The Bioscan 15(4): 525-528, 2020
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES
www.thebioscan.com

IDENTIFICATION OF *BRADYRHIZOBIUM JAPONICUM* STRAIN ISOLATED FROM ROOT NODULES OF SHANKAPUSHPI (*Clitorea ternatea* L.)

P.DHANRAJ[1]*., A. P. MALLIKARJUNA GOWDA [2]., R .MUTHURAJU [3]., T.H. SHANKARAPPA[4]., S. ANIL KUMAR [5]., Y.S. PRANEETH[6] AND M. AVINASH[6]

[1]Department of Plantation, Spices, Medicinal and Aromatic crops, KRC college of Horticulture, Arabhavi-591218 Karnataka
[2]ICAR- KVK, Hadonahalli, Tubgere Hobli, Doddaballapura, Taluk, Bengaluru Rural District-561205 , Karnataka
[3]Department of Agriculture Microbiology ,UAS, GKVK-Bengaluru North Taluk - 560065,Bengaluru
[4]Department of Agriculture Microbiology,[5]Department of soil science and agriculture chemistry,[6]Department of Plantation, Spices, Medicinal and Aromatic crops
College of Horticulture, UHS Campus Bettahalli layout,GKVK- 560065
[7]Shanthipura, Puradalu post, Shivamogga - 577205, Karnataka
e-mail: dhanrajpnaik96@gmail.com

KEYWORDS
Shankapushpi
Bradyrhizobium japonicum
16s rRNA
YEMA

Received on :
10.09.2020

Accepted on :
09.10.2020

***Corresponding author**

ABSTRACT
The study was under taken to isolate native strain of *Rhizobium* from *Clitoria ternatea* L. Known as shankapushpi, cultivated at College of Horticulture, Bengaluru. *Rhizobium* has been identified by following 16S rRNA analysis procedure. The objective of this study was to isolate and identify native strains of Rhizobium in shankapushpi root nodules on YEMA media and characterized for morphological properties. Partial 16S rRNA gene sequence from the isolates was amplified by PCR using 27F and 1492R primers. Purification and sequencing of the amplified fragments were done at macrogen laboratory Bengaluru. Sequences were analyzed using the programmed BLAST and results of BLAST queries showed that, the isolates have scored the maximum score, 100% query coverage, E value of 0.0 and 100% identity were selected as the probable identities of the isolate. Results of present study confirmed that, the presence of Bradyrhizobium japonicum strain in root nodules of Shankapushpi and deposited in the NCBI Gene Bank database under a unique accession number (KY864921). Identified strain from shankapushpi is of great importance because the symbiosis relationship between rhizobia and legumes is one of the effective agronomic practice to ensure an adequate supply of nitrogen for legumes, while improving fertility status of soils.

INTRODUCTION

Plants of the legume family can effectively make their own fertilizers by forming symbioses with a diverse group of nitrogen-fixing soil bacteria known as rhizobia. This cross-kingdom collaboration is characterized by the formation of the root nodule, a specialized plant organ that provides an optimized environment in which the bacteria convert atmospheric nitrogen into ammonia. The symbiotic interactions between leguminous plants and Rhizobium bacteria show high species specificity, which is determined by the exchange of signal molecules (Faruque *et al*., 2015). Rhizobia contribute 65 % of total atmospheric nitrogen fixed in an environment and thus reduces the requirement of nitrogenous fertilizer, which in turn reduces adverse environmental effects (Somasegaran and Hoben, 1994). The agricultural, ecological and economic importance of legume plants besides quality and chemical composition is reflected in the ability of these plants to fix atmospheric nitrogen in the community with the root nodulating bacteria (Sengupta and Reddy, 2011). Most of the legume rhizobia symbiosis studies have been carried out on either agriculture crops (soybean, mungbean, pea and chickpea) and pasture legumes (alfalfa). From last two decades such studies have been extended to wild legume plants from tropical to subtropical zones that resulted in discovering several novel species and genera of an organism (Peix *et al*., 2015).

