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New options in drug discovery: Endophyte technology

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Abstract

Antimicrobial discovery has traditionally focused on sources from plants, animals and microorganisms. However, with the reoccurring incidences of multidrug-resistant organisms (MDROs) towards the in-use antibiotics and the problem of over- exploration of plants that is heavily altering the ecosystem, the need for other sustainable means of acquiring novel drugs becomes imperative. In a bid to ameliorate these issues, researchegrs have resorted to the use of some endosymbiotic microorganisms, known as endophytes. These are microorganisms inhabiting the internal part of plants, which have been verified to possess great potentials to bioengineer novel products, for therapeutic purposes. The discovery of endophytic microorganisms has come with so many promises of alleviating the challenges of an increasingly daunting multidrug- resistant strain of pathogenic microbes, which have become a global issue in health care delivery. They can be harnessed and utilized in the generation of active ingredients for drug production, instead of the incessant cutting down or harvesting of plants or plant parts, which destroys nature, resulting in global warming and world climate problems. This systematic review is on endophytic technology as a new alternative in drug discovery.

Keywords: Plants; Endophytes; Resistant Organisms; Antimicrobial; Drug Discovery; New Technology

1. Introduction

Plant sources, for a very long time now have been continually explored for medicinal uses. Endophytic microbes inhabiting these plant tissues compete favorably as important sources of new drug compounds. As microorganisms, the novel strains could be harnessed, manipulated or bio-engineered using biotechnological techniques to produce desired therapeutic products, which could become possible arsenals for the fight against multi- drug resistant organisms that have rendered some current in-use antimicrobial agents worthless. There are reports that highlighted the importance and benefits of production of antimicrobial compounds using micro-organisms via biotechnological methods, which are laxity of season, climatic and geographical constraints, controllable scale-up procedure, as well as improving yield at low cost, and these are all feasible with endophyte [1-3].

The word endophyte was first mentioned in 1866 by de Bary. It's a Greek word, which stands as endo, meaning "within", whereas phyte means "plant" [4]. It was first seen and reported in 1898 by Vogl as mycelium residing in a grass of *Lolium temulentum* and by Perotti, who first observed the occurrence of non-pathogenic bacteria flora, in the tissue of a root of the plant [5, 6]. Endophyte is a term broadly used for the endo-symbiotic group of microorganisms that colonize and

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multiply in the internal part of a healthy plant. This cohabitation between the plant and endophytes can be for the entirety or part of their life, without causing any immediate overt negative effect [7- 9]. These microorganisms and their plant hosts mutually benefit from each other. While the endophytes provide the plant with numerous substances that help to protect the plant from hazardous environmental conditions and promote plants growth, the plants in turn provide a conducive environment and the substances needed for maximum growth and development of endophytes [10-12]. Mengistu (2020) cited that endophytes became interesting when they showed potentials/capacity to produce various important lead compounds from their metabolic activities [13]. These compounds differ in their molecular structures, and can barely be reproduced from chemical synthesis. Endophytes can be isolated from all parts of plants, such as leaves, stems, roots, seeds, fruit [14-16] and the biomass of endophytes found in them varies to a great extent and is dependent on host species, plant distribution, availability of nutrients, inoculums density and environmental condition of the plant [17-19]. However, research work by Rana *et.al* (2020) documented that endophytes are more prominent in roots than any other plant parts [20].

Endophytic microorganisms that naturally possess bioactive products could be bacteria, fungi, actinomycetes, and mycoplasm which can be found in all plant species and serve as a promising source of novel drug molecules [21-24]. These micro flora found in plants are highly diversified, especially the fungi whose presence in the plants has a lot to do with the type of host and nutrients available, as well as other microorganisms that make up the community and the interactions among them. They act as chemical reservoirs of so many novel bioactive secondary metabolites, such as alkaloids, phenol, quinones, steroids, saponins, tannins, and terpenoids and other therapeutic novels bioactive complex, such as, xanthones, methoxyphenols,dep-sipeptides, bicyclic , lactones,depsidoenes, butenolides, maleimide-bearing compounds, ergosterol, spirobisnaphthalenes, benzopyran derivatives,isofuranonaphthalenone, butyrolactones, diketopiperazine, sesquiterpenoids, cytochalasin-related compounds, pestalols and cyclic pentapeptides [25-27]. These secondary metabolites are known to harbor a great variety of chemical substances that are significantly important in both pharmaceuticals and allied fields of biopharmaceuticals. The usefulness of these lead drug candidates cut across, not only in medicines but in agriculture, food and cosmetic industries and environmental management. These compounds possess activities such as antimicrobial, insecticidal, anticancer, anti-diabetic, immunosuppressive, neuroprotective, hepatoprotective and host of other properties [28-30]. Furthermore, it was reported that endophytes found in plants that grow in tropical rain forests yield much more bioactive compounds than those that grow in temperate regions [31]. Concerning season and diversity, a comparative study revealed that endophytes colonize more in samples obtained during the rainy season when compared to ones of the dry season which may have resulted from the increase in sporulation, enhanced by rain and high humidity, while there are no much variations regarding diversity [32, 33]. Some scientific works revealed that in the fermentation process, endophytic microbes yield more bioactive secondary metabolites with rice as the substrate than when corn was used [34,36]. Although, many investigative studies have been carried out on bioactive compounds from endophytes that brought about several contributions by several scientists, regarding endophytic microbes, a review study conducted in 2021 with some others stated that, to date, an insignificant proportion of 7% of 1.5 million fungal species have been identified [37,38,39] and with other findings, using next-generation sequencing, it was reported that 3.5 to 5.1 million fungal endophytes are in existence as was proposed by Hawksworth and Lücking, 2017 [38]. This is a pointer to the fact that a plethora of endophytic species that can be employed to handle new emerging and re-emerging infections, as well as multi- drug resistant infectious diseases are yet to be discovered and harnessed.

2. Novel Endophytic Antimicrobials

Several natural products from endophytic microorganisms have been observed to exhibit biostatic or biocidal action on a wide range of pathogenic organisms, such as bacteria, fungi, and viruses infecting or infesting man, animals and plants as illustrated in Fig. 1. If the antimicrobial activities of these novel molecular substances are adequately explored, they can serve as a better choice of treatment for these infectious diseases. This appears more important because multi- drug resistant strains are on the increase.

