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Screening of antibacterial metabolites from the endophytic bacteria isolated from the medicinal plant *Saraca Asoca*

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Abstract
Bacterial endophytes are prolific producers of novel bioactive compounds. New antimicrobial compounds and antibiotics are necessary to combat growing antimicrobial resistance. The present study focuses on the isolation of endophytic bacteria and extraction of bioactive compounds from the medicinal plant *Saraca asoca.* Two endophytic bacterial isolates were obtained from the flower and leaf tissues of *Saraca asoca.* The screening of endophytic bacteria based on their antimicrobial activity against the test organisms, *Staphylococcus aureus, Pseudomonas* sp., *Escherichia coli* and *Klebsiella* sp. revealed that the isolate As from leaves of *Saraca asoca* exhibited significant antimicrobial activity. The isolate was characterized based on its morphological, cultural and biochemical properties. Molecular analysis revealed that the isolate As showed 99.52% similarity to *Enterobacter cloacae* strain ALD4.5. Bioactive compound was extracted from the isolate As using ethyl acetate and it showed significant growth inhibition against *Staphylococcus aureus.*

Keywords: Endophytes, *Saraca asoca*, *Enterobacter cloacae*, Bioactive compounds, Antimicrobial compounds

1. Introduction
All investigated plant taxa have well established symbiosis with microorganisms [1, 2] and these associations can be neutral, pathogenic or beneficial to the host [3]. Endophytes are regarded as a group of microorganisms that colonize the inter-and/or intracellular locations of plants [4, 5]. They are present in various forms inside a plant, such as bacteria (actinomycetes or mycoplasma) or fungi. More than 200 genera of bacterial species have been recognized to be associated with endophytes and most of the species belong to the phyla Actinobacteria, Proteobacteria and Firmicutes [6]. Gram negative to gram positive bacteria such as *Achromobacter, Acinetobacter, Agrobacterium, Bacillus, Brevibacterium, Pseudomonas, Xanthomonas* etc. are present as endophytes [7]. Bacterial endophytes produce various bioactive metabolites that act as antimicrobial and anticancer compounds [8].
Endophytes produce several secondary metabolites that possess antimicrobial properties which belong to different structural groups such as peptides, steroids, alkaloids, phenols and flavonoids [9, 10]. Endophytic bacteria produce a range of antibiotics including ecomycin, pseudomycins and kakadumycins [11]. Medicinal plants are rich sources of natural products and have been used in the treatment of various ailments. Plant bioactive compounds are present in low levels, long growth periods are required for plant maturation and there is great difficulty in extraction and purification of compounds from various other plant derived compounds [12]. Hence, alternative approaches to produce such bioactive compounds are essential. By exploiting endophytes residing in such plants, the production of similar or same bioactive compounds as their host is attainable [13].
In this study, the medicinal plant *Saraca asoca* (Ashoka) that belongs to the Detarioideae subfamily of the Fabaceae family was selected. It is one of the most significant ayurvedic drug used in the treatment of several feminine disorders. It is known to purify blood and prevent skin allergies. Several properties such as uterine tonic activity, antihemorrhagic activity, anti-diabetic, analgesic, antimicrobial, anti-helminthic and anti-oxidant activity are also possessed by it [14]. Novel antimicrobial compounds produced by endophytes are alternatives to chemically synthesized ones and are highly effective against multi drug resistant strains [15, 16]. The aim of the current study was the isolation and molecular characterization of endophytic bacteria from *Saraca asoca* and its screening for antimicrobial properties.

2. Materials and methods
2.1. Sample collection
The plant was identified on the basis of botanical characteristics. Healthy leaves and flowers of the plant was collected from Kothamangalam, Kerala, India for the study. Plant materials were brought to the laboratory in polythene bags and washed thoroughly in running tap water [17].