Shankapushpi one such novel wild leguminous medicinal plant, botanically known as *Clitoria ternatea* L. belonging to the family Fabaceae. The plant originated from tropical Asia and distributed widely in South and Central America (Barik *et al*., 2007). Mainly used as a forage as it is highly palatable for live-stock apart from its various medicinal usage (Gomez *et al*., 2003). The genus Clitoria comprises of about 60 species distributed mostly within the tropical belt with a few species found in temperate areas. The most frequently reported species is *Clitoria ternatea* L. The plant is considered as Madhya-Rasayana in Ayurveda and used for its action on the CNS (Central Nervous System), especially for boosting memory and improving intellect (Sethiya *et al*., 2009). The leaves of shankapushpi conatains glycosides *viz*., kaempferol-3-

glucoside, kaempferol-3-rutinoide and kaempferol-3-neohesperidoside. The seeds have nucleoprotein with its aminoacid sequence similar to insulin, delphinidin-3,3,5-triglucoside, essential amino-acids, and water soluble mucilage (Zingare *et al*., 2013). The root powder of clitorea is used as one of the ingredients in the preparation of the drug "SULAK" and its ointment to treat leprosy. The flower is also being used traditionally as diuretic, anthelmintic, purgative, demulcent and remedy for rheumatism, bronchitis, urinogenital, disorderand cancer (Subramanian and Prathyusha, 2011).

The sequence of the 16S rRNA gene has been widely used as a molecular clock to estimate relationships among bacteria but more recently it has also become significant as a means to identify an unknown bacterium to the genus or species level. Limited number of 16S rRNA sequences have been available at Gene Bank. Although those sequences are of different lengths and qualities, in complementary regions they differ from each other by no more than a few nucleotides (Claudio *et al*., 2002). Therefore, minimal level of diversity seen in Bradyrhizobium japonicum was thought to be an obstacle for using 16S rRNA gene sequencing to identify and differentiate species.

Bacteria form root nodules of legumes have long been placed in a common genus *Rhizobium* and these species of organisams are symbiotically associated with several leguminous plants are characterised as gram negative, motile, non-endospore forming bacteria. These bacteria are generally cultured in Yeast Mannitol Agar medium (Priya and harish, 2013). Morever, the application of molecular techniques in microbiology enabled a simple, fast and reliable genotypic characterization of rhizobia and pointed to their great genetic diversity and divergence. The search for effective strains capable of eliciting and invading root nodules on leguminous plants require isolation and identification of a large number of desirable species (Marinkovic, 2012). Therefore, the aim of the present investigation is to isolate and identify native strains of *Rhizobium* in root nodules of shankapushpi on the basis of molecular characteristics.

MATERIALS AND METHODS

A experiment was conducted at College of Horticulture, University of Horticultural Sciences Campus, Gandhi Krishi Vignana Kendra (Post), Bengaluru during 2016-17. The native *Rhizobium* strain of shankapushpi was identified by following 16S rRNA procedure at macrogen laboratory Bengaluru.

Root nodules collection

The root nodules of *Clitorea ternatea* L. were randomly collected at bloom stage during monsoon season. The whole plant of shankapushpi were excavated with intact root system and then thoroughly washed with tap water later nodules with roots were kept in moist soil and brought to the laboratory for preservation of nodules and isolation of native strain of rhizobia (Indu *et al*., 2018).

Isolation of native strain

The root nodules were washed thoroughly with sterile water and surface treatment of nodules done with 70% alcohol for 30 seconds and then they were further washed 5-6 times with sterile distilled water (Sonali *et al*., 2017) and crushed with the help of sterile glass rod and streaked on yeast extract mannitol agar media (YEMA) plate. The plate were incubated at 28 ± 2 ºC for 2-3 days (Biswajit *et al*., 2015). Followed by several successive isolation of *Rhizobium* and pure individual colonies are sub-cultured on the same medium, the isolates were further characterized for morphological properties (Vincent, 1970). The process of isolation was carried in the laminar airflow instrument to maintain the purity.