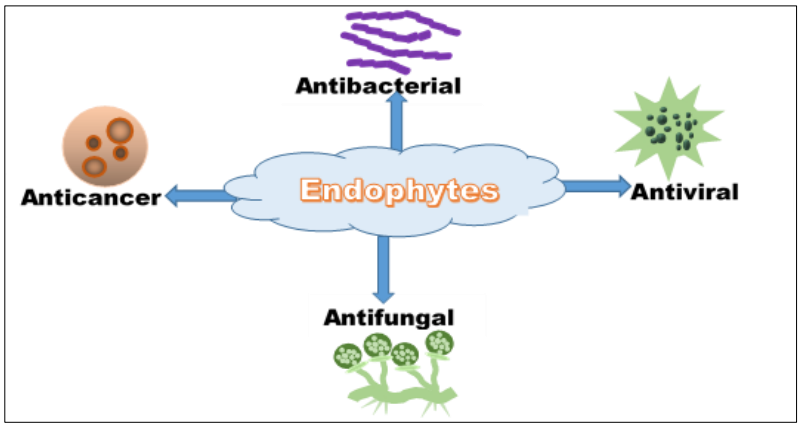

Figure 1 Bioactivities of endophytes

3. Endophytes as sources of Anti-bacterial agents

The capacity of microorganisms to compromise antibiotic actions is a growing trend and has become a global public health challenge, which portends future health catastrophe, if not properly handled. Interestingly, the discovery of endophytes was welcomed by the scientific community, as an additional arsenal for the fight against pathogenic organisms. This has made the past few decades witness an increased reports on endophytes in the mycological literature [40]. Being presented as microorganisms present in the plant that are of great biodiversity that generate promising molecules of drug candidates with versatile pharmacological activities [40, 41]. These promising outcomes have raised the interest of more scientists to delve into the study of endophyte [42, 43]. Recent pieces of literature on endophytes have showcased some attributes that present them as good alternative source/provider of novel antibiotics in which antibacterial activity was indicated heavily [44,45]. Correspondingly, a review by Falade *et al*. (2021) cited endophytes as a promising bioresources for isolating novel bioactive molecules that have antibiofilm activity [46]. The formation of biofilms is one among many mechanisms of drug resistance by microorganisms, in which aggregates of microbial cells are enclosed by an extracellular matrix made up of polymeric substances which oppose the penetration of antimicrobial agents [47]. This confers resistance to microorganisms that are formerly susceptible to a particular antimicrobial agent. Endophytic isolates of Nocardiopsis sp DMS2 as demonstrated by Nadar *et al*. 2020 produced antibiofilm activities that were capable of inhibiting *Klebesilla pneumoniae* [48]. In another study, it was observed that oral and dental pathogens were inhibited by the antibiofilms activites elicited by endophytic isolates of *Bacillus firmus* PT18 and *Enterobacter asburiae* [49]. Isolates of the endophytic compound of *Eurotinum chevalieri* KUFA000 from *Rhizophor amucronate* were reported to have exhibited antimicrobial activity against *Staphylococcus auerus* and *Escherichia coli* by producing antibiofilms that disrupts the matrix formed by those bacterial cells [50]. *Lasiodiplodia pseudotheobromae* IBRL OS-64 an endophytic isolate from *Ocimum Sanctum* was reported to have shown antibiofilm activity against MR *S. aureus* ATCC 33591 [51]. Some clinical pathogens, such as *S. aureus* ATCC 25923, *S. aureus* ATCC 29213, MR *S. aureus* ATCC 43300, MR *S. aureus* 562 were more than 90 percent dose-dependent sensitive to isolate from metabolic extract of *Datura metel* [51]. Ikechukwu *et al* (2021) reported that out of the six endophytic fungi isolates from *Annona senegalensis*, RT1 metabolite had a significant antimicrobial effect against ESβL *E. coli* [53]. Furthermore, methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most stubborn antibiotic- resistant pathogens that compromise the action of drugs by the formation of biofilm. Its effect constitutes a big menace in the application of antibiotics [54]. However, with the emergence of endophytic microbes, many literature reviews on them revealed that the bioactive moieties produced by these microbes are good producers of anti-MRSA compounds [55]. Scientific evidence-based research on Heritiera fomes (an endophyte) from *Pestalotia sp* produced methicillin-oxysporone and xylitol, with strong inhibitory activity against six strains of MRSA [56]. Another study by El-Gendy *et al*. (2018) showed how an extended number of methicillin-resistant *Staphylococcus aureus* were antagonized by two metabolic extracts of endophytic Streptomyces [57]. Additionally, the leaves of the *Irvingiag abonensis*, produced endophytes which showed a noteworthy antibacterial activity against the standard strains of gram positive and negative bacteria, as well as an antifungal action on mycotic hyphae [58]. Another investigative report revealed that endophytic actinomycetes are potential producers of antimicrobials, as a handful of them have produced novel bioactive molecules that showed remarkable pharmacological activities at a notable low concentration against bacteria, fungi and viruses [59]. Also a study on isolation and characterization of the bioactive principles of roots of *Piper nigrum*, produced protease as one of the enzymes isolated from the *Piper nigrum*- derived endophytes [60]. Protease is an enzyme that breaks peptide bonds in proteins that make up the cellular structure of peptidoglycan found in both gram- positive and negative microorganisms. This enzyme

showed antagonistic activity against *Fusarium oxysporum* and *Meloidogyne incognita.* Although, this study was on the biocontrol of plant pathogens, it could be further assessed to profile efficacy against human pathogens.