2.2. Surface sterilization
The samples were cut into small pieces using a sterile blade and washed in distilled water. The samples were immersed in 0.1% $HgCl_2$ for about 5 minutes and were washed in sterile distilled water 3-4 times to remove the surface sterilizing agent. The samples were then placed in 70% ethyl alcohol for 5 minutes and again washed in sterile distilled water 3-4 times. After sterilization, the samples were dried on blotting sheets. The efficiency of the surface sterilization procedure was confirmed by plating the final rinse water on nutrient agar medium [18].

2.3. Isolation of endophytes
The sterilized pieces were placed aseptically on Nutrient agar medium and the plates were incubated at 37ºC for 24-36 hours. At the end of the incubation, the bacterial colony was subcultured on nutrient agar. Each bacterial colony was checked for purity and subcultured again [17].

2.4. Primary screening of endophytic bacteria with antibacterial activity
Cross-streak method was used for the primary screening of isolates based on their inhibitory action against the test organisms, *Staphylococcus aureus, Pseudomonas* sp, *Escherichia coli* and *Klebsiella* sp. The test organisms were streaked at right angles to the line of growth of the producer isolate which was previously inoculated and incubated at 37ºC for 24 hours. The inoculated plates were re-incubated at 37ºC for 24 hours. The extent of inhibition against different test organisms were noted [19].

2.5. Secondary Screening
Agar well diffusion method was used for secondary screening. The strain As isolated from leaves of Ashoka (*Saraca asoca*) was selected for secondary screening on the basis of inhibition against the test organisms in primary screening. Samples of the culture filtrate was assayed for antimicrobial activity. The Mueller- Hinton agar plate surface was inoculated by spreading the test organism over the entire agar surface using a cotton swab. Two holes with a diameter of 6-8mm were punched aseptically using a sterile tip on the agar plate. Culture filtrate (50 µl) was transferred to a well using a micropipette. The plate was incubated at 37ºC for 24 hours and observed for zone of growth inhibition. Control along with a standard antibiotic amikacin (AK) dic was placed in the plate [19].

2.6. Morphological and Cultural Characterization
The size, shape, margin, elevation, consistency, opacity, pigmentation, Gram staining and motility and biochemical activity of As was estimated [20].

2.7. Molecular Analysis
2.7.1. Isolation of genomic DNA
The organism was inoculated into 3ml of Luria Bertanni Broth (LB) and incubated at 37ºC for 16 hours. DNA was isolated using NucleoSpin® Microbial DNA isolation kit. Cells were harvested from the culture by centrifugation in a microcentrifuge tube and discarded the supernatant. 100µl Elution Buffer BE was added and resuspended the cells. The cell suspension was transferred into the NucleoSpin® Bead Tube Type B. 40µl Buffer MG and 10µl Liquid Proteinase K was added and closed the tube. Agitated and centrifuged the NucleoSpin® Bead Tube 30s at 11,000 x g to clean the lid. 600µl Buffer MG was added, vortexed and centrifuged for 30s at 11,000 x g. The supernatant was centrifuged for 30s at 11, 000 x g. 500µl Buffer BW was added and centrifuged for 30s at 11,000 g. Placed the NucleoSpin® Microbial DNA column into a 1.5ml nuclease-free tube and added 100 µl Buffer BE onto the column. Incubated at room temperature for 1 minute and centrifuged for 30s at 11,000 g to elute the DNA [21].

2.7.2. Qualitative analysis
Agarose Gel Electrophoresis
Agarose gel electrophoresis was used for the separation and visualization of the extracted DNA. 0.8% agarose gel was prepared by dissolving 0.16 g of agarose in 20 ml 1x TAE buffer. The solution was heated until agarose melted and allowed to cool and 2µl Ethidium Bromide was added to it. Comb was placed in the gel tray and agarose was poured into a gel tray and allowed to set. After the gel was solidified, the comb was removed. The gel tray was transferred to electrophoresis tank containing 1x TAE buffer. 5 µl of sample mixed with 1µl 6 X loading dye and loaded into well. After electrophoresis, the gel was visualized under UV Transilluminator to detect the presence of DNA [21].