16S rRNA analysis Procedure

In order to study the genetic diversity of shankapushpi isolate was carried out by following 16S rRNA analysis procedure. Pure cultured bacterium was taken and colonies are picked up with a sterilized toothpick and suspended in a 1.5μl effendorfftube. Centrifuge run at 10,000 rpm for 10 min. and supernatant was separated. PCR was carried out by using 1μl of template DNA and the universal primer 27F (AGAGTTTGATCMTGGCTCAG)and1492R(TACG-GYTACC TTGTTACGACTT) were used for amplification reaction (Weisburg *et al*., 1991). And total PCR reaction mixture was made to 20 μl. The PCR reactions were performed with an initial denaturation step at 95 ºC for 3 min, followed by 35 cycles of denaturation at 94 ºC for 1 min, at 55 ºC primer annealing for 1 min and at 72ºC extension for 2 min, followed by a final extension step at 72 ºC for 3 min (Laguerre *et al*., 1994). PCR products were purified by using montage PCR clean up kit (Millipore). Purified PCR products were sent to macrogen laboratory Bengaluru for sequencing. The sequencing were performed by using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA). The high quality reads were aligned and nucleotide sequences were BLAST in the gene bank database at the NCBI.

RESULTS AND DISCUSSION

In order to find out an efficient nitrogen-fixing bacteria from Shankapushpi, based on the morphological characteristics of isolates, the species of Bradyrhizobium japonicum are characterized as rod-shaped, aerobic, non-spore forming and motile by polar or subpolar flagellum. Colonies are circular, opaque, rarely translucent, white and convex, with entire margins (Figure 1). Strains are usually slow growing, not exceeding 1 mm in diameter within 5–7 days incubation on YEMA (Jelana *et al*., 2017).

Strains are characteristically able to invade the root hairs of leguminous plants and incite the production of root nodules, whereas bacteria occur as intracellular symbionts with host specificity (Gage, 2004). The bacteria are present in root nodules as swollen forms which are normally involved in fixing atmospheric nitrogen into combined forms utilizable by the host plant, while some strains fix nitrogen in the free living state under special conditions (Holt *et al*., 1994).

The genus *Rhizobium* is the most heterogeneous group of the family Rhizobiaceae, and comprises most of the species which form symbiotic relationships with leguminous plants, and also

Table1 : BLAST result of 16S rRNA Sequence showing similarity with *Bradyrhizobium japonicum*

Accession	Description	Maximun Score	Total Score	Query Cover	E value	Percent Ident
KY864921.1	Bradyrhizobium japonicum strain gkvk2 16S ribosomal RNA gene	2479	2479	100%	0	100
KY412844.1	Bradyrhizobium japonicum strain L16 16S ribosomal RNA gene	2479	2479	100%	0	100
KY000644.1	Bradyrhizobium japonicum strain Bj17 16S ribosomal RNA gene	2479	2479	100%	0	100
KY000633.1	Bradyrhizobium japonicum strain Bj6 16S ribosomal RNA gene	2479	2479	100%	0	100
KF995110.1	Bradyrhizobium japonicum strain NP-184 16S ribosomal RNA gene	2479	2479	100%	0	100
KF995103.1	Bradyrhizobium japonicum strain MN-115 16S ribosomal RNA gene	2479	2479	100%	0	100
KF995091.1	Bradyrhizobium japonicum strain M-35 16S ribosomal RNA gene	2479	2479	100%	0	100
KF995088.1	Bradyrhizobium japonicum strain CK-12 16S ribosomal RNA gene	2479	2479	100%	0	100
KF995087.1	Bradyrhizobium japonicum strain CK-12 16S ribosomal RNA gene	2479	2479	100%	0	100
KF995111.1	Bradyrhizobium japonicum strain NP-185 16S ribosomal RNA gene	2473	2473	100%	0	99.93
KF995097.1	Bradyrhizobium japonicum strain M-55 16S ribosomal RNA gene	2473	2473	100%	0	99.93
KF995095.1	Bradyrhizobium japonicum strain M-49 16S ribosomal RNA gene	2468	2468	100%	0	99.85
KY072863.1	Bradyrhizobium japonicum strain Bj28 16S ribosomal RNA gene	2477	2477	99%	0	100
KY072862.1	Bradyrhizobium japonicum strain Bj27 16S ribosomal RNA gene	2477	2477	99%	0	100
KP768791.1	Bradyrhizobium sp. 61S1 16S ribosomal RNA gene, partial sequence	2477	2477	99%	0	100
KF527971.1	Bradyrhizobium sp. CTS7 16S ribosomal RNA gene, partial sequence	2477	2477	99%	0	100
HG940531.1	Bradyrhizobium sp. VUPME50 partial 16S rRNA gene, strain VUPME50	2477	2477	99%	0	100
KF995119.1	Bradyrhizobium japonicum strain N2-225 16S ribosomal RNA gene	2477	2477	99%	0	100
KF995115.1	Bradyrhizobium japonicum strain N2P2-205 16S ribosomal RNA gen	2477	2477	99%	0	100
KF995105.1	Bradyrhizobium japonicum strain MN-130 16S ribosomal RNA gene	2477	2477	99%	0	100
KF995096.1	Bradyrhizobium japonicum strain M-52 16S ribosomal RNA gene	2477	2477	99%	0	100
KF836044.1	Bradyrhizobium sp. SCAUS20 16S ribosomal RNA gene, partial	2477	2477	99%	0	100
KC736658.1	Bradyrhizobium japonicum strain 5329 16S ribosomal RNA gene	2477	2477	99%	0	100
FR753136.1	Bradyrhizobium japonicum partial 16S rRNA gene, strain R-45771	2477	2477	99%	0	100

