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Isolation and Characterization of Endophytes from *Citrus limon.*

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ABSTRACT

This study explores the endophytes from commonly available fruits and vegetables which are involved in the production of anti-diabetic property and anti-inflammatory property obtained from *Citrus limon*. The study involves screening of the bacteria from *Citrus limon,* screening for anti-diabetic and anti-inflammatory property of the compounds from the endophytic organism and genus identification of the organism. Species identification was performed by gram-staining and morphological identification. HRBC membrane stabilization method and protein denaturation method were performed to determine anti-inflammatory property while alpha-amylase inhibitory assay was performed to determine anti-diabetic property. Isolated endophytes belong to the *bacillus* species. Anti-inflammatory and anti-diabetic assays showed low by significant anti-diabetic and anti-inflammatory property. Further purification of the endophytic metabolites are needed to have more deterministic results. Preliminary analysis has yielded positive results on the presence of anti-inflammatory and anti-diabetic properties in endophytic organisms. Further analysis has to be done to obtain other industrially important compounds. Endophytes could also be isolated from other fruits and vegetables and could be tested for many other properties which could pave the way for safer and cheaper drugs.

Keywords: Endophytes, *Citrus limon, Bacillus* sp., anti-inflammatory property, anti-diabetic property.

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INTRODUCTION

Endophytes are symbiotic bacteria and fungi that colonize and live in intercellular and intracellular locations of plants exhibiting complex interactions with their hosts. They have ubiquitous symbiotic relationship with host both harmful and beneficial effects depending on the environmental conditions with balanced antagonism [1, 5]. The beneficiary effects would be production of bioactive compounds that cannot be synthesized by chemical reactions[4] The endophytic compounds draw the attention of the researchers with their therapeutic uses in pharmaceutical, cosmetic, food and textile industries[6]. Free radicals in our body contribute to cell damage; antioxidants balance the generation and free radicals and neutralize them. Thus offers protection to our system against various disorders [8]. Bright coloured fruits and vegetables are known to be enriched with antioxidants [7]. Inflammation is a kind of body's non-specific defence system against microbial damage or due to chemical and physical agents. These stimulants trigger a stress which lead to inflammation. The process of inflammation involves increase in vascular permeability, membrane alteration and protein denaturation [10]. During inflammation there is accumulation of fluid, leukocytes and cytokines [11]. Some of the symptoms involved in inflammation are redness, pain, heat, swellings and loss of function in injured region. Vasodilation is triggered by the release of kinins, prostaglandins and histamines. These chemical substances not only trigger vasodilation but also act as messengers to elicit chemotaxis [10]. Fighting the unstable molecules (free radicals) protects the cell damage caused by various carbohydrate, protein and fat metabolic disorders. In case of diabetes, improper food habits and genetic factors lead to failure in insulin production or insulin action or both resulting in conditions called hyperglycemia and hyperlipidemia [13]. Inflammation, ageing, cancers, arthritis, heart disease, diabetes, alzheimer's, parkinson's autism, hypertension are some of the conditons, diseases and disorders associated with oxidative stress. This study aims to explore bacteria from citrus fruit and screen for its anti-diabetic and anti-inflammatory property.

MATERIALS & METHODS

Selection of fruit sample

Citrus limon samples were procured from the local market in Katpadi. The samples were stored at a low temperatures to prevent spoilage.

Surface sterilization of plant sample and plating of sample

Citrus limon samples obtained was thoroughly washed to remove any dirt and surface sterilized with 70% ethanol. The sample was cut into smaller pieces and washed in 1% sodium hypochlorite for 20 min and then with 10% sodium carbonate and Tween 20 for complete sterilization.

The plates were then incubated at 37ºC for 24 h.

Isolation and Morphological Identification of Endophytic Organism

Single differentiated colony isolates obtained from spread plating the *citrus limon* sample was used for growing endophytes in a nutrient broth.

Production

The nutrient broth containing the endophytic organism was used for this process. Endophytes were allowed to grow for 24hr to reach stationary phase when several metabolites conferring anti-diabetic and anti-inflammatory property are produced. The nutrient broth was centrifuged for 5 min at 10,000rpm.