2.7.3. PCR amplification and detection of PCR product
The DNA was amplified using forward and reverse primers. The PCR product was detected by agarose gel electrophoresis [21].
Forward primer A2:
5' AGAGTTTGATCCTGGCTCAG 3'
Reverse primer S8:
5' TCTACGCATTTCACCGCTAC 3'
The 16s rRNA genes were analyzed using NCBI Basic Local Alignment Search Tool (BLAST) and highly similar sequences were found.

2.7.4. Phylogenetic analysis
MEGA- X was used to construct a neighbor-joining phylogenetic tree using the Kimura two-parameter and the reliability of the branching and clustering pattern was estimated from 1000 bootstrap replicates [23].

2.8. Determination of the effect of plant extract on growth of endophytic bacteria:
This was performed to determine the effect of plant extract on the growth of endophytic bacteria. The leaves of Ashoka (*Saraca asoca*) were boiled in water to prepare the plant extract. 1 ml of bacterial suspension of As was mixed with 1 ml of peptone water (control). Again, 1 ml of bacterial suspension of As was mixed with 1ml of plant extract of *Saraca asoca*. The tubes were incubated for a total of 120 hours. And readings were taken at a wavelength of 600 nm at 24 hours, 36 hours and 120 hours respectively [17].

2.9. Extraction of Bioactive Compound
Growth from a mature slant culture of the strain As was inoculated into 5ml of nutrient broth and incubated for 37ºC for 24 hours. The solvents; ethyl acetate, chloroform, acetone and hexane were selected fro the extraction of bioactive compound. 1ml of each solvent was transferred into 4 sterile test tubes. 1ml of the suspension of strain was added to each of the test tubes and vortexed. Aqueous and solvent layers were separated into sterile test tubes and dried in a water bath. Dimethyl sulfoxide (DMSO) was added to each tube. The zone of inhibition against the test organism *Staphylococcus aureus* was determined using agar well diffusion method and the solvent which exhibited the largest zone was noted [19].

3. Results and discussion
Medicinal plants are producers of pharmacologically important secondary metabolites and essential oils. They are used in food preservation and in reducing the dose of antibiotic for treatment of infections [24]. Hence they are appropriate targets for the isolation of endophytic bacteria which are rich sources of secondary metabolites with antibacterial activity [25]. This is the first report on the bioactive potential of *Saraca asoca*. The isolate discovered in this study belongs to the phylum Proteobacteria, which is the most predominant phylum isolated from plants [26].
Two endophytic bacterial isolates Af and As were obtained from the flower and leaf tissues of the selected medicinal