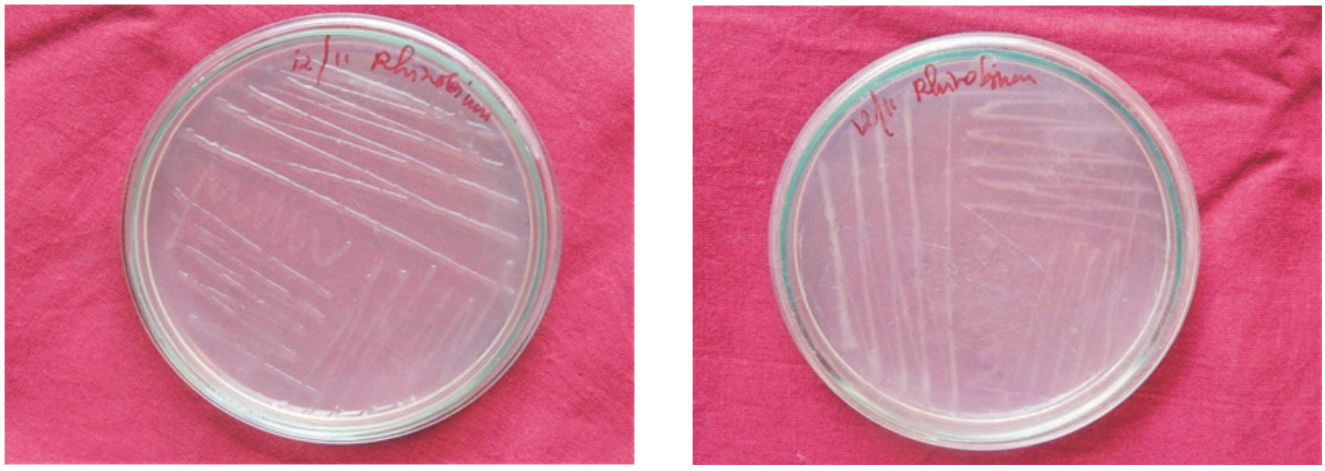

Figure 1:Bacterial colonies of *Bradyrhizobium japonicum* strain isolated from root nodules of shankapushpi (*Clitorea ternatea* L.)

Table 2: Isolate of *Bradyrhizobium japonicum* from root nodules of shankapushpi (*Clitorea ternatea* L.).

Crop	Year of Isolation	NCBI gene bank Accession number
Shankapushpi	2016-17	KY864921

includes plant pathogenic bacteria (Weir 2016). Characterization of rhizobia based on genetic characteristics is more precise and more informative compared to the morphological and physiological classification. Until 1992, only one species was known within the genus Bradyrhizobium that is *Bradyrhizobium japonicum* (Jordan, 1982). While, the application of molecular methods in the past 20 years enabled the separation of several new species (Ramirez-Bahena *et al*., 2009).

