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RESEARCH ARTICLE

ENDOSYMBIONTS: A CONTINUING SOURCE OF CYTOTOXIC METABOLITE TAXOL.

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Abstract

Taxol® (generic name: paclitaxel) is a naturally occurring diterpenoid cytotoxic metabolite. Approved by FDA, this drug has been widely used for the treatment of various kinds of cancers. Plants belonging to *Taxus* sp. are the primary natural source of Taxol®. In 1960 it was isolated from bark of the Pacific yew tree (*Taxus brevifolia*) for the first time. However, due to the environmental constraints and to protect the yew trees, alternatives to the use of trees were sought. Microbiologists screened the novel fungus capable of producing Taxol®-precisely two decades back. This was followed by a plethora of investigations on other endophytes possessing similar biosynthetic potential. Till date, highest level of Taxol® has been synthesized after 7 days of incubation period from *Cladosporium* sp. F3 isolated from Iranian yew (*Taxus baccata*) producing paclitaxel at 139.2 mg/kg. However, industrial-scale of Taxol® production using fungal endophytes, although ostensibly capable, has not happened at a practical level yet. This review examines the potential for production of Taxol® from fungi. The biology of Taxol® synthesis in fungi and different methods which may improve Taxol® [1]yield is also discussed.

Significance of the study:
Endophytic fungi have ability to synthesize diverse classes of secondary metabolites originally from plants, one such classical example being paclitaxel. Better understanding and exploration of host-endophyte lifestyle will provide considerable knowledge that can be utilized to increase and improve production of desired pharmaceutical or industrial product by employing biotechnological manipulations. Therefore, our present dependency on medicinal plants for paclitaxel production can be minimized with the help of this endophytic fungus derived end product.

[1] Taxol® is the trademark of Bristol-Meyers-Squibb Inc.

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Introduction:-

14.5 lakh of Indian population are presently cancer-inflicted and around 7 lakh cases get registered and 5,56,400 deaths occur every year. National cancer registry quotes breast cancer followed by cervical as most common cancer prevalent in Indian women. In a recent report, Indian Council of Medical Research (ICMR) has projected 17.3 lakh new cases with cancer by 2020. In Indian male population oral cancer tops the list (http://icmr.nic.in/icmrsql/archive/2016/7.pdf).

Historically, plants have been used in folklore and traditional medicinal system for treatment of cancer. Natural products play a major role as active substances, model molecules for the discovery and validation of drug targets. Structural diversity, highly specific biological activities with selective modes of action is the basis of natural product discovery. Regardless of the intensive investigation of earthly flora, close to 6% of the approximately 400,000 plants species have been elaborately examined for both pharmacologically and phytochemically (Cragg and Newman, 2013). Vinca alkaloids i.e vinblastine and vincristine are remarkable plant-derived anticancer metabolites in clinical use, initially isolated from the Madagascar periwinkle, *Catharanthus roseus*. In fact, about 50% of the drugs introduced to the market during the last 20 years are derived directly or indirectly from small biogenic molecules (Vourela et al., 2004). Fascinatingly, an extract from the bark of the *Taxus brevifolia* Nutt, was identified as one of the most promising anticancer agent in 1970's (Wani et al., 1971). Paclitaxel success stirred extensive studies on the other taxane analogues for example docetaxel (Taxotere®) (Kingston and Newman, 2007). Novel albumin-bound paclitaxel formulation having brand name Abraxane® has been clinically-approved in India. (Green et al., 2006). Recently, Cabazitaxel (Jevtana®) was approved in the USA (Paller, and Antonarakis 2011). The present review focuses on commercial availability of one such cytotoxic drug Taxol® and various attempts made by scientific fraternity for its sustainable production.

