the regeneration of roots. Extracts of wild plant as well as callus in five different solvents (acetone, chloroform, ethyl acetate, hexane and methanol) were studied for the antimicrobial activity of the plant against microorganisms such as *Bacillus subtilis, Salmonella typhi, Staphylococcus aureus, Escherichia coli, Vibrio cholera* and *Candida krusei*. All the extracts were effective against the microorganisms. Ethyl acetate extract were alone used for callus, as ethylacetate extract of wild plants were effective against three microorganisms such as *Candida krusei, Escherichia coli* and *Salmonella typhi*.

TABLE 1 : Effect of Plant growth hormones on callus, shoot and root formation.

Hormones	Callus of leaf explants (%)	Shoot elongation (%)	Root elongation (%)
IAA	75	82	24
NAA	42	25	80
2,4-D	51	32	31
BAP	43	27	27



8Days 22 Days

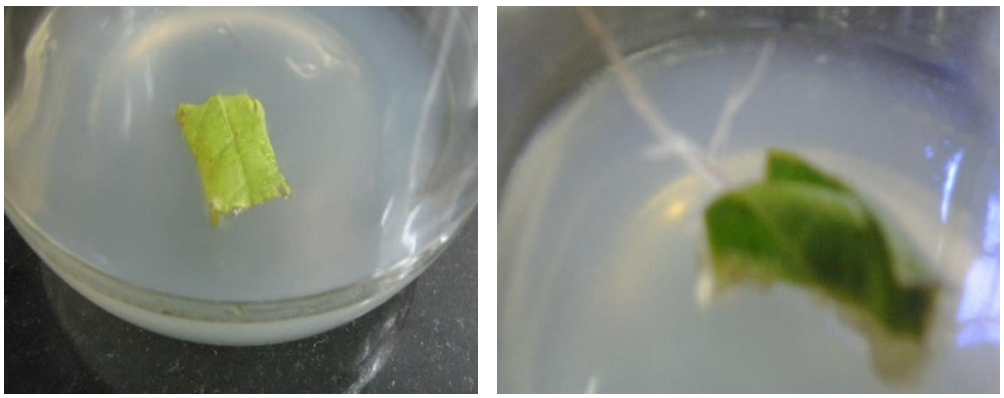
Fig 1: Callus from leaf explants

The callus formation in leaf explants was observed on MS medium amended with different hormones of same concentration (1.0mg/mL). The MS medium amended with IAA showed significant callus growth of 75% when compared to other hormones.

8 Days 15 Days 20 Days

Fig: 2: Shoot elongation from nodal explants

Different hormones were used to induce shoot elongation in the nodal explants. The maximum percentage of shoot elongation was found in IAA (1.0mg/mL) which induced elongation of about 82%. Whereas the medium amended with 2, 4-D, NAA and BAP showed lesser elongation.



2 Days 20 Days

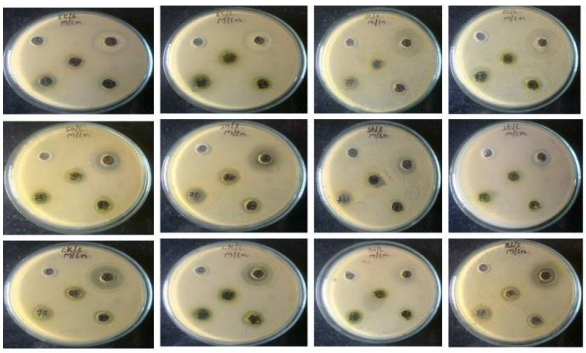
Fig 03: Root elongation from leaf explants

The root of *Lippia nodiflora* was induced using different growth hormones. The highest root induction from leaf explants was observed to be 80% in MS medium supplemented with NAA (1.0mg/mL) when compared to the roots induced with other hormones.