plant, *Saraca asoca*. The bacterial colonies were isolated and streaked over the surface of Nutrient agar and maintained.
The primary screening of isolates based on their inhibitory action against the test organisms, *Staphylococcus aureus, Pseudomonas* sp., *Escherichia coli* and *Klebsiella* sp. revealed that the isolate As from leaf tissues of *Saraca asoca* inhibited the growth of *Staphylococcus aureus* whereas the isolate Af did not inhibit the growth of any test organisms (Table 1).
Based on primary screening, As was selected for secondary screening of the isolate to confirm its inhibitory activity. It inhibited the growth of *Staphylococcus aureus* and *Pseudomonas* sp. The largest zone of growth inhibition was observed against *Staphylococcus aureus* (Table 2).
Metabolites containing antibacterial activity can be defined as low molecular substances made by microorganisms that are active at low concentrations against other microorganisms [9]. Endophytes are known to resist pathogenic invasion by producing such secondary metabolites [27].
Colony characteristics of the endophytic bacteria As on nutrient agar was observed and noted (Table 3)**.** Gram staining, motility and various biochemical tests were performed using As (Table 4).
The DNA of the isolate As was extracted using NucleoSpin® Microbial DNA isolation kit. The DNA obtained was analyzed by Agarose Gel Electrophoresis and clear bands were observed under UV transilluminator. The 16s rRNA genes were amplified using the forward and reverse primers A2 and S8.
The sequence of As was analyzed using NCBI Basic Local Alignment Search Tool (BLAST) and highly similar sequences were found. The trimmed sequence of the isolate As consisted of 629bp and showed 99.52% similarity to *Enterobacter cloacae* strain ALD4.5.
The results of the phylogenetic analysis showed that the isolate As belonged to the genus *Enterobacter*. These have been previously isolated from various plant species such as *Vochysia divergens* [28], *Musa* sp. [29], *Ocimum sanctum* [30] and *Brassica chinensis* L [31]. This indicates that the isolate is present as endophyte within a variety of plant species which makes them more interesting for further studies such as extraction of bioactive compounds.
The effect of plant extract on growth and antibacterial activity of endophytic bacteria was determined by taking readings at a wavelength of 600 nm at 24 hours, 36 hours and 120 hours respectively. Even though, initially plant extracts showed a positive effect on the growth of bacteria, on prolonged incubation it did not enhanced the growth of the endophytic bacteria (Table 5, Figure 8).
On the solvent extraction of the bioactive compound, ethyl acetate exhibited the largest zone of growth inhibition. The aqueous layer showed inhibition of test organism which indicated the presence of bioactive compound in the aqueous layer (Table 6). It has been previously reported that plant endophytes produce compounds with antimicrobial activity [32, 33, 34]. Endophytes are reported to be chemical synthesizers within plants capable of synthesizing bioactive compound that provide defense against pathogens. These compounds have been reported to be valuable for drug discovery [9, 35].
These reports supports the inhibitory action observed by the sisolated endophyte As. It was also observed that Gram positive bacteria appear to be more susceptible than Gram negative bacteria which could be due to the presence of outer membrane in Gram negative organisms. It is known that Gram negative bacteria are more resistant to antibiotics than Gram positive bacteria [34].

4. Tables and figures

Table 1: Primary screening of bacterial endophytes with antimicrobial activity

Endophytic bacteria	Zone of inhibition against test organisms (mm)			
	Staphylococcus aureus	***Pseudomonas* sp.**	***Escherichia coli***	***Klebsiella* sp.**
As	1	0	0	0
Af	0	0	0	0

Table 2: Secondary screening of endophytic bacteria with antimicrobial activity

Test organisms	Zone of growth inhibition (mm)		
	As	Amikacin (AK)	Control (C)
Staphylococcus aureus	**15**	**23**	**0**
Pseudomonas sp	10	25	0
Escherichia coli	0	18	0
Klebsiella sp.	0	20	0

As- Endophytic bacteria from leaves of *Saraca asoca,* Control- peptone water

Table 3: Colony characteristics of As on nutrient agar

Characteristics	As
Size	Small
Shape	Irregular
Pigmentation	No pigment
Margin	Undulate
Elevation	Flat
Opacity	Opaque
Consistency	Mucoid

Table 4: Morphological and biochemical characteristics of As

Tests	As
Gram stain	-
Shape	Bacilli
Motility	Motile
Indole test	-
Methly red test	-
Voges Proskauer test	-
Citrate utilization test	+
Urease test	-
TSI test	A/A, Gas
Nitrate reduction test	+
Catalase test	+
Oxidase test	-

Positive (+), Negative (-)

Table 5: Growth of Endophytic bacteria

Time	Growth of Bacteria (600nm)		
	Blank	Plant Extract + Bacteria (As)	Bacteria (As)
24 hours	0	0.6057	0.5547
36 hours	0	0.6305	0.2835
120 hours	0	0.6449	1.1740

Table 6: Antimicrobial activity of Bioactive Compounds extracted with different solvents

Solvent	Zone of inhibition (mm)	
	As	Control
CS	0	0
CA	0	0
HS	0	0
HA	0	0
ES	0	**0**
EA	25	**0**
AS	0	0
AA	0	0
D	0	0