Chemistry and mode of action:-

Chemically, it is a polyoxygenated diterpene alkaloid having empiric formula of $C_{47}H_{51}NO_{14}$ and a molecular weight of 853.9. It is highly lipophilic and thus highly insoluble in water. Its melting point is around 216-217°C. Its taxane nucleus to which an uncommon four-membered oxetane ring was linked to C-4 and C-5, and an ester was attached at the C-13 position (Baloglu and Kingston, 1999). Paclitaxel drug targets tubulin stabilization, a unique mode of action to impede uncontrolled cellular growth in cancer cells. It has been proposed that Taxol stabilizes lateral contacts between protofilaments in microtubules (Arnal and Wade, 1995). Photoaffinity labeling studies and mutation-induced drug resistance revealed stabilization of the microtubule due to a bridging effect of the N-terminal and a second domain in a relative position, enhancing lateral contacts between subunits leading to non-GTP hydrolysis. The total mass of assembled microtubules remains unchanged when Taxol® binds to microtubules and suppresses the tubulin microtubule dynamics. Furthermore, it binds to Bcl-2 protein which normally blocks the process of apoptosis, or cell death (Fang et al., 1998). Bristol-Meyers-Squibb Inc. started commercial production of taxol from plant cell fermentation (PCF) however, the maintenance of tissue cultures for Taxol® production is highly time consuming and expensive.

Paclitaxel producing Endophytic fungi:-

Fungal endophytes are regarded as a fascinating group of organisms that colonize the living internal tissues of their host - usually higher plants without causing any evident symptoms of disease in the host cells. They produce natural bioactive compounds that act as elicitors for plant secondary metabolites production. Hypothesis describing the asymptomatic existence of endophytes as balanced antagonism is depicted in figure 2 (Schulz 1999; Schulz and Boyle 2005). Being able to reside in a specialized niche, fungal endophytes are constantly in a state of "metabolic aggressiveness" thereby synthesizing inimitable array of metabolites (Aly et al., 2011). These metabolites have exhibited a plethora of biological activities such as antimicrobial, antineoplastic, immunosuppressive and cytotoxic activities as indicated in literature. In 1993 Taxol® production from an endophytic fungus, *Taxomyces andreanae* isolated from *Taxus brevifolia* was reported by Stierle and his group with an extremely low yield of 25-50ng L^{-1}. Remarkably, Taxol® produced by endophytes is identical to that produced by *Taxus* sp., chemically and biologically (Stierle et al., 1993). Since then, over a span of two decades various *Taxus* plant species such as *T. baccata*, *T. wallachiana*, *T. mairei*, *T.cuspidata* among others have been host to different Taxol® producing endophytic fungi, collected mostly from bark tissues (contains highest concentration of Taxol®) followed by leaf and stems tissues (Table 3). More recently, gene mining approach has been employed as molecular marker for screening taxol-producing endophytic fungi i.e.

dbat (encoding 10-deacetylbaccatin III-10-O-acetyltransferase) gene and *bapt* phenylpropanoid side chain-CoA acyltransferase.

Figure 1 Structure of Taxol®
Figure 2: Balanced antagonism hypothesis (adapted from Schulz 1999)

Strain improvement attempts:-
Protoplast fusion technology also plays an important role in the genetic breeding of microorganisms. Production of taxol-producing endophytic fungi by inactivating the parents' protoplast fusion experiment led to 468.62 μg/L, increased yield (Zhao et al, 2008a). The same group researched the mutation effects of UV, NTG, and UV + NTG treatments on HD1-3, a strain of taxol-producing endophytic fungi and found that the combined treatment resulted into an yield that was1.41 times that of the original (Zhao al, 2008b). A high yielding endophytic strain of *Fusarium maire* K178, capable of producing upto 225.2 mg/L Taxol® was developed by protoplast mutation using UV radiation and diethylsulfate (Xu et al, 2006). Genome shuffling was introduced in *Nodullisporum sylvi-forme* as a means to enhance Taxol® production which was 64.41% higher than that of the starting strain NCEU-1. In Ozonium sp. EFY-21 isolated from *T. chinensis* var. *mairei*, overexpression of Taxus TS gene under a fungal specific promoter resulted in about fivefold increase in Taxol® production as compared to control (Wei et al, 2012). Heterologous gene expression successfully occurred in *Saccharomyces cerevisiae* by insertion of Taxol® producing plant host (*Taxus chinensis*) gene taxadiene synthase. This breakthrough research led to high-level expression of the taxadiene synthase gene resulting in a 40-fold increase in taxadiene upto 8.7 mg/L alongwith considerable increase in geranylgeraniol (Engels et al, 2008). In a recent study from India authors demonstrated that fungal taxol isolated from *Cladosporium oxysporum* could inhibit breast cancer cell line by regulating multiple apoptotic signalling pathways (Raj et al., 2015).