TABLE 2: Methanol leaf and stem extract of *Lippia nodiflora*

Organisms	*L. nodiflora (*leaf)- Methanol								*L. nodiflora* (stem)- Methanol							
	S	I%	25	I%	50	I%	75	I%	S	I%	25	I%	50	I%	75	I%
B. subtilis	22	24.44	12	13.33	13	14.44	14	15.55	22	24.44	13	14.44	15	16.66	18	**19.99**
S. aureus	19	21.10	11	12.22	13	14.44	14	15.55	23	25.55	11	12.22	14	15.55	15	16.66
E. coli	26	28.88	12	13.33	14	15.55	16	**17.77**	22	24.44	13	14.44	14	15.55	15	16.66
S. typhi	25	27.77	10	11.11	11	12.22	12	13.33	24	26.66	12	13.33	13	14.44	14	15.55
V. cholera	28	31.10	12	13.33	15	16.66	16	**17.77**	28	31.10	14	15.55	15	16.66	17	18.88
C. krusei	28	31.10	13	14.44	14	15.55	15	16.66	29	32.21	13	14.44	14	15.55	15	16.66

Methanol stem extract of *Lippia nodiflora* showed a significant result 19.99% against the *Bacillus subtilis* while the methanol leaf extract showed 17.77% of effectiveness against *Escherichia coli* and *Vibrio cholera*.



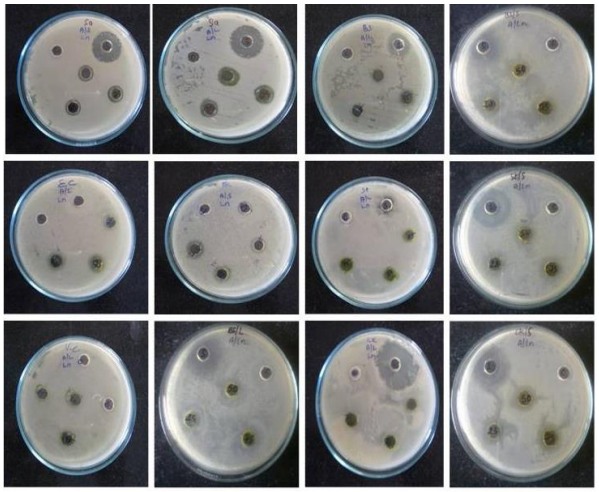
Bs-Bacillus subtilis, St-Salmonella typhi, Ec-Escherichia coli
Vc-vibrio cholerae, Sa-Staphylococcus aureus, Ck-Candida krusei

Fig 4: Well diffusion assay of methanol extract of *Lippia nodiflora*

TABLE 3: Acetone leaf and stem extract of Lippia nodiflora

Organisms	*L. nodiflora (*leaf)- Acetone								*L. nodiflora* (stem)- Acetone							
	S	I%	25	I%	50	I%	75	I%	S	I%	25	I%	50	I%	75	I%
B. subtilis	28	31.10	12	13.33	16	17.77	21	**23.33**	22	24.44	13	14.44	14	15.55	18	19.99
S. aureus	28	31.10	-	-	18	19.99	20	22.22	22	24.44	17	18.88	18	19.99	21	**23.33**
E. coli	25	27.77	11	12.22	12	13.33	15	16.66	24	26.66	14	15.55	15	16.66	16	17.77
S. typhi	24	26.66	24	26.66	15	16.66	18	19.99	28	31.10	14	15.55	16	17.77	17	18.88
V. cholera	29	32.21	14	15.55	16	17.77	17	18.88	28	31.10	16	17.77	18	19.99	19	21.10
C. krusei	25	27.77	13	14.44	16	17.77	17	18.88	25	27.77	15	16.66	16	17.77	20	22.22

Both leaf and stem extracts of Lippia nodiflora were effective (23.33%) against Bacillus subtilis and staphylococcus aureus



Bs-Bacillus subtilis, St-Salmonella typhi, Ec-Escherichia coli
Vc-vibrio cholerae, Sa-Staphylococcus aureus, Ck-Candida krusei