However, in this study the isolate showing resemblance with Bradyrhizobium sp. were grown on YEMA plates for 72 hours and identification of native organism based on 16S rRNA homology was performed using PCR with the help of universal primer 27F and 1492R, probably the most widely used primer pair for amplification of a taxonomically diverse eubacteria 16S rRNA gene fragments by PCR (Weisburg *et al*., 1991). The purified PCR products of approximately 1,342 bp were sequenced by using primer (27F and 1492R) and identified as *Bradyrhizobium japonicum*. The results of sequencing (BLAST queries of Gene Bank) showed that, the isolate have scored the maximum score (2479 bits), 100% query coverage, E value of 0.0 and 100% identity were selected as the probable identities of the isolate. Further, the BLAST resultes were revealed that, the bacterial isolate showed 100% similarity with *Bradyrhizobium japonicum* strain are presnted in Table1. The bacterial isolates of Bradyrhizobium japonicum strain comes under the member of rhizobia group leads to endophytic association with some plant species providing

benefites on plant growth (Vernans *et al*., 2017). Morever, the isolate of *Bradyrhizobium japonicum* sequence strain of shankapushpi were deposited in the NCBI GenBank database under a unique accession number KY864921 (Table 2).

The partial sequencing of 16S rRNA made a significant step in the phylogeny and classification of rhizobia and allowed to description of several new genera and species (Germano *et al*., 2006). However, the conservative nature of 16S rRNA gene allows the characterization to the species level, while the differences between the strains of the same species cannot be determined. More molecular procedures enable the identification and classification of bacteria at a high level of taxonomic resolution, such as using rep-PCR genomic fingerprinting to achieve genetic differences at subspecies and strain levels (Melchiorre *et al*., 2011).

ACKNOWLEDGMENT

The author is thankful to Dr. A.P.Mallikarjuna Gowda, Seinor Scientist and Head KVK Doddabalapura, GKVK, Bengaluru and Dr. Muthuraju, R Assistant Professor of Microbiology UAS, Bengaluru for their constant inspiration and immense support.

REFERENCES

Barik, D. P., Naik, S. K., Mudgal, A. and Chand, P. K. 2007. Rapid plant regeneration through in vitro axillary shoot proliferation of butter-fly pea (*Clitoria ternatea* L.) a twinning legume, In Vitro Cell. *Dev. Biol. Plant*. **43:** 144- 148.

Biswajit, S., Pranab, B.M. and Piyush, P. 2016. Characterization of plant growth promoting rhizobacteria from root nodules of Crotolaria pollida grown in Assam. *Ind. J. Biotechnol*. **15:**210-216.

Claudio, T., Sacchi, A. M., Whitney, L. W., Mayer, R. M., Arnold, S. A., Boras, R. S. and Tanja, P. 2002. Sequencing of 16S rRNA Gene: A Rapid Tool for Identification of Bacillus anthracis. Emerging Infectious Diseases. Vol. 8, No. 10.

Faruque, O. M., Miwa, H., Yasuda, M., Fujii, Y., Kaneko, T., Sato, S. and Okazaki, S. 2015. Identification of Bradyrhizobium elkanii genes involved in incompatibility with soybean plants carrying the Rj4 allele. *Appl. Environ. Microbiol*. **81:**6710 –6717.

Gage. D. 2004. Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. *Microbiol. mol. Biol. Rev*. **68:** 280–300.

Germano, M.G., Menna, P., Mostasso, F. L. and Hungria, M. 2006. RFLP analysis of the rRNA operon of a Brazilian collection of bradyrhizobial strains from 33 legume species. *Int. J. Syst. Evol. Microbiol*. **56:** 217–229.

Gomez, S. M. Kalamani, A. 2003. Butter-fly Pea (*Clitoria ternatea* L.) A Nutritive Multipurpose Forage Legume for the Tropics An Overview. *Pak. J. Nutr*. **2:** 374-379.

Holt, J. G., Krieg, N. R., Sneath, P. H., Staley, J. T. and Williams, S. T. 1994. Bergey's manual of determina-tive bacteriology, ninth ed. Williams and wilkins pub., MD, USA.

Indu, S. S., Raju, R. M., Sunil, C., Sonam, R., Nisha, T., Alakesh, T. and Hukam, S.G. 2018. Molecular characterization of microsymionts associated with root nodules of certain Burhia- Ham.ex benth a native keystone legume species from thar desert of India. *Ind. J. Exp. Biol*. **56:** 373-384.