Conclusive and future perspectives:-
The daunting and challenging task of optimization of culture conditions and bioengineering techniques to develop fungal overproducers of taxol is the future goal. The desired and pending industrial utilization of Taxol® producing fungal endophytes despite numerous investigations of such fungi from plethora of different plants. These limitations attest the imminently cryptic lifestyles of endophytes (alternating between endophyte-pathogen-epiphyte lifestyles), their complex and varying physiology under various environmental and culture conditions, and our presently progressive yet insufficient knowledge about their biochemistry, molecular controls, and regulatory networks. Taxol® producing endophytic fungi like *Metarhizium anisopliae* and *Cladosporium cladosporioides* MD2 reportedly secreting Taxol® up to 800 mg/L in liquid culture, should further be investigated in an elaborative manner for strain improvement (Liu et al., 2009; Zhang et al., 2009). Genetic engineering and recombinant DNA technology may add a new dimension to the goal of maximizing yield of taxol from fungi. These techniques are most commonly applied in fungi for the production of homologous as well as heterologous enzymes, biochemicals, and pharmaceuticals at the commercial level.

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Conflict of interest:-
Authors have no conflict of interest to declare.

Figure legend:-
Figure 1 Balanced antagonism hypothesis (adapted from Schulz 1999)
Figure 2 Structure of Taxol®

Table legend:-
Table 1 Taxol producing endophytic fungi isolated from *Taxus* sp. and other Plants

Endophytic Fungi	Host Plant	Host Tissue	Taxol® Yields (µg/L)	Growth Medium	References
Pestalotiopsis microspora	*T. wallichiana*	Bark	60-70	MID	Strobel et al., 1996a,b
Nodulisporium sylviforme	*T. cuspidata*	Phloem	51.06-125.70	S7	Zhou and Ping, 2001
Fusarium maire Y1117	*T. mairei*	Bark	20	COMPLEX	Xu et al., 2006
Ozonium sp.	*T. chinensis* var. *mairei*	Twig	4-7	PDB	Guo et al., 2006b
Botrytis sp. XT2	*T. chinensis* var. *mairei*		161.24	-	Hu et al., 2006
Papulaspora sp. XT17	*T. chinensis* var. *mairei*		10.25	-	Hu et al., 2006
Botrytis sp. HD181-23	*T. cuspidata*		206.34	-	Zhao et al., 2008a
Fusarium solani	*T. celebica*	Stem	1.6	PDB	Chakravarthi et al., 2008
Botryodiplodia theobromae BT115	*T. baccata*		280.50	MID	Raja et al., 2008
Fusarium arthrosporioides	*T. cuspidata*	Bark	131	PDB	Li et al., 2008
Fusarium solani Tax-3	*T. chinensis*	Bark	163.35	PDB	Deng et al., 2009
Metarhizium anisopliae	*T. chinensis*	Bark	846.1	COMPLEX	Liu et al., 2009
Aspergillus niger var. *taxi*	*T. cuspidata*	Bark	273.46	PDB	Zhao et al., 2009
Phomopsis sp. BKH 27	*T. cuspidata*	Leaf	418	MID	Kumaran and Hur, 2009
Phomopsis sp.3 (BKH 35)	*Larix leptolepis*	Leaf	334	MID	Kumaran and Hur, 2009
Phomopsis sp.2 (BKH 30)	*Ginkgo biloba*	Leaf	372	MID	Kumaran and Hur, 2009
Cladosporium cladosporioides MD2	*T. media*	Bark	800	PDB	Zhang et al., 2009
Didymostilbe sp.	*T. chinensis* var. *mairei*	Bark	8-15	PDB	Wang and Tang, 2011
Paraconiothyrium sp. SSM001	*T. media*	Bark	80	YPDB	Soliman et al., 2011
Stemphylium sedicola SBU-16	*T. baccata*	Bark	6.9 ±0.2	PDB	Mirjalili et al., 2012
Fusarium redolens	*T. baccata L. subsp.*	Bark	66	S7	Garyali et al., 2013
Pestalotiopsis microspora CP-4	*Taxodium distichum*	Bark	1.487	MID	Li et al., 1996
Bartalinia robillardoides AMB-9	*Aegle marmelos*	Leaf	187.6	MID	Gangadevi and Muthumary, 2008a
Pestalotiopsis pauciseta CHP-11	*Cardiospermum halicacabum*	Leaf	113.3	MID; PDB	Gangadevi et al., 2008