Fig 5: Well diffusion assay of acetone extract of *Lippia nodiflora*

TABLE 4: Hexane leaf and stem extract of Lippia nodiflora

Organisms	*L. nodiflora (*leaf)- Hexane								*L. nodiflora* (stem) Hexane							
	S	I%	25	I%	50	I%	75	I%	S	I%	25	I%	50	I%	75	I%
B. subtilis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S. aureus	-	-	-	-	-	-	-	-	23	25.55	21	23.33	24	26.66	25	**27.77**
E. coli	26	28.88	12	13.33	15	16.66	18	19.99	28	31.10	18	19.99	21	23.33	22	24.44
S. typhi			16	17.77	14	15.55	19	**21.10**	21	23.33	17	18.88	18	19.99	18	19.99
V. cholera	24	26.66	14	15.55	12	13.33	16	17.77	22	24.44	11	12.22	13	14.44	16	17.77
C. krusei	28	31.10	12	13.33	14	15.55	16	17.77	21	23.33	13	14.44	15	16.66	20	22.22

Hexane stem extract of *Lippia nodiflora* showed the highest result of 27.77% against *Staphylococcus aureus*. While the leaf extract showed 21.10% of effectiveness against the *Salmonella* typhi

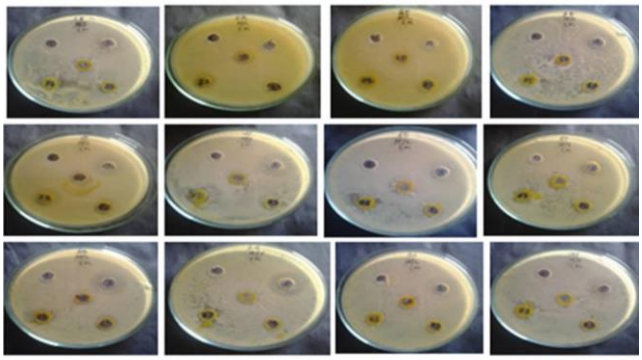


Fig 6: Well diffusion assay of hexane extract of *Lippia nodiflora*

TABLE 05: Chloroform leaf and stem extract of *Lippia nodiflora*

Organisms	*L. nodiflora (*leaf)- Chloroform								*L. nodiflora* (stem) Chloroform							
	S	I%	25	I%	50	I%	75	I%	S	I%	25	I%	50	I%	75	I%
B. subtilis	21	23.33	12	13.33	13	14.44	15	16.66	26	28.88	13	14.44	16	17.77	18	19.99
S. aureus	24	26.66	12	13.33	12	13.33	13	14.44	29	32.21	12	13.33	15	16.66	19	**21.10**
E. coli	29	32.33	14	15.55	21	23.33	24	**26.66**	-	-	-	-	-	-	-	-
S. typhi	30	33.33	14	15.55	16	17.77	17	18.88	26	28.88	13	14.44	-	-	16	17.77
V. cholera	29	32.21	12	13.33	13	14.44	15	16.66	28	31.10	13	14.44	15	16.66	17	18.88
C. krusei	25	27.77	11	12.22	12	13.33	14	15.55	28	31.10	13	14.44	15	16.66	18	19.99

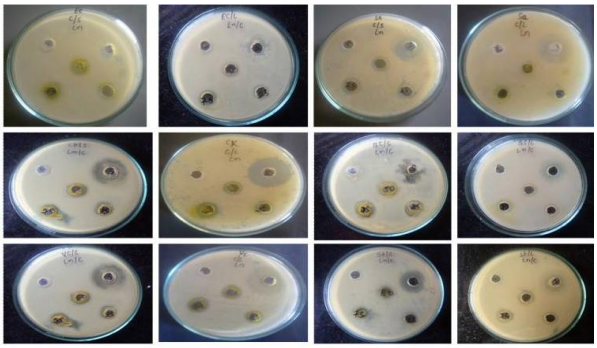
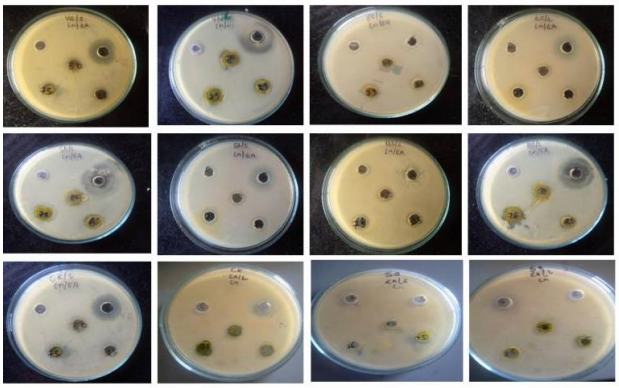


