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Introduction to Phytochemicals: Secondary Metabolites from Plants with Active Principles for Pharmacological Importance

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Abstract

Phytochemicals are substances produced mainly by plants, and these substances have biological activity. In the pharmaceutical industry, plants represent the main source to obtain various active ingredients. They exhibit pharmacological effects applicable to the treatment of bacterial and fungal infections and also chronic-degenerative diseases such as diabetes and cancer. However, the next step in science is to find new ways to obtain it. In this chapter, we discuss about the main groups of phytochemicals, in addition to presenting two case studies. One of the most important secondary metabolites is currently Taxol, which is a natural compound of the taxoid family and is also known for its antitumor activity against cancer located in breasts, lungs, and prostate and is also effective with Kaposi's sarcoma. Our case studies will be about Taxol, extracted from an unexplored plant species, and the production of Taxol by its endophytic fungi.

Keywords: phytochemicals, biosynthesis, alkaloids, flavonoids, taxoids, Taxol, *Taxodium mucronatum*

1. Introduction

In the history of humanity, plants have always been present as a source of health. The knowledge of the various healing properties of plants has been transmitted in an empirical way. However, over time, man has been interested in knowing where the properties of plants come from. In the process of knowledge generation, man has developed many methodologies to know the structures of organic compounds responsible for the healing properties of plants. This is the birth of phytochemistry that is defined as the science responsible for the study of

the compounds contained in plants. In this field, various techniques have been developed, ranging from the preparation of the plant tissue sample to sophisticated techniques for the elucidation of organic structures. The search for new products for the pharmaceutical and agrochemical industries is an ongoing process that requires continual optimization [1]. Previously, the screening of 10,000 natural products resulted in one commercial product. In the advent of combinatorial chemistry, this relationship changed. Presently, the screening of 100,000 structures day⁻¹ from combinatorial chemistry together with the natural products screened yields less than one commercial product year⁻¹ (F. Hansske, pers. comm.). Its development takes approximately 12 years and costs ~\$350 M [2]. Considering that 6 out of 20 of the most commonly prescribed medications are of fungal origin [3] and only ~5% of the fungi have been described [4], fungi offer an enormous potential for new products.

Endophytic fungi, a polyphyletic group of highly diverse, primarily ascomycetous fungi defined functionally by their occurrence within asymptomatic tissues of plants, are found in aboveground tissues of liverworts, hornworts, mosses, lycophytes, equisetopsids, ferns, and seed plants from the arctic to the tropics, and from agricultural fields to the most biotically diverse tropical forests. Their cryptic lifestyle, ubiquity and richness within individual plants, coupled with emerging evidence of their often overlooked ecological importance, have inspired growing enthusiasm regarding these little known fungi over the past four decades. In particular, David Hawksworth's much discussed estimates of fungal diversity at a global scale [4, 5] engendered tremendous enthusiasm for understanding endophyte diversity. Comprising interactions that range from mutualism to antagonism, fungal symbioses with plants are key determinants of biomass, nutrient cycling and ecosystem productivity in terrestrial habitats from the poles to the equator [6, 7]. Most plant-associated fungi catalogued to date have been recognized because of the fruitbodies they produce in association with their hosts (e.g., plant pathogens, mycorrhizal fungi). Yet plants in all major lineages, including liverworts, mosses, seed free vascular plants, conifers, and angiosperms, also form cryptic symbioses with fungi that penetrate and persist within healthy aboveground tissues such as leaves. Foliar fungal endophytes (i.e., endophylls or mycophyllas) are a fundamental but frequently overlooked aspect of plant biology: all plant species surveyed thus far harbor one or more endophytic symbionts in their photosynthetic tissues [8]. Plants live in association with microorganisms with different levels of interaction. This assumption stimulates insights on plant microbiome, intended as the collective genome of microorganisms living in contact with plants [9], and new concepts in plant evolution have been developed considering a basic role of the associated fungal endophytes [10]. Regarded as an underexplored niche of chemo diversity [11], endophytic fungi have a recognized ability to produce bioactive compounds which may play a role in plant protection against pathogens and pests [12, 13]. Colonization by endophytes may offer significant benefits to their host plants by producing various metabolites that protect against pathogen attack, promote plant (or vegetative) growth, improve crop yields, show herbicide activity and induce resistance. Fungal natural products are currently used in agriculture as active ingredients of different bioformulates [14] and several endophytes are known to have anti-insect properties [15]. Although bioinsecticides currently occupy only a small amount of the market, these compounds are very interesting and their use is constantly increasing [16].

In 1991, researchers began studying the microbial endophytes of the Northwest Pacific yew tree *Taxus brevifolia*, in search for a fungus or bacterium that could produce paclitaxel in de

novo fashion [17]. At that time, there were few reports describing the chemistry of plant endophytes, although there was a rich literature cataloguing the secondary metabolism of plant pathogenic fungi and bacteria. Phytotoxins, secondary metabolites produced by plant pathogenic microorganisms, have been studied for almost a century as virulence factors and the initiators of diseases in susceptible plants. Three well-known examples are the host-specific toxins produced by three different *Cochliobolus* species, all of which caused severe blight diseases of economically important crops [18]. *C. carbonum* (*Helminthosporium carbonum*) produces host-specific HC-toxin, which causes Northern leaf blight of maize and inhibits maize histone deacetylase [18]. *C. heterostrophus* (*H. maydis*) produces T-toxin which caused Southern Corn Leaf Blight, one of the worst plant disease epidemics in modern history, and which was especially virulent toward maize carrying Texas male sterile cytoplasm [18]. *C. victoriae* produces victorin which caused a devastating epidemic in the Victoria race of oats that was developed by plant breeders in an effort to produce oats that were resistant to crown rust [18].

Plant endophytes are subtler, however, rarely causing problems and coexisting with their hosts under most circumstances. Hirsch and Braun provided an inclusive and widely accepted definition of endophytes: “microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effects” [19, 20]. They are generally nonpathogenic in nature, but may produce secondary metabolites that enable them to survive in the competitive world of plant interstitial space without harming their host. Microorganisms in most ecosystems establish and define their ecological niches by their ability to control fellow microbes with only their cell walls or membranes and chemical arsenals to defend them. But these chemical arsenals have provided many of the important chemotherapeutics used to date. The potent antifungal agent griseofulvin is of fungal origin [21] and both the antibiotic streptomycin [21] and the anticancer agent calicheamycin are produced by actinomycetes [22].

Plant endophytes, however, received less attention until the discovery of a Taxol-producing fungus in the bark and needles of the Northwest Pacific yew tree. In 2011, we published a review of cytotoxic or anticancer compounds produced by plant endophytes [23]. Over 100 compounds with demonstrated cytotoxicity or anticancer activity had been isolated from endophytic fungi—including several compounds originally isolated from higher plants [8]. Less than 10% of these compounds were isolated from coniferous species [24]. Our own work with the fungal endophytes of conifers has shown them to be rich producers of bioactive secondary metabolites.

Two reasons led us to start this research project: produce secondary metabolites of pharmaceutical application to reduce a type of cancer and reduce the cutting of trees and apply biotechnology to produce taxanes by endophytic microorganisms. The main aim of this book chapter is to present case studies of isolation, characterization and application of secondary metabolites: Taxoles.

2. Metabolism

Metabolism is a set of chemical reactions carried out by the cells of living beings, to synthesize complex substances from simpler ones, or to degrade complexes and obtain simple ones [25]. Plants, autotrophic organisms, have two metabolisms, the primary metabolism present in