Morphological Identification

The isolates were subjected to gram's staining and biochemical tests. Biochemical tests were performed with KB002 & KB005A kits from HiMedia.

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Anti-inflammatory property-

Standard methods were followed to study anti-inflammatory property [17].

Anti-diabetic property

Standard method was followed to study Anti-Diabetic property [14].

RESULTS & DISCUSSION

The *citrus limon* samples were plated on nutrient agar medium and endophytic organisms were isolated from the sample.

Biochemical Tests

The results obtained from the biochemical tests have confirmed that the endophytic organism belong to the *bacillus* species.

Anti-Inflammatory Property

It can be inferred from table 6 that the percentage inhibition found through HRBC membrane stabilization method is quite less as compared to the standard diclofinac sodium. Since the value is more than 10% hence we can infer that the endophytes thriving within *citrus limon* has anti-inflammatory property though not very significant. On purification of the products obtained from the endophytes there might be an increase in the property studied.

It can be inferred from table 7 that the percentage inhibition found through protein denaturation method is quite less as compared to the results standard. Also the percentage inhibition found by HRBC membrane stabilization method is higher than that found by protein denaturation method.

Anti-Diabetic Property

The isolated endophyte had very less or minute anti-diabetic property. But the *activity was* quiet close and comparable of the property of *Lycopersicon esculentum*

In *Fig1.* Shows the mother culture which was obtained by following proper plating protocol with Citrus limon sample obtained from Katpadi. The organism has to be analysed a characterized to know about the various properties and benefits we can obtain.

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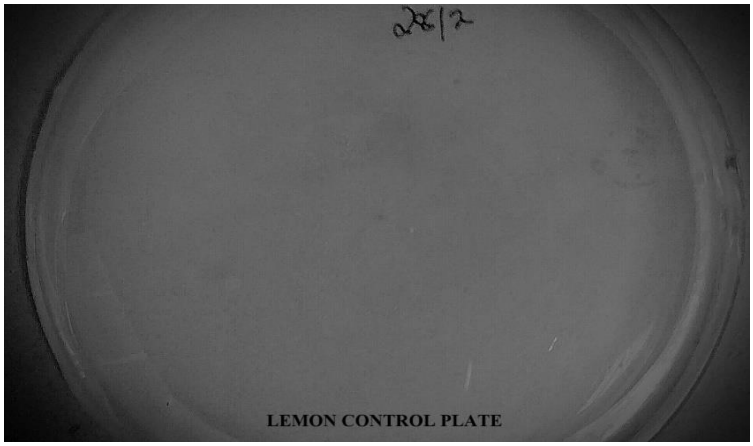
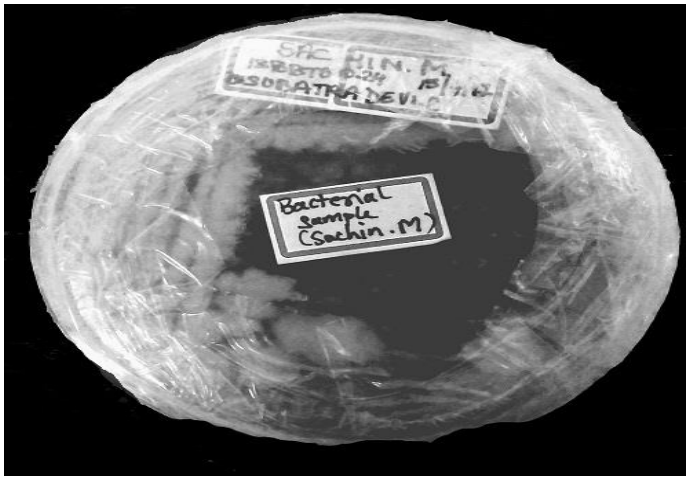
In Fig2. Shows the control plate which is devoid of growth of any organism, as only pure distilled water was platted in it, hence shows that the organism grown in the previous image is not a possible contaminant as the sterilization of the samples were carried out efficiently.