Fig 7: Well diffusion assay of chloroform extract of *Lippia nodiflora*

TABLE 6: Ethyl acetate leaf and stem extract of *Lippia nodiflora*

Organisms	*L. nodiflora (*leaf)- Ethyl acetate								*L. nodiflora* (stem)- Ethyl acetate							
	S	I%	25	I%	50	I%	75	I%	S	I%	25	I%	50	I%	75	I%
B. subtilis	26	28.88	14	15.55	18	19.99	19	21.10	22	24.44	-	-	10	11.11	18	19.99
S. aureus	28	31.10	12	19.99	13	14.44	18	19.99	22	24,44	-	-	12	13.33	15	16.66
E. coli	25	27.77	16	17.77	18	19.99	20	**22.22**	24	26.66	13	14.44	15	16.66	16	17.77
S. typhi	28	31.10	14	15.55	18	19.99	20	**22.22**	24	26.66	16	17.77	18	19.99	20	**22.22**
V. cholera	29	32.21	12	13.33	16	17.77	17	18.88	20	22.22	-	-	14	15.55	18	19.99
C. krusei	21	23.33	14	15.55	18	19.99	20	**22.22**	18	19.99	12	13.33	14	15.55	18	19.99

Ethyl acetate leaf extract of *Lippia nodiflora* showed effective result of 22.22% against three microorganisms such as *Escherichia coli*, *Salmonella typhi* and *Candida krusei*. The ethyl acetate stem extract also showed 22.22% against *Salmonella typhi*.



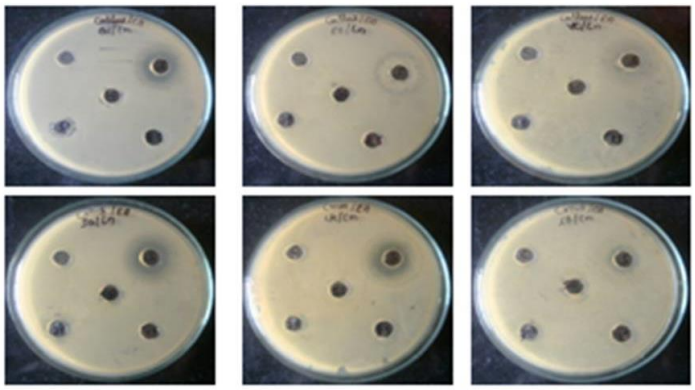
Bs-Bacillus subtilis, St-Salmonella typhi, Ec-Escherichia coli
Vc-vibrio cholerae, Sa-Staphylococcus aureus, Ck-Candida krusei

Fig 8: Well diffusion assay of ethyl acetate extract of *Lippia nodiflora*

TABLE 7: Ethyl acetate callus extract of *Lippia nodiflora*

Organisms	*L.nodiflora* (Callus)-Ethyl acetate							
	S	I%	25	I%	50	I%	75	I%
B. subtilis	26	28.88	_	_	11	12.22	14	15.55
S. aureus	28	31.11	12	13.33	14	15.55	19	**21.11**
E. coli	23	25.55	_	_	14	15.55	15	16.66
S. typhi	25	27.77	_	_	13	14.44	15	16.66
V. cholera	23	25.55	11	12.22	14	15.55	15	16.66
C. krusei	29	32.22	13	14.44	16	17.77	19	**21.11**

The ethyl acetate callus extract of *Lippia nodiflora* were effective (21.10%) against *Staphylococcus aureus* and *Candida krusei* than the ethyl acetate extract of wild plant.



Bs-Bacillus subtilis, St-Salmonella typhi, Ec-Escherichia coli
Vc-vibrio cholerae, Sa-Staphylococcus aureus, Ck-Candida krusei

Fig 9: Well diffusion assay of ethyl acetate callus extract of *Lippia nodiflora*

TABLE 8: Effect of Plant growth hormones on immobilized beads of *Lippia nodiflora*

Hormones	Growth of immobilized beads (%)
IAA	83
NAA	29
2,4-D	26
BAP	16

Fig 10: Regenerated immobilized beads of *Lippia nodiflora*

The sodium alginate immobilized beads were stored in sterile distilled water for 3 days and when inoculated in the MS medium supplemented with different growth hormones, showed viable regeneration of the roots in MS medium supplemented with IAA after 23 days. Very less percentage of regeneration ability was observed in other hormones.