3.1. Anti-mycotic activities of endophytes

Endophytic fungi are so far the largest group of all the microbiota that scientists have explored, yet a bio-prospection study on endophytic fungi revealed that a very minute strain of endophytic fungi has been extensively studied. Endophytic antifungal compounds have been reported from various scientific researches and, so far, these fungi have been known to be the largest isolated endophytes on record. Healthy *Moringa oleifera* leaves was a source of an endophyte *Aspergillus terreus* whose ethylacetate extract produced significant inhibitory activity against fungi implicated in Mucormycosis, such as *Rhizopus oryzae*, *Mucor racemosus*, *and Syncephalastrum racemosum*, with inhibition zones diameter of 20, 37, and 18 mm, respectively, at concentration of 10 mg/ml [61]. In another study, the isolate of the endophytic fungus *Phomopsis cassia* produced some metabolites that inhibited *Cladosporium cladosporioides* and *Cladosporium sphaerospermum* [62]. Furthermore, *Rhodomyrtu tomentosa* a medicinal flowering plant from the family of Myrtaceae, which has been in use traditionally to treat amoebic dysentery and wound healing [63]. when subjected to study for identification and isolation of endophytic secondary metabolites resulted in the identification of a handful of antifungal compounds that were active against many human pathogens such as *C. albicans*, *Cryptococcus neoformans, Microsporum gypseum* and *Penicillin marneffi* [64]. In a research report documented by Xin *et al*. (2018), the inhibitory effects of endophytes isolated from*Vaccinium dunalianum* varurophyllum (Ericaceae) against clinical yeast after a comprehensive screening revealed that ethyl acetate and dichloromethane extracts of the filtrates from the *Colletotrichum sp.* VD001, *Epicoccum nigrum* VD021 and *E. nigrum* VD022 strains displayed good inhibitory activity against test microorganisms from the disc diffusion assays and minimal inhibitory concentration (MIC) results [65]. The antimicrobial investigation of endophytes isolated from the root, stem, inflorescences and leaves of a Chinese plant- *Spiranthes sinensis (Pers)* produced broad spectrum antimycotic effects against some tested fungi [65]. Additionally, evaluation of the antifungal activities of the secondary metabolites obtained from *Paecilomyces sp.*anisolate from *Moringa oleifera* leaves against a clinical isolate *Rhizoctonia solani*revealed that it has a significant antagonistic impacts of 76.25% on the test pathogen [66].

3.2. Anti-viral endophytic bioactive agents

The emergence of life-threatening viral infections forms part of the challenges facing medical practitioners. Several attempts have been made by scientists to develop drugs that can inhibit the activities of these human pathogenic viruses. Much scientific- based research done on endophytes revealed that they possess great bioactive substances with antiviral activities against some of these life- threatening pathogens [67]. A hypothetical report resulting from scientific evidence-based research conducted by Thatol et al*,* (2015) and Chávez *et al*, (2015), stated that extreme habitats of natural environments have been discovered to be great harbors of novel drugs with viral inhibitory effects [68,69]. In other studies, it was interestingly discovered that copious fungal species of different varieties, occupy habitats such as, mangrove ecosystems and deep-sea sediments and a good number of ascomycetous species found in them have antiviral activity and other pharmacological actions [70]. An endophytic strain found in *Pleospora tarda* when subjected to investigative study exhibited an inhibitory action against HSV-2virus and VZV viruses,through its metabolites [71,72]. In addition, another endophytic strain isolated from *Streptomyces spp* inhabiting the mangrove tree *Bruguierga gymnorrhiza* produced xiamycin, which is a selective HIV antagonist [73]. Also, research on another mangrove plant *Aegicera corniculatum* yielded an endophytic isolate *Emericella spp* which when subjected to preliminary antimicrobial screening, produced various bioactive isoindolone compounds, in which two of them showed a moderate antagonistic effect on influenza A virus (H1N1) [74]. Bioactivities of fungal tree-pathogens such as white-root fungi, soft-root fungi, blue-stain and insect-symbionts, were investigated for antimicrobials and it was found that they can serve as good sources of antiviral agents, though there is a need for in-depth exploration [75]. The antiviral chemical compound-2-(furan-2-yl)-6-(2S, 3S, 4-trihydroxybutyl) pyrazine isolated from alkaloids extracted from mangrove actinomycetes of a plant species (*Jishengella endophytica* 161111) displayed good antiviral activity against the influenza A (H1N1) virus [15, 76]. An endophytic fungus isolated from phyllosphere of an oak tree (*Quercus coccifera*) produced the antiviral chemical Hinnuliquinone, which has inhibitory activity against HIV-1 protease [77]. Likewise a documented research work by Singh *et al*. (2023) ; Raekiansyah *et al*. (2017) produced two novel compounds (cytonic acid A and cytonic acid B) which were extracted from *Cytonaema* spp and upon structurally elucidation ptrideside isomers a novel antagonistic of the protease activity of human cytomegalovirus was revealed [78,79]. In another study, nigranoic acid, a HIV-1 reverse transcriptase inhibitor, was produced by the endophytic *T. Harzianum* isolated from *Kadsura angustifolia* [80]. *Vernonia amygdalina* was revealed in a recent research by Khiralla *et al*. (2020) to harbor an endophyte *Curvularia papendorfii* whose crude extract produced anti-corona virus activity with about 40% reduction in corona virus induced complications [81].

3.3. Other benefits from endophytes

Beside the production of novel chemicals by the endophytes, there are other gold mines found in endophytic technology. These natural products are less toxic and the process of their discovery is cheaper, once the isolates are generated, strategic processes are applied for its large- scale production by manipulation of its biosynthetic pathway [82]. Arora&Ramawat (2017) was able to outline the procedures for isolation, genomic data mining and sequence matching, which have played important role in the identification of endophytes, as well as other diverse studies [83]. Endophytic fungi have a great prospect to secrete so many lead compounds some of which are phytonutrients, and phytochemical substances like polyphenol and anthocyanin that possess the ability to reduce mortalities associated with diseases, such as malignancies and cardiovascular diseases [84]. In a review done to ascertain the bioprospects of endophytes, it was hypothesized that some secondary metabolites like polyketides and peptides act against *Mycobacterium tuberculosis* [85, 26]. In another investigative study on *Taxus brevifolia*, which brought about the discovery of taxol, a highly active anti-cancer agent, a lot of attention was brought to endophytic microbes as it gave a higher yield of taxol than the extract obtained from the bark of the yew tree.[76] In addition, from this same study it was cited that endophytic microbes have an independent biosynthetic pathway different from the host which is even more skilled. This results from their genetic recombination from prolonged co-evolution and genetic recombination, as was observed by the way the taxadiene synthase gene is sequenced by endophytes that produce taxol [76]. Equally important, the rampant harvesting of pacilitaxel extract from yew trees is highly reduced, thus nature is conserved. Microbial biotechnology technique has grown far and above production of only metabolites; the production of chemicals such as ethanol and butanol and the biotransformation of many chemicals are also included [86]. Its application employed in the production of secondary metabolites has given room for the mass production of industrially relevant products such as antibiotics, enzymes, riboflavin etc. [87].