CS- Chloroform solvent layer, CA- Chloroform aqueous layer, HS- Hexane solvent layer, HA- Hexane aqueous layer, ES- Ethyl acetate solvent layer, EA- Ethyl acetate aqueous layer, AS- Acetone solvent layer, AA- Acetone aqueous layer, D- Dimethyl sulfoxide

Fig 1: *Saraca asoca* [36]

Fig 2: Bacterial endophytes from *Saraca asoca*

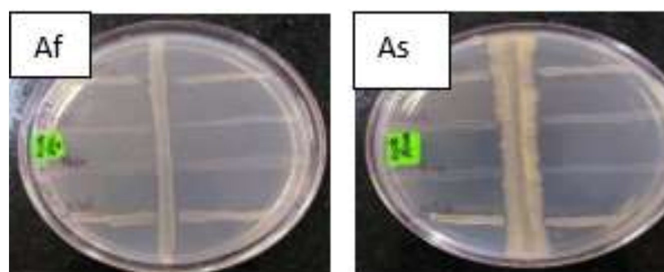

Fig 3: Primary screening for antibacterial activity

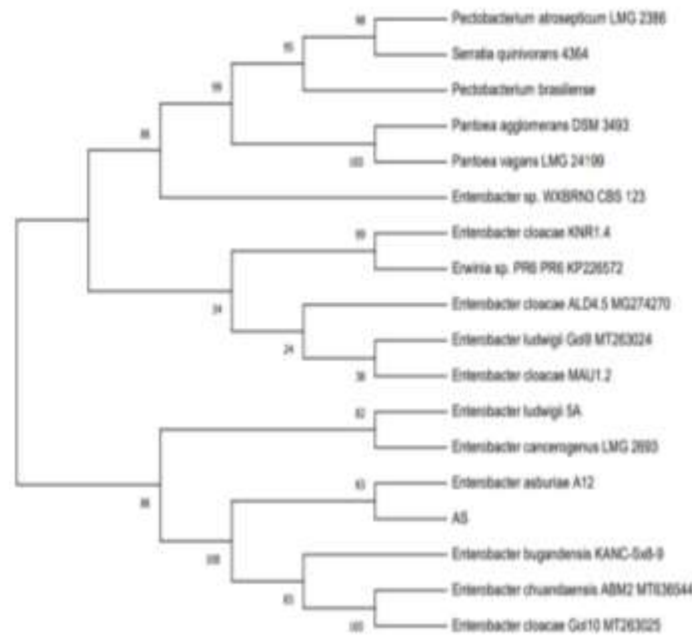

Fig 4: Phylogenetic tree of As

Fig 5: Antimicrobial activity of Bioactive Compounds extracted with different solvents

CS- Chloroform solvent layer, CA- Chloroform aqueous layer, HS- Hexane solvent layer, HA-Hexane aqueous layer, ES- Ethyl acetate solvent layer, EA- Ethyl acetate aqueous layer, AS- Acetone solvent layer, AA- Acetone aqueous layer, D- Dimethyl sulfoxide