Colletotrichum gloeosporioides	*Justicia gendarussa*	Leaf	163.4		Muthumary, 2008a
Phyllosticta spinarum	*Cupressus sp.*	Leaf	235; 125	MID	Kumaran et al., 2008a
Phyllosticta melochiae	*Melochia corchorifolia*	Leaf	274		Kumaran et al., 2008c
Aspergillus fumigatus EPTP-1	*Podocarpus sp.*	Leaf	560	MID;	Sun et al.,2008
Pestalotiopsis terminaliae	*Terminalia arjuna*	Leaf	211.1	PDB	Gangadevi and Muthumary, 2009a
Chaetomella raphigera TAC-15	*Terminalia arjuna*	Leaf	79.6	MID	Gangadevi and Muthumary, 2009b
Phomopsis sp. BKH 30	*Ginkgo biloba*	Leaf	372	PDB	Kumaran and Hur, 2009
Phomopsis sp. BKH 35	*Larix leptolepis*	Leaf	334	MID	Kumaran and Hur, 2009
Lasiodiplodia theobromae	*Morinda citrifolia*	Leaf	245		Pandi et al., 2011
Fusarium oxysporum	*Rhizophora annamalayana*	Leaf	172.3	PBD	Elavarasi et al., 2012
Phoma betae	*Ginkgo biloba*	Leaf	795	MID	Kumaran et al., 2012
Chaetomium sp.	*Michelia champaca*	Leaf	77.23	MID	Rebecca et al., 2012
Guignardia mangiferae HAA11	*Taxus X Media*	-	0.7	PBD	Xiong et al 2013
Penicillium aurantiogriseum NRRL 62431	*Corylus avellana*	Nut	70.00	MID	Yang et al., 2014
Cladosporium sp. F1 and F3	Iranian *Taxus baccata*	-	129 and 139.2 mg/kg	-	Kasaei et al., 2017
Cladosporium oxysporum	-	-	-	MID (Soytone amended)	Raj et al., 2015
Botryosphaeria rhodina, Aspergillus niger, Coriolopsis caperata	*Salacia oblonga*	-	-	PDB	Roopa et al., 2015