Table 1 List of endophytes, plant source, compound generated and their therapeutic applications

S/N	Endophyte	Plant source	Compound	Therapeutic applications	Reference
1	*Chaetomium ovatoascomatis*	*Euphorbia milii*	Coumarin glycoside, esculin	Antimicrbial	[88]
2	*Aspergillus aculeatus*	*Carica papaya*	Secalnoic acid D-F Aculeatine A-J	Cytotoxicity	[89]
3	*Phomopsis* sp	*Distylium chinense*	DR46-1	Anti-cancer	[90]
4	*F. solani*	*Chloranthus multistachys*	9α-dihydroxy-5α-methoxyergosta-7,22-diene (**56**) and 2β,6β-dihydroxy-5α-methoxyergosta-7,22-diene	Antimicrobial	[91]
5	*Fusarium solani*	*Camptotheca accuminata*	9-methoxycamptothecin 10-methoxycamptothecin	Anti. Cancer	[92]
6	*Aspergillus fumigatus*	*Taxus sp*.	*Paclitaxel*	Cytotoxicity	[93]
7	*Penicillium brasilianum*	*Melia Azedarach*	phenylpropanoid amide	Anti-cancer	[94]
8	*Juniperus communis* *Phialocephala fortinI* *Trametes hirsute*	*Uniperus recurve* *Podophyllum peltatum*	Podophyllotoxin	Anticancer	[94]
9	*Alternaria sp*	Nothapodytesp nimmoniana	Camptothecin	Anti-cancer	[92]
10	*Nocardio psissp*	*Zingiber officinale*	Phenol, 2,4-bis (1,1-dimethylethyl) and trans cinnamic acid	Anti-bacteria	[95]
11	*Colletotrichum gloesporoides*	*Artemisis mongoli*	colletotric acid	Antimicrobial	[39]

12	*Xylaria spp*	*Gingko biloba*	7-amino-4-methylcoumarin	Anti-fungal	[96]
13	*Aspergillus minisclerotigens*	*Mangifera casturi Kosterm*	Dihydropyran and 4- H-pyran-4-one	Antioxidant	[55]
14	*Pencillium citrinum, pencillium citrinum Cladosporium sp*	*Tragia involucrate linn*	L-ascorbic acid	Antioxidant	[97]
15	*Chaetomium globosum*	*Adiantum capillus*	Phenolic	Antioxidant	[72]
16	*EC3*	*Carica papaya.L.*	Gallic acid	Phenolic	[98]
17	*Methylobacterium radiotolerans*	*Combretum erthrophyllum*	Alkaloids,Flavonoids	Antioxidant	[99]
18	*Aspergillus spp.*	*Moringa oleifera*	4-hydroxyphenyacetic acid	Antimicrobial	[100]
19	*Aegicera corniculatum*	*Emericella spp*	Isoindolone	Anti-viral	[74]
20	*Phoma multirostrata XJ-2-1*	*Aconitum vilmorinianum*	14-nordrimane sesquiterpenoid	Anti-influenza	[101]
21	Phoma multirostrata XJ-2-1	*Phoma multirostrata XJ-2-1*	Ergocytochalasin A (1)	Anti-Influenza	[102]
22	*Phomopsis sp. CGMCC No. 5416*	*Achyranthes bidentata*	Chromanones	Anti-Viral	[103]
23	*Pleospora tarda strain*	*Ephedra aphylla*	Alternariol and alternariol-(9)-methy	Anti-Viral	[72]
24	*Aspergillus terreus*	*Soyabeans*	Two dereplicated metabolites, aspergillide B1 and 3α-Hydroxy-3, 5-dihydromonacolin L	anti-COVID-19	[104]
25	*Aspergillus versicolor*	*Sea crab (Chiromantes haematocheir)*	four novel indolyl diketopiperazines, aspamides A–E (1–4) and two novel diketopiperazines, aspamides F–G (5–6), in addition to 11 existing diketopiperazines and intermediates	Coronavirus 3-chymoretpsin	[105]
26	*Penicillium citrinum* TOPEF34(Pc)	*Phoenix dactylifera* (date palm tree roof)	Benzodiazepine alkaloid analogue cydopentin A and B, dehydro-cyclopeptin and cyclopeniol	SARS-COV	[106]
27	*Cladosporium sp.* 7951	*Paris polyphylla var. yunnanesis*	Eight new aspulvinone analogues, aspulvins A–H (1–8) and aspulvinones D, M, O, and R (9–12)	Inhibitor of ARS-CoV-2	[107]
28	*Cochliobolus* spp.	*Piptadonia adiantiodes*	Cochlioquonone A	Anti-parastic Anti-malarial	[108]
29	*Nemania* spp	*Torreya taxifolia*	9,20-epoxycytochalasins C and D, cytochalasins and 18-deoxy-19,20-epoxy-cytochalasin C.	Anti-malaria	[109]
30	*Mycosphaerella* spp.	*Psychotoria horizontalis*	Cercosporin	Anti-*Trypanosomaiasis* *Anti-malaria*	[108]

4. Isolation of endophytes

Endophytic microbes can be isolated from various parts of plant such as the root, stem and leaves [53]. These parts serve as a reservoir for endophytic bioactive molecules, but they have to be healthy and not diseased to give a good yield of endophytes, and it is also important to know that endophytes are isolated from the surface of plant tissues (after disinfecting the surface) or from internal part of a plant tissue [110]. To isolate the purest state of endophytes, the method of sterilization established by Okezie *et al*. (2017) has been followed, though with a little modifications [111]. This procedure involves washing off any plant part under study, diligently, under running tap water, followed by sterile double distilled water. This is paramount to get rid of any dirt. Then, this sample is subjected to four steps sterilization method which can be summarized as washing immensely under running tap water-ethanol-sodium hypochlorite-distilled water. The sterilization properly involves, flooding the sample sufficiently under a running tap, then submerged in 70% ethanol for three minutes, and washed twice with distilled water. Furthermore, the washed sample is further soaked in 4% sodium hypochlorite solution for five minutes and adequately rinsed thrice in distilled water and 70% ethanol for three minutes, before a final rinse in sterilized double distilled water. This is then dried in a laminar flow on a sterile filter paper. All these processes of four standard steps of sterilizing samples for isolation of endophytes are in a bid to get rid of epiphytes/rhizosphere and any other contaminants that could interfere with the product of isolation, and this makes this process quite crucial, though difficult.