IV. DISCUSSION

Phyla nodiflora (*Lippia nodiflora*) belongs to Verbenaceae family, distributed in India, Ceylon, Baluchistan, South Africa and Central America. The plant is used as gastro protective effect, anti inflammatory, antineoplastic, antioxidant and diuretic. In addition, the compounds halleridone and hallerone serve as anti-cancer, antitumor, anti-malarial and anti-fungal.

Tissue culture is a potential tool and has opened extensive areas of research for biodiversity conservation. Plant tissue culture techniques offer a viable tool for mass multiplication and germplasm conservation of medicinal plants in order to meet the needs of pharmaceutical industry [20].

Drug resistant human pathogenic microorganisms have developed due to indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious diseases. This condition has forced scientist to search for new antimicrobial substances from various sources [23] .In India, large section of people especially in villages using the herbal medicine to combat the infectious diseases and disorders. Gradually people move towards the traditional medicine. The reason for that is, trusts on herbal medicine, which improve the diseases conditions, after the herbal medicine treatment. No side effect or fewer side effects is observed due to herbal medicine. Another reason is the cost of the drugs and cost of the treatment is low. People in developing countries now prefer the herbal medicine [24]. Plant metabolites and plant based pesticides appear to be one of the better alternatives as they are known to have minimal environmental impact and danger to consumers in contrast to synthetic pesticides [25].

The explants such as node, internodes and leaves were cultured on Murashige and Skoog (MS) medium supplemented with 0.1mL of different growth regulators (IAA, 2,4-D, NAA and BAP) for the initiation of root, shoot and callus. The maximum 82% of shoot growth were observed in IAA (1.0mg/mL) and this is in accordance with Evelyne Priya *et.al.,* [26] where the shoot growth range from 80 -100% with concentration of 3.0mg/mL of BAP**.** It also concluded that BAP without supplementation of auxins was able to induce maximum number of shoots in both shoot tips and nodal explants. The maximum of 75% callus growth was observed in MS medium amended with IAA of 1.0mg/mL from leaves explants and this is in contrast with Ahmed *et.al.,* [27] where the better callus biomass was observed from the stem explants when cultured in MS medium containing NAA

(1.5 mg/L). The maximum of 80% root growth was developed in NAA (1.0mg/mL) in the present study was similar to Ahmed *et al.,* [28] observation were highly effective root production on half strength MS medium with IBA (1.0mg/mL). But Julians *et al.,* [29] in *L. junelliana* and Gupta *et.al*., [30] in *L. alba had* better response of root formation in full strength MS medium without growth regulators whereas Evelyne Priya *et.al.,* [26] observed maximum roots produced in half strength MS medium containing IBA of 1mg/mL.

Antimicrobial activity of leaf and stem extract of *Lippia nodiflora* in different solvent extracts (acetone, chloroform, ethyl acetate, hexane and methanol) was studied against human pathogenic microorganisms *Bacillus subtilis, Salmonella typhi, Staphylococcus aureus, Escherichia coli, Vibrio cholera and Candida krusei.* The hexane stem extract showed maximum effect of 27.77% against *S. aureus*. Kunle *et.al*., [31] studied the antimicrobial activity of hexane, dichloromethane and methanol extract of *Lippia multiflora* and observed the hexane extract to be most active, while the methanol extract exhibited no antimicrobial activity. Chloroform leaf extract showed 26.66% effectiveness against *E. coli* and this is related with the work of M. Veera *et.al.,* [32] where the chloroform leaf extract was shown to be effective against *E. coli* and in contrast the ethyl acetate extract was effective only against *S. typhi* but the present study shown to be effective against *S. typhi* , *E. coli* and *C. krusei.* The methanol extract of stem is effective against both Gram positive and Gram negative as of Patel *et.al.*, [33] in Methanol seed extract.

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