Jelena, B. M., Dragana, D. B., Branislava, B., Tintor, R., Maja, V., Jgnjatov, Z. T., Dukic, S. N. and Balessevic, T. 2017. Molecular identification of *Bradyrhizobium japonicum* strains isolated from root nodules of soybean (*Glycine max* L.). J. Nat. Sci. Novi, Sad. 132 : 49-56.

Jordan. D. C. 1982. Transfer of rhizobium japonicum buchanan 1980 to bradyrhizobium gen. Nov., a genus of slow-growing, root nodule bacteria from leguminous plants. *Int. J. Syst. Bacteriol*. **32:** 136-139.

Laguerre, G., Allard, M.R., Revoy, F. and Amarger, N. 1994. Rapid Identification of Rhizobia by Restriction Fragment Length Polymorphism Analysis of PCR-Amplified 16S rRNA Genes. Appl. Environ. *Microbiol*. **60:** 56–63.

Marinkovic, J. 2012. Biochemical and molecular characterization of the symbiosis between soybean and different strains of Bradyrhizobium japonicum (Kirchner) Jordan under drought conditions agriculture (in Serbian). PhD Thesis, University of Belgrade, Serbia .

Melchiorre, M. D., Luca, M. J., Anta, G. G., Suarez, P., Lopez, C., Lascano, R. and Racca, R.W. 2011. Evaluation of bradyrhizobia strains isolated from field-grown soybean plants in Argentina as improved inoculants. *Biol. Fertil. Soils*. **47:** 81-89.

Peix, A., Ramirrz, M. H., Velazqueze, M. H. and Bedmar, E. J. 2015. Bacterial association with legumes, *Crit. Rev. Plant. sci*. **34:** 42-46.

Priya, K. and Harish, K. D. 2013. Isolation and characterization of PHB producing micro-organisms isolated from root nodules of leguminous plants, *The Bioscan*. **8:** 109-113.

Ramirez-bahena, M. H., Peix, A., Rivas, R., Camacho, M., Rodriguez-navarro, D. N., Mateos, P. F., Mar-tinez, M. E., Willems, A. and Velazquez, E. 2009. Bradyrhizobium pachyrhizi sp. Nov. And Bradyrhizobium jicamae sp. Nov. isolated from effective nodules of pachyrhizus erosus. *Int. J. Syst. Evol. Microbiol*. **59:** 1929-1934.

Sengupta, D. and Reddy, A.R. 2011. Water deficit as a regulatory switch for legume root responses. *Plant Signal. Behav*. **6:** 914-917.

Sethiya, N. K., Nahata, A., Mishra, H. and Dixit, V. K. 2009. An update on Shankhpushpi, a cognition- boostingAyurvedic medicine. *J. Chi. Integr. Med*. **7:** 1001-1022.

Somasegeran, P. and Hoben, H. J. 1994. Handbook for rhizobia methods in legume-rhizobium technology, springer-verlang, new York.

Sonali and Priya. 2017. Isolation, biochemical characterization and metabolic fingerprinting of Rhizobium from root nodules of Clitorea ternatea. *Int. J. of Applied biology and pharm. tec*. **8:**19- 30.

Subramanian, M. S. and Prathyusha, P. 2011. Pharmaco-phytochemical characterization of *Clitoria ternatea* L. *Int. J. Pharm. tech. Res*. **3:** 606-612.

Vernans, V. B., Rosario, G. M. and Akira, Y. 2017. Identification of Bacteria from Root Nodules of Philippine Legumes Using 16S rRNA Gene Sequencing. Philipp. *Agric. Sci*.**100:** 0031-7454.

Vincent. J. M. A. 1970. Manual for the practical study of the root nodule bacteria. Int. Biol. Prog. Handbook, vol. 15. Blackwell scientific publications, oxford, usa.

Weir. B.S. The current taxonomy of rhizobia. NZ Rhizobia website. Last updated: January 2016.

Weisburg, W. G., Barns, S. M., Pelletier, D. A. and Lane, D. J. 1991. 16s ribosomal DNA amplification for phylogenetic study. *J. Bacteriol*. **173:** 697–703.

Zingare, M. L., Prasana, L. Z., Ashish, K. D. and Aslam, M. D. A. 2013. Review of antioxidant, antidiabetic and heptaoprotective potentials *Clitorea terntea* L. *Int. J. Pharm. Bio. sci*. **3:** 203-213.