Then, isolation process presented in Fig. 2 entails cutting the sterile samples into 1cm length, with sterile knife and then each fragment is enrooted in an already prepared sterile media (malt extract agar) containing 5mg/l of chloramphenicol (a total of 30 at 3-6 segments petri dish). These petri dishes are correctly covered with paraffin and incubated at 25^{o}C and routinely checked every other day, until one week. Thereafter, the hyphal tips of actively growing fungi in the petri dish are sub-cultured on another sterile malt extract agar (MEA) and incubated for another 5-7 days with a routine check to ascertain the purity. This procedure is repeated till the purest isolates of endophyte/s under study are obtained (an interval of two weeks sub-culturing is required to maintain the purity of the cultures). Culture characterization of the samples is studied morphologically by observing their colors, nature of the growth of colony and texture. It is noteworthy to use freshly sub-cultured samples (pure samples) as the starting material for production/cultivation of metabolites to avoid contamination.

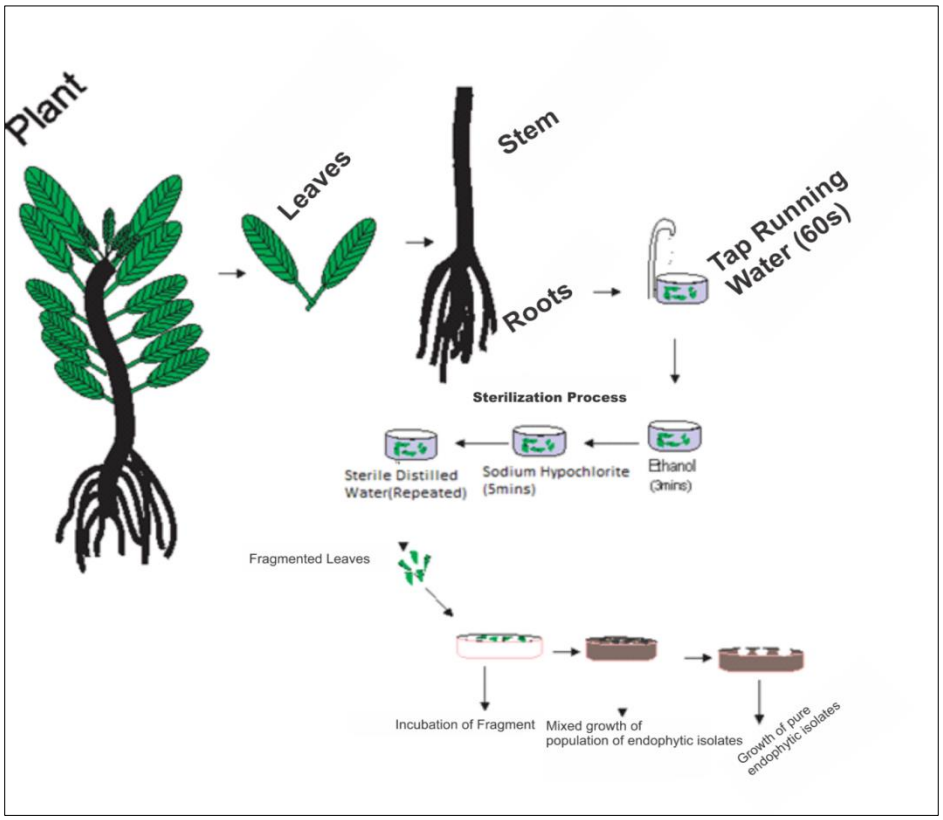

Figure 2 Isolation of the endophytes

5. Identification and Characterization of Endophytic Isolates

Traditionally, identification and characterization of endophytic microbes are carried out based on their morphological characteristics, microscopy, gram-staining method, and biochemical characteristics.

5.1. Morphological Characterization

The morphological characterization is carried out by microscopy. The following features are usually investigated: colonies, form of growth, elevation, size, color, margin edge, consistency, opacity and change in medium established.

5.2. Gram Staining

For further classification of the endophytes, Gram staining technique is used to characterize endophytic cells based on their ability to take up some dyes as a means of differentiating them as either gram-positive or gram-negative based on the content of their cell wall.

5.3. Biochemical Characterization

Microorganisms just like endophytes produce exo-enzymes during degradation of large polymers into smaller compounds. The detection of such enzyme activities is of much importance to the identification of endophytes into their various genus and species. This is achieved by analyzing the nutrient utilization and metabolic capacities of the endophytic microbes. Different biochemical tests such as, catalase test, coagulase test, indole test, etc are carried out to ascertain the taxonomic identification.

5.4. Molecular characterization

The techniques mentioned above are culture and microscopy-based which do not give complete information on the characteristics of the endophytes. It was Kumar *et al.* (2019b) that reported that these approaches have failed to give detailed or comprehensive characterization of these microorganisms found in plant tissues [112]. However, to accurately identify and characterize an endophytic microorganism, there is need to trace it down to its genetic makeup. In this regard, molecular techniques have been employed. This approach entails extraction of the sample DNA using standard protocol and subjecting it to DNA sequencing analysis of ribosomal RNA genes using PCR. Gene sequencing for bacteria uses 16s rRNA gene sequence, whereas fungi uses ITS/18s rRNA gene sequence. Other methods that have more resolution power than 16s rRNA gene sequencing, such as multi locus sequence typing (MLST), fatty acids methyl esters chromatography (FAME-GC) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF), can identify endophytic bacteria down to its strain level [112].

5.5. Culturing of the Endophytes to Yield Metabolites

This process could otherwise be called fermentation of endophytic isolates to generate metabolites. A procedure described by Okezie *et al*. (2017) is also very much adequate for the production of metabolites from endophytes [111]. This is a biotechnological technique that involves using an inoculum that was purified by picking and cultivating the tip of hypha/mycelium of fungi species sprouting out of previously sub-cultured fungi and incubating at 25^{o}C or 30^{o}C for 48hours, if the samples are of bacteria species, and all transfers are done aseptically to control contamination.

The whole process of fermentation is done by using local rice or corn as the culture medium. It was aforementioned that endophytes tend to yield more metabolites when the fermentation medium is prepared with local rice than when it is corn or parboiled rice. Home grown rice of 100g in 200 ml sterile water in sterilized 5000ml Erlenmeyer flask, sterilized at 121^{o}C for 30 minutes and allowed to cool is a very favorable condition to ferment the endophytes. Then, on the fermentation medium prepared, actively growing pure isolates of endophytes are aseptically cut off and inoculated on MEA and properly sealed with sterile cotton wool, and kept on the shelf. This will be allowed to ferment for 21 days at 30^{o}C under static conditions.