In *Fig3.* Shows the streaked pure culture plate. Mother inoculum from the previously incubated plates were used to streak the given plates to get individual pure culture colonies. These isolated colonies can then be further studied.

In *Fig4.* A single colony was taken from the previous plate and four quadrant streaking was done with the colony on an agar plate. This plate was used for further sequencing and analysis.



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In *Fig5.* Gram staining was done for the colony obtained so as to get the actual morphology of the bacterial culture. The morphology shows thin rod shaped structure which can be concluded as bacillus.

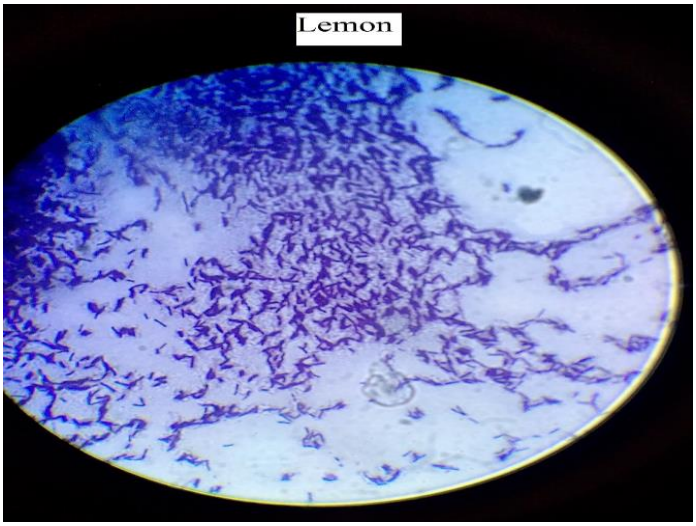

***Fig 6.* Shows the colour change that happened when the bacterial culture was incubated with KB002 kit.**

***Fig 7.* Shows the colour change that happened when bacterial sample was incubated with KB005A kit.**

***Table 1.* Shows the various tests present in KBOO2 kit and the positive and negative results that could be obtained on incubation with the bacterial culture.**

Test	Reagent to be added	Original colour of medium	Positive reaction	Negative reaction
Citrate Utilization	-	Green	Blue	Green
Lysine Utilization	-	Olive green to light purple	Purple/ Dark Purple	Yellow
Ornithine Utilization	-	Olive green to light purple	Purple/ Dark Purple	Yellow
Urease	-	Orangish Yellow	Pink	Orangish Yellow
Phenylalanine Deamination	2-3 drops TDA Reagent	Colourless	Green	Colourless
Nitrate Reduction	1-2 drops of sulphanillic acid and 1-2 drops of N,N-	Colourless	Pinkish Red	Colourless

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	Dimethyl-1-Napthylamine			
Hydrogen Sulphide Production	-	Orangish Yellow	Black	Orangish Yellow
Glucose	-	Pinkish Red/Red	Yellow	Pink/ Red
Adonitol	-	Pinkish Red/Red	Yellow	Pink/ Red
Lactose	-	Pinkish Red/Red	Yellow	Pink/ Red
Arabinose	-	Pinkish Red/Red	Yellow	Pink/ Red
Sorbitol	-	Pinkish Red/Red	Yellow	Pink/ Red

Table 2. **Shows the colour change and the inferences that can be drawn by comparing with the table 1, these inferences helps us in determining the genus.**

Test	Colour Change	Inference
Citrate Utilization	Green	Negative
Lysine Utilization	Purple	Positive
Ornithine Utilization	Purple	Positive
Urease	Pink	Positive
Phenylalanine Deamination	Colourless	Negative
Nitrate Reduction	Pinkish Red	Positive
Hydrogen Sulphide Production	Orangish Yellow	Negative
Glucose	Red	Negative
Adonitol	Red	Negative
Lactose	Red	Negative
Arabinose	Red	Negative
Sorbitol	Red	Negative