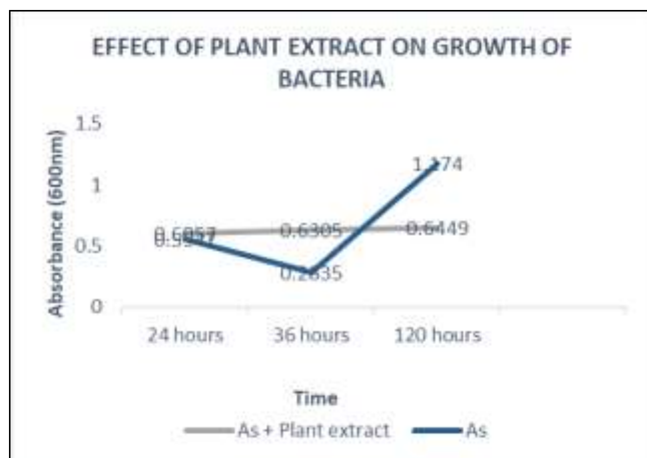

Fig 6: Determination of the effect of plant extract on growth of bacteria

5. Conclusion

This study indicates the presence of a plant-microbe relationship in the flowers and leaves of *Saraca asoca*. And this is the first report on occurrence of bacterial endophytes in *Saraca asoca*. In brief, the endophytic bacteria isolated from the leaves of *Saraca asoca* was identified as *Enterobacter cloacae* strain ALD4.5. The endophytic bacteria as well as the bioactive compound extracted from the endophytic bacteria demonstrated antibacterial activity, notably against *Staphylococcus aureus*. It can be concluded that the endophytic bacteria can be used for the production of novel bioactive compounds and can be considered as a potential source for the development of new drugs. Further research is needed to isolate and characterize pure bioactive compounds produced by the endophyte.

6. Reference

1. Nicolson TH. "Vesicular-arbuscular mycorrhiza—a universal plant symbiosis." Science Progress. 1933 (1967):561-581.
2. Brundrett Mark C. "Mycorrhizal associations and other means of nutrition of vascular plants: understanding the global diversity of host plants by resolving conflicting information and developing reliable means of diagnosis." Plant and Soil. 2009; 320:1-2:37-77.
3. Bary Anton de. "De la symbiose." Revue internationale des Sciences. 1879; 3:301-309.
4. Pimentel, Mariana Recco, *et al*. The use of endophytes to obtain bioactive compounds and their application in biotransformation process." Biotechnology research international, (2011).
5. Singh Radha, AK Dubey. "Endophytic actinomycetes as emerging source for therapeutic compounds." Indo Global J Pharm Sci. 2015; 5.2:106-116.
6. Golinska Patrycja. *et al*. "Endophytic actinobacteria of medicinal plants: diversity and bioactivity." Antonie Van Leeuwenhoek. 2015; 108.2:267-289.
7. Sun Hui. *et al*. "Isolation, characterization, and antimicrobial activity of endophytic bacteria from Polygonum cuspidatum." African Journal of Microbiology Research. 2013; 7.16:1496-1504.
8. Bérdy, János. "Thoughts and facts about antibiotics: where we are now and where we are heading." The Journal of antibiotics. 2012; 65.8:385-395.