5.6. Isolation of Metabolites

Okezie *et al*. (2017) described a standard procedure to recover metabolites from endophytic fermentation.[111]The process of fermentation is terminated by adding extraction solvent (ethyl acetate). The fermented mixture is cut in smaller lumps into a 1litre Erlenmeyer flask with the aid of a sterile glass rod, and 50ml of ethyl acetate is added, then the mixture is homogenized and kept for two days with intermittent shaking, after which a Whatman filter paper (pore size, 11µm) is used to filter the mixture. Furthermore, the filtrate obtained is concentrated under 50^{o}C at reduced pressure employing a rotary evaporator and the concentrated extract obtained is allowed to evaporate to dryness in a desiccator that contains sodium hydroxide. Then, the resultant extract (metabolites) is normally weighed and stored,

to be reconstituted in DMSO (Dimethyl sulfoxide) whenever any study is needed to be carried out on it. The scheme for the isolation is presented in Fig. 3.

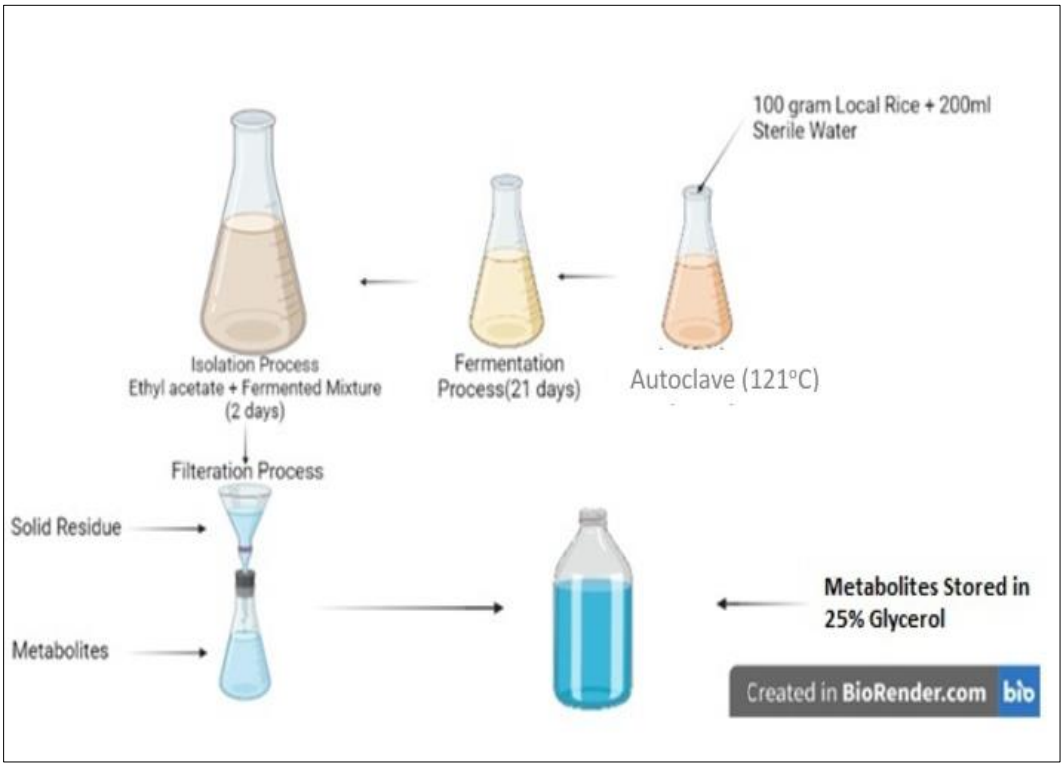

Figure 3 Production and isolation of metabolites from endophytes[132]

5.7. Challenges of Endophyte Technology as an Alternative Drug Discovery Route

There is a handful of reports of the entire plant species harboring diverse microorganism with high potent secondary metabolites, yet a few of them have been explored due to some hurdles associated with the process of extraction of those metabolites (fig 4), which are discussed below.

5.8. Uncultivable Microbes

It has been estimated that over 70% of environmental microbes with an estimated population of about 2500×10^{26} cannot be cultivated in the laboratories [113]. These results from the difficulties associated with understanding the growth requirements of these microorganisms which also applies to endophytic microbes. Growth requirements such as culture media type, temperature, pH, culture time, inoculums size and other environmental conditions are hurdles to culturing and characterizing endophytic microorganisms. These conditions can increase or decrease the yield of endophytes or hinder the growth of most endophytes [114]. A noteworthy scientific work by Murphy *et al*. (2015) on the effect of different types of media for growth such as corn meal extract cazpek sox, malt extract, potatoes dextrose and Sabouraud dextrose media, showed that growth variance of the isolates of fungi together with their biomass are heavily influenced by the type of culture media used [115]. The majority of experimental studies on endophytes do not diversify culture media during isolation of endophytic microorganisms, thus there is the likelihood of failing to get an adequate amount of endophytes residing in the plant tissues [115]

5.9. Surface sterilization procedure

This is also one of the major limitations of endophyte technology as an alternative drug discovery route. The first and foremost step in isolating the true endophyte from the plant tissues is getting rid of the surface microflora (epiphytes and rhizosphere) through a process called surface sterilization. It is a very crucial step in isolation of endophytes from the plant tissues, as it determines to a great extent, what grows (surface microflora or endophyte) on Petri dishes of isolation process hence, knowing the right solution for sterilization, right amount of the sterilization solution and right time of exposure to enable thorough surface disinfection, with little or no damage on endophytic cells is important to ensure isolation of a true endophyte [116].