S-7-Glucose 1 g/L, fructose 3 g/L, sucrose 6 g/L, peptone 1 g/L, sodium acetate 1 g/L, yeast extract 250 mg/L, thiamine 1 mg/L, biotin 1 mg/L,pyridoxal 1 mg/L, $Ca(NO_3)2$ 6.5 mg/L, phenylalanine 5 mg/L, $MgSO_4$ 3.6 mg/L, $CuSO_4$ 1 mg/L, $ZnSO_4$ 2.5 mg/L, $MnCl_2$ 5 mg/L, $FeCl_3$ 2 mg/L, benzoic acid 100 mg/L, 1M KH_2PO_4 buffer (pH 6.8) 1 mL/L; MID-Sucrose 30 g/L, yeast extract 0.25 g/L, ammonium tartrate 5 g/L, $Ca(NO_3)_2\$4H_2O$ 0.5 g/L, KNO_3 80 mg/L, $MgSO_4\$7H_2O$ 360 mg/L, KCl 60 mg/L, $NaH_2PO_4\$H_2O$ 20 mg/L, $FeCl_3\$6H_2O$ 2 mg/L, $MnSO_4\$H_2O$ 5 mg/L, $ZnSO_4\$7H_2O$ 3 mg/L, H_3BO_3 1.4 mg/L, KI 0.7 mg/L (pH 6.2); PDB-Potato Dextrose Broth; YPDB-Yeast extract 10.0 g/L, Peptone 20.0 g/L, Dextrose 20.0 g/L

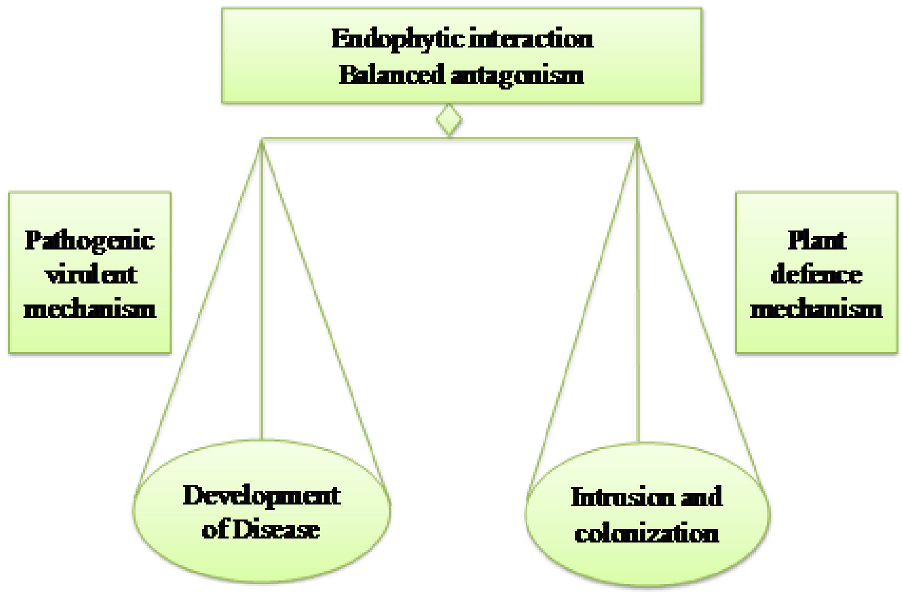

Figure 1:- Balanced antagonism hypothesis (adapted from Schulz 1999)

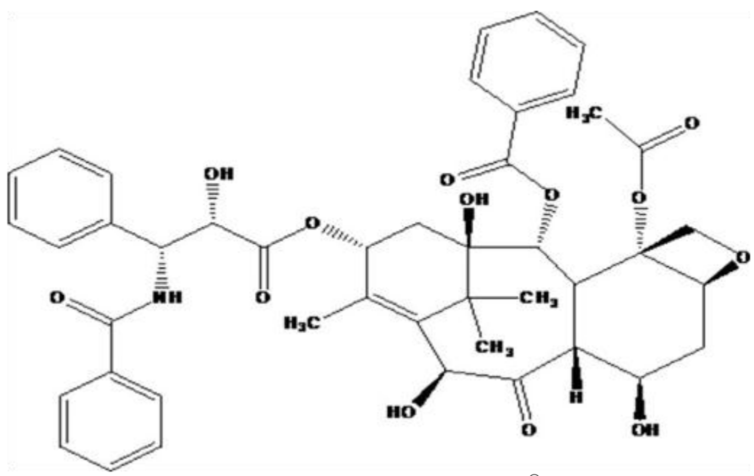

Figure 2:- Structure of Taxol®

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