9. Guo B. *et al*. "Bioactive natural products from endophytes: a review." Applied Biochemistry and Microbiology. 2008; 44.2:136-142.
10. Yu Hongsheng. *et al*. "Recent developments and future prospects of antimicrobial metabolites produced by endophytes." Microbiological research. 2010; 165.6: 437-449.
11. Christina Ambrose, Varghese Christapher, Subhash J. Bhore. "Endophytic bacteria as a source of novel antibiotics: an overview." Pharmacognosy reviews. 2013; 7.13:11.
12. Staniek Agata. *et al*. "Natural products–learning chemistry from plants." Biotechnology journal. 2014; 9.3:326-336.
13. Zhao Ke. *et al*. "The diversity and anti-microbial activity of endophytic actinomycetes isolated from medicinal plants in Panxi plateau, China." Current microbiology. 2011; 62.1: 182-190.
14. Bhalerao, Satish A., et al. "Saraca asoca (Roxb.), de. Wild: an overview." Annals of plant sciences 3.7 (2014): 770-775.
15. Ferlay Jacques. *et al*. "Estimates of worldwide burden of cancer in 2008: Globocan 2008." International journal of cancer 127.12. 2010, 2893-2917.
16. Taechowisan, Thongchai. *et al*. "Anti-inflammatory effect of 3-methylcarbazoles on RAW 264.7 cells stimulated with LPS, polyinosinic-polycytidylic acid and Pam3CSK." 2012.
17. Das I. *et al*. "Bioactivities of bacterial endophytes isolated from leaf tissues of Hyptis suaveolens against some clinically significant pathogens." J Appl Pharm Sci. 2017; 7:131-136.
18. Aneja KR. Experiments in microbiology, plant pathology and biotechnology. New Age International, 2007.
19. Balouiri Mounyr, Moulay Sadiki, Saad Koraichi Ibnsouda. "Methods for in vitro evaluating antimicrobial activity: A review." Journal of pharmaceutical analysis 6.2. 2016, 71-79.
20. Cappuccino James G, Natalie Sherman. "Microbiology: a laboratory manual, 2005.
21. https://www.mn-net.com
22. Sambrook HC. "Molecular cloning: a laboratory manual. Cold Spring Harbor, NY, 1989.
23. Beiranvand Maryam. *et al*. "Antimicrobial activity of endophytic bacterial populations isolated from medical plants of Iran." Iranian journal of microbiology 9.1, 2017, 11.
24. Katoch Meenu. *et al*. "Diversity, Phylogeny, anticancer and antimicrobial potential of fungal endophytes associated with Monarda citriodora L." BMC microbiology 17.1, 2017, 44.
25. Mohamad Osama AA. *et al*. "Evaluation of the antimicrobial activity of endophytic bacterial populations from Chinese traditional medicinal plant licorice and characterization of the bioactive secondary metabolites produced by Bacillus atrophaeus against Verticillium dahliae." Frontiers in Microbiology. 2018; 9:924.
26. Afzal Imran. *et al*. "Plant beneficial endophytic bacteria: Mechanisms, diversity, host range and genetic determinants." Microbiological research. 2019; 221:36-49.
27. Tan Ren Xiang, Wen Xin Zou. "Endophytes: a rich source of functional metabolites." Natural product reports 18.4. 2001, 448-459.
28. Diale Mamonokane O, Eunice Ubomba-Jaswa, Mahloro H. Serepa-Dlamini. "The antibacterial activity of bacterial endophytes isolated from Combretum molle." African Journal of Biotechnology 17.8. 2018, 255-262.
29. Macedo-Raygoza, Gloria M. *et al*. "Enterobacter cloacae, an endophyte that establishes a nutrient-transfer symbiosis with banana plants and protects against the black Sigatoka pathogen." Frontiers in microbiology. 2019; 10:804.
30. Panigrahi SS, Mohanty CC. Rath. "Characterization of endophytic bacteria Enterobacter cloacae MG00145 isolated from Ocimum sanctum with indole acetic acid (IAA) production and plant growth promoting capabilities against selected crops." South African Journal of Botany, 2019.
31. Wang Wenfeng. *et al*. "Application of an Endophyte Enterobacter sp. TMX13 to Reduce Thiamethoxam Residues and Stress in Chinese Cabbage (Brassica chinensis L)." Journal of Agricultural and Food Chemistry 68.34, 2020, 9180-9187.
32. Huang Yaojian. *et al*. "Antitumor and antifungal activities in endophytic fungi isolated from pharmaceutical plants Taxus mairei, Cephalataxus fortunei and Torreya grandis." FEMS Immunology & Medical Microbiology 31.2, 2001, 163-167.
33. Li Jia Yao, Gary A. Strobel. "Jesterone and hydroxy-jesterone antioomycete cyclohexenone epoxides from the endophytic fungus Pestalotiopsis jesteri." Phytochemistry 57.2, 2001, 261-265.
34. Uche-Okereafor Nkemdinma. *et al*. "Antibacterial activities of crude secondary metabolite extracts from Pantoea species obtained from the stem of Solanum mauritianum and their effects on two cancer cell lines." International journal of environmental research and public health 16.4, 2019, 602.
35. Sadrati Nouari. *et al*. "Screening of antimicrobial and antioxidant secondary metabolites from endophytic fungi isolated from wheat (Triticum durum)." Journal of plant protection research, 2013.
36. https://commons.wikimedia.org/wiki/File:Saraca_asoca_(Roxb.)_Willd._(420111730).jpg 6 October 2020