5.10. Time and resources consumption factor

This has shown to enormously contribute to the constraints associated with implementing endophyte technology as an alternative route of drug discovery. Endophyte technology is a culture-dependent approach which requires elaborate laboratory practical in which much time and surplus materials are used for culturing and sub-culturing of endophytes to ensure generation of pure isolates. For instance, some fungi endophytes take 35 days to grow in culture media and 21 days to fully excrete their secondary metabolites. These will involve huge amount of time and resources with endurance on the path of the scientist to have a conclusive result of the research. In furtherance, some researchers have reported that sub- culturing of endophytes reduces the growth and yield of endophytic microbes, as well as the metabolites overtime, and in some cases, the growth of targeted microbes becomes negligible after several process of sub culturing all in a bid to get a pure isolate. It was cited that *Periconia sp.,* an isolate from *Tarreya grandifolia* plant produced anti-cancer drug paclitaxel of concentration 350 $ng.\mu l^{-1}$. However this was reduced significantly to 118 $ng.\mu l^{-1}$ after third rounds of *in vitro* sub-culturing [117]. In addition, a landmark study by Kusari *et al*. (2009) reported a dramatic reduction of yield of CPT (camptothecin) from 400 – 800 µg to less than 100 µg per 100 g dry weights of mycelia on 3rd and 4th round of subculturing and became negligible on 7th round [118]

5.11. Endophytes interactions within the host and the surrounding environment

What is described by Mishra *et al*. (2021) as co-culture is the interactions of endophytes with other endophytes/plant cells and other environmental conditions, when cultured together, play a vital role in the activation of the biosynthetic gene clusters (BGCs) - a class of organized genes that control the biosynthetic pathways of secondary metabolites required for expression of the targeted metabolites [116]. This technique has brought a worthwhile improvement on the yield of endophytes. In the same study, it was highlighted that these microbial cells could be attenuated under axenic monoculture resulting from no signals or stimulus from host plant or other co-existing endophytes and other conditions in which the endophytic isolates are cultured. Hence, identifying and addition of the plant extract that help the endophyte express the target metabolites are important, as some endophytes lose the plant extract that originally aid their ability to get the required metabolites

5.12. Mutation

Ochi *et al*. (2014) showed in their study that mutation could also be a drawback in this technology, as several rounds of sub-culturing could alter the quantity as well as the quality of the secondary metabolites produced by endophytic microbes [119]. It was observed that random, non-synonymous substitutions during surplus sub-culturing results in loss of enzymes that coordinate the activities of endophytic biosynthesis [120]. Houde *et al*. (2021) discussed other factors such as BGC identification, cryptic BGC, stability of metabolites as determinants to the quantity of secondary metabolites produced [121]

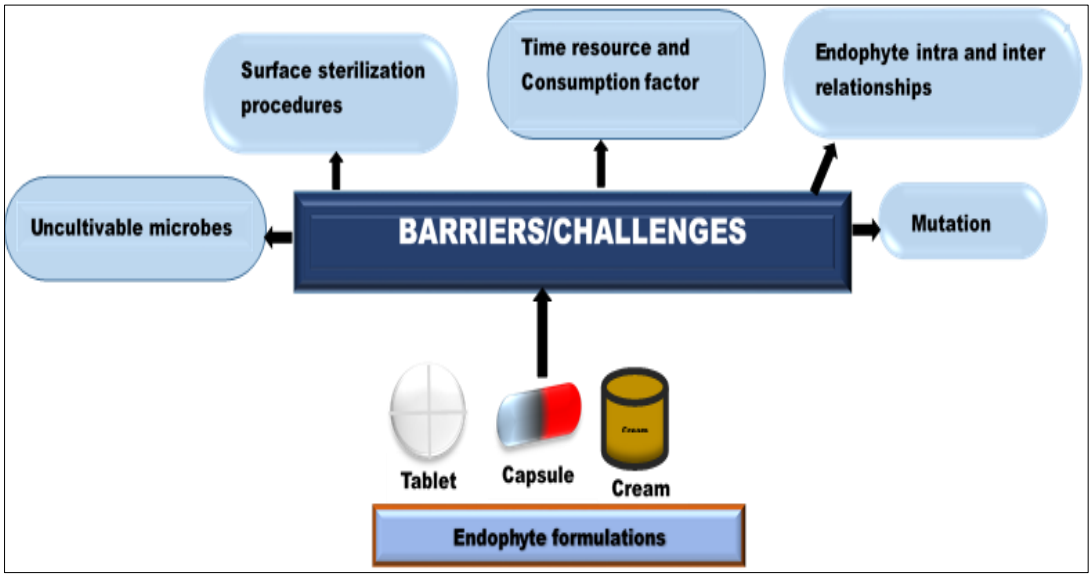

Figure 4 Possible Contributors to Poor Yield of Endophytes

5.13. Techniques for amplification of the yield of endophytic metabolites

Poor yield has been a huge gap in the implementation of endophyte technology as an alternative drug discovery route; hence improvement of the yield of endophytic metabolites for adequacy and sustainability has become paramount. To bridge this gap, some biotechnological methods have been employed (fig. 5).

5.14. Genome mining

This is a very important biotechnological tool that takes advantage of bioinformatics knowledge, whole genome information and gene exploitations targeted at identifying gene clusters responsible for the production of an important bioactive compound [122]. The identification of gene clusters will make for easy manipulation of the path to the desired products.

5.15. Omics approaches

Metagenomics, metatranscriptomics and metabolomics have given a new understanding of plant microbiome interactions. Omics are advanced biotechnological techniques that are currently leading the way in the development of vaccines, new therapeutics and other important industrial products [123]. These approaches can be utilized to improve the yield of endophytic secondary metabolites, both qualitatively and quantitatively and can also ensure sustainability as they can rule out the tedious uncertainties associated with processes of elaborate laboratory practical. Mishra *et al*, (2021b) have demonstrated that via omics techniques, spikes on *Piper longum* plant houses commercially adequate endophytic actinobacteria and fungi [117].