Test	Reagent to be added	Original colour of medium	Positive reaction	Negative reaction
Voges Proskauer's	1-2 drops of Baritt Reagent A and 1-2 drops of Baritt Reagent B	Colourless/ Light Yellow	Pinkish Red	Colourless/ slight copper
Esculin Hydrolysis	-	Cream	Black	Cream
PYR	-	Cream	Cherry Red	Cream
ONPG	-	Colourless	Yellow	Colourless
Arginine Utilization	-	Olive green to light purple	Purple/ Dark Purple	No change in colour or yellow
Glucose	-	Pinkish Red/Red	Yellow	Red/ pink
Lactose	-	Pinkish Red/Red	Yellow	Red/ Pink
Arabinose	-	Pinkish Red/Red	Yellow	Pink/ Red
Sucrose	-	Pinkish Red/Red	Yellow	Pink/ Red
Sorbitol	-	Pinkish Red/Red	Yellow	Pink/ Red
Mannitol	-	Pinkish Red/Red	Yellow	Pink/ Red
Raffinose	-	Pinkish Red/Red	Yellow	Pink/ Red

Table 3. **Shows the various tests in KB005A kit and the positive and negative results that can be obtained on incubation with bacterial culture.**

Test	Colour Change	Inference
Voges Proskauer's	Slight Copper	Negative
Esculin Hydrolysis	Black	Positive
PYR	Cream	Negative
ONPG	Cream	Negative
Arginine Utilization	Colourless	Negative
Glucose	Purple	Positive
Lactose	Pink	Negative

Arabinose	Yellow	Positive
Sucrose	Yellow	Positive
Sorbitol	Yellow	Positive
Mannitol	Pink	Negative
Raffinose	Pink	Negative

Table 4. **Shows the colour change and the inferences that can be drawn from the colour change in the biochemical kit (KB005A), these inferences helps us in determining the genus of the organism.**

Sample	Percentage Inhibition
Citrus limon	12.2% ± 0.054
Diclofenac Sodium	87%

Table 5. **Shows the percentage inhibition by HRBC membrane stabilisation method.**

Sample	Percentage Inhibition
Citrus limon	4%±0.925
Diclofenac Sodium	87%

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able *6*. Shows the percentage inhibition by protein denaturation.

Sample	Percentage Inhibition
Lemon	4%±0.426

Table 7. **Shows the percentage inhibition of alpha amylase.**

CONCLUSION

Citrus limon (lemon) has previously shown various pharmacological activities in various studies hence we chose to characterise the endophytes thriving in *Citus limon,* The isolated organism was found out to be a gram positive, rod shaped (*Bacillus*) bacterium as concluded through gram staining procedure. Additional studies have to be supported out to find the species in various varieties of *citrus limon* during its growth stages. The characterisation of selected endophytic bacterial isolates were performed and evidently verified through various assays. The study evidenced that the bacillus isolates possess certain properties like anti-inflammatory (4%±0.925) and anti- diabetic (4%±0.426) which were found out by HRBC membrane stabilisation method and alpha amylase inhibition method respectively. The percentages are less but not negligible, hence we can conclude that the *bacillus* that is the endophytes thriving inside the *citrus limon* plays an important role in production of certain bioactive compounds that imparts these characteristic properties to *citrus limon*. Detailed investigations on the isolated endophytic bacteria from *citrus limon* are needed to evidence its prospective further. Also further research might lead to the encountering of several other endophytic species like fungi having various other characteristic properties. Endophytes like any other organism undergo mutation from time to time hence their study becomes very interesting and time consuming and also leaves us huge scope to study them further.

REFERENCES

[1] D. Dutta[1]*, K. C. Puzari[2], R. Gogoi[3], and P. Dutta[4], "Endophytes: Exploitation as a tool in plant protection," *Braz. Arch. Biol. Technol*, vol. 57, no. 5, pp. 621–629, 2014.

[2] G. E. Aiken, M. D. Flythe, I. A. Kagan, H. Ji, L. P. Bush, and G. E. Aiken, "Mitigation of ergot vasoconstriction by clover isoflavones in goats (Capra hircus)," *Front. Vet. Sci.*, vol. 3, no. March, pp. 1–10, 2016.