5.16. Advanced biotechnological methods

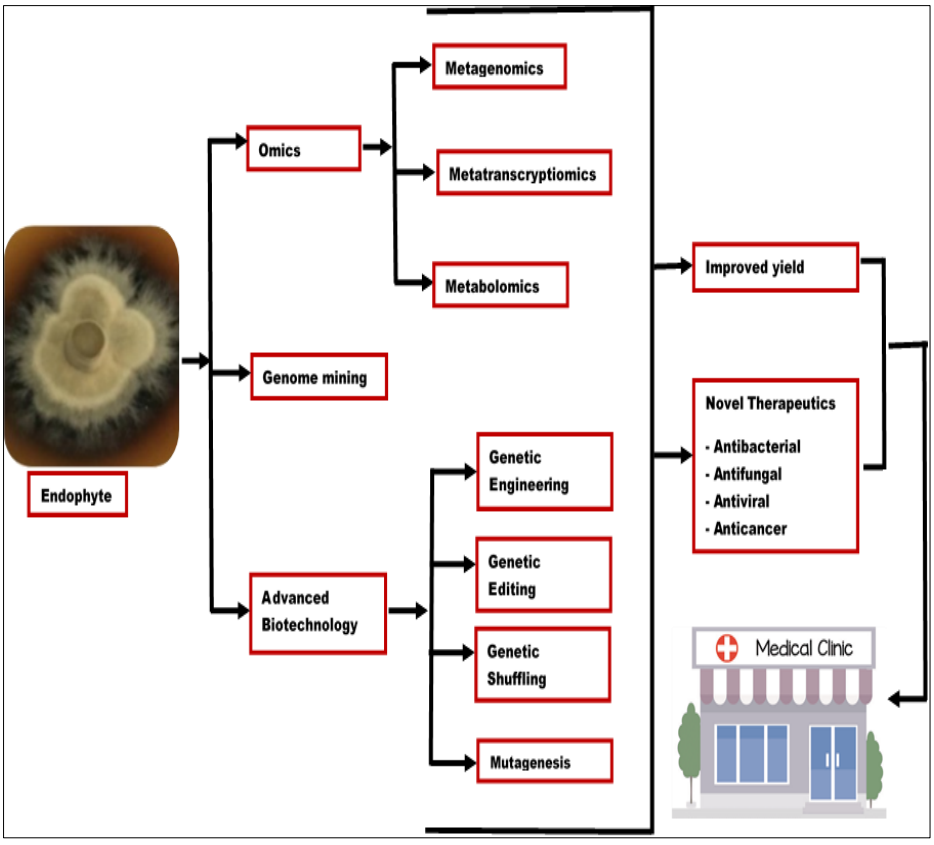

Figure 5 Biotechnologies of Improvement of Endophytic Yield

These methods have been highlighted in literature as methods that can enhance metabolite yield by genetic modification and they include genetic engineering, [124,117] genome editing [8] genome shuffling [125] as well as mutagenesis method [126]. These biotechnological techniques work by either over-expressing the transcription factors, promotes engineering genes, or creation of knock-out genes which have been proven to improve endophytic strain by inducing the expression of silent biosynthetic gene clusters (BGC) [127]. Genetic engineering technique was utilized to produce over expression of *txs* gene that encodes the first main rate-limiting step in the biosynthesis of taxol, which gave four-

fold increase of taxol, compared to the original yield. Similarly, one thousand-fold productions of two novel compounds/strains of *Aspergillus niger* was achieved by over-expression of a cluster-linked transcription factor, found within the biosynthetic gene cluster which triggered the activation of the expressor in the adjacent secondary metabolites encoding gene. The use of deletion of key genes involved in the biosynthesis of some strains and expression of silent BGC has been documented as a way of increasing the targeted endophytic metabolites. Synthesis of mutagens from the exposure of fungal proplasts to UV-ray and N-methyl-N"-nitro-N-nitrosoguanidine followed by further exposure to genome shuffling gave rise to the generation of many novel drug candidates [128].

6. Expert opinion/Way forward

The application of endophyte technology in the discovery and production of pharmaceuticals for the treatment of emerging and re-emerging infectious diseases has been documented. Therapeutic agents such as, antimalarials, antidiabetics, anti-inflammatory, anti-cancerous, anti-hypertensives, anti-microbials agents and many others, can be employed in treatments of malaria, cancer, HIV/AIDS, diabetes, polio, cholera, inflammatory responses, infectious diseases and others that traditionally could be obtained from plants are now possible through endophyte technology as a credible alternative. Due to the growing need for pharmaceuticals and biopharmaceutical products in modern medicine to handle these diseases, more aggressive attention on the exploration of these highly potent biomolecules has become increasingly needed, as they are endowed with natural bio-actives with potentials as novel drug moieties. Moreover, employing endophyte technology as a good option for the production of these new bioactive compounds will not only facilitate their productions but will enormously boost the market of pharmaceuticals/biopharmaceuticals products. This technology is known to improve the availability of targeted material through mass production (biotech approach) via submerged fermentation technology in 1940 [129]. This biotech technique led to the manufacturing of some bioactive molecules such as, acid, alkaline, neutral proteases, cellulose, diastase, invertase, lactase, lipase and pectinase which are all products of endophytes [130]. Besides, the gain of massive production of the targeted drug component has presented endopyte technology to be profit-oriented, cost-effective and environmentally friendly, thus, will make it very attractive to pharmaceuticals/biopharmaceuticals and R&D companies. However, the advent of biotechnology was heralded with issues of uncertainties as it concerns ethics and safety. The ethics and safety issues include the implications of gene manipulation/ engineering of organisms, but following series of debates and other academic programs such as, conferences it was established that biotechnology was undoubtedly safe, as it has contributed positively towards handling serious challenges of humanity like: hunger, diseases, environmental destruction and many more [131]. A report from WHO working group was summarized by stating that biotechnology in general, is a safe industry [131]. This has cleared the ethical issues that could have arisen in the course of endophyte technology.It is also important to know that there is a great need for small pharmaceutical industries to scale-up their bioreactor to accommodate the arduous efforts coupled with the time-tasking activities of fermentation/extraction involved in endophytic technology, which may fail in small-scale or start-up fermentation companies [3]

7. Conclusion

For some decades, exploring microorganisms colonizing the internal part of plants has gained so much attention as credible sources of new drugs other than sourcing from animals, and plants for the discovery of new drugs. These results are from the fact that these exceptional plant microflora are naturally endowed with interesting bioactive novel compounds that can serve as new drug candidate. Endophytes which could be described as benign microorganisms living within the plant`s healthy tissues have been found to possess a great amount of genetic diversity of bioactive species that secrete bioactive compounds which could be harnessed and enhanced for therapeutic purposes. These natural products generated from these plant microfloras have been scientifically confirmed to possess inhibitory or lethal effects on pathogenic microbes that are daunting to humans. They have been found promising to curb the rising incidences of multidrug-resistant menace that is financially draining the healthcare system globally. Additionally, the exploration of endophytes for the treatment of infections is coming as a cheaper and better alternative, since any identified and isolated drug candidate can be mass-produced using biotechnological techniques, thereby promoting availability and less production cost. Expectedly, mass production will not only make drug moieties abundant, but also solves the world problem of climate change, resulting from rampant cutting down of plant for production of drugs, thus conserving nature, in agreement with United Nations millennium development goals numbers 6 and 7.

Compliance with ethical standards

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This article does not contain any studies with human participants or animal performed by any of the authors.

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