[3] A. Alvin, A., Kristin, I., Miller, B., and Neilan, "Exploring the potential of endophytes from medicinal plants as sources of antimycobacterial compounds.," *Microbiol. Res.*, vol. 169, pp. 483–498, 2014.

[4] D. N. Nair and S. Padmavathy, "Impact of endophytic microorganisms on plants , environment and humans," *Sci. World J.*, vol. 2014, p. 11, 2014.

[5] B. R. Vázquez-de-aldana, A. García-ciudad, B. García-criado, and S. Vicente-, "Fungal endophyte (*epichloë festucae*) alters the nutrient content of festuca rubra regardless of water availability," *PLoS*

One, vol. 8, no. 12, pp. 1–14, 2013.

[6] M. Jia, L. Chen, H. Xin, C. Zheng, K. Rahman, and T. Han, "A friendly relationship between endophytic fungi and medicinal plants : a systematic review," *Front. Microbiol.*, vol. 7, no. June, pp. 1–14, 2016.

[7] C. Kaur and H. C. Kapoor," Antioxidants in fruits and vegetables–the millennium's health". Int J Food Sci Tech. 2001; 36:703–725..

[8] L. Azeez, M. D. Adeoye, T. A. Majolagbe, A. T. Lawal, and R. Badiru, "Antioxidant activity and phytochemical contents of some selected nigerian fruits and vegetables," *Am. J. Chem.*, vol. 2, no. 4, pp. 209–213, 2012.

[9] S. Li, G. Chen, C. Zhang, M. Wu, S. Wu, and Q. Liu, "Research progress of natural antioxidants in foods for the treatment of diseases," *Food Sci. Hum. Wellness*, vol. 3, no. 3–4, pp. 110–116, 2014.

[10] G. Leelaprakash, S. M. Dass, and B. Road, "Invitro anti-inflammatory activity of methanol extract of enicostemma axillare," *Int. J. Drug Dev. Rsearch*, vol. 3, no. 3, pp. 189–196, 2011.

[11] Y. Nalli, S. Gupta, V. Khajuria, V. P. Singh, M. Sajgotra, Z. Ahmed, N. L. Thakur, and A. Ali, "Chemistry TNF- α and IL-6 inhibitory effects of cyclic dipeptides isolated from marine bacteria *streptomyces sp*," *Med. Chem. Res.*, pp. 0–1, 2016.

[12] Madhusudhan Telagari and Kirankumar Hullatti, "Invitro alpha -amylase and alpha-glucosidase inhibitory activity of *adiantum caudatum linn*. and *celosia argentea linn.* extracts and fractions," *Indian J Pharmacol*, vol. 47, no. 4, pp. 425–429, 2015.

[13] I. G. Tamil, B. Dineshkumar, M. Nandhakumar, M. Senthilkumar, and A. Mitra, "In vitro study on α -amylase inhibitory activity of an indian medicinal plant , *Phyllanthus amarus*," *Indian J Pharmacol*, vol. 42, no. 5, pp. 280–282, 2010.

[14] G. Keerthana, M. K. Kalaivani, and A. Sumathy, "In-vitro alpha amylase inhibitory and anti-oxidant activities of ethanolic leaf extract of *croton bonplandianum*," *Asian J. Pharm. Cult. Res.*, vol. 6, pp. 2–6, 2013.

[15] M. A. Bhutkar and S. B. Bhise, "In vitro assay of alpha amylase inhibitory activity of some indigenous plants," *Int. J. Chem.*, vol. 10, no. 1, pp. 457–462, 2012.

[16] N. Babbar and H. S. Oberoi, "Influence of different solvents in extraction of phenolic compounds from vegetable residues and their evaluation as natural sources of antioxidants," vol. 51, no. October, pp. 2568–2575, 2014.

[17] G. Kishore, G. Siva, and E. S. Sindhu, "In vitro anti-inflammatory and anti-arthritic activity of leaves of *physalis angulata* l.," *Int. J. Pharm Ind. Res*, vol. 1, no. 3, pp. 211–213, 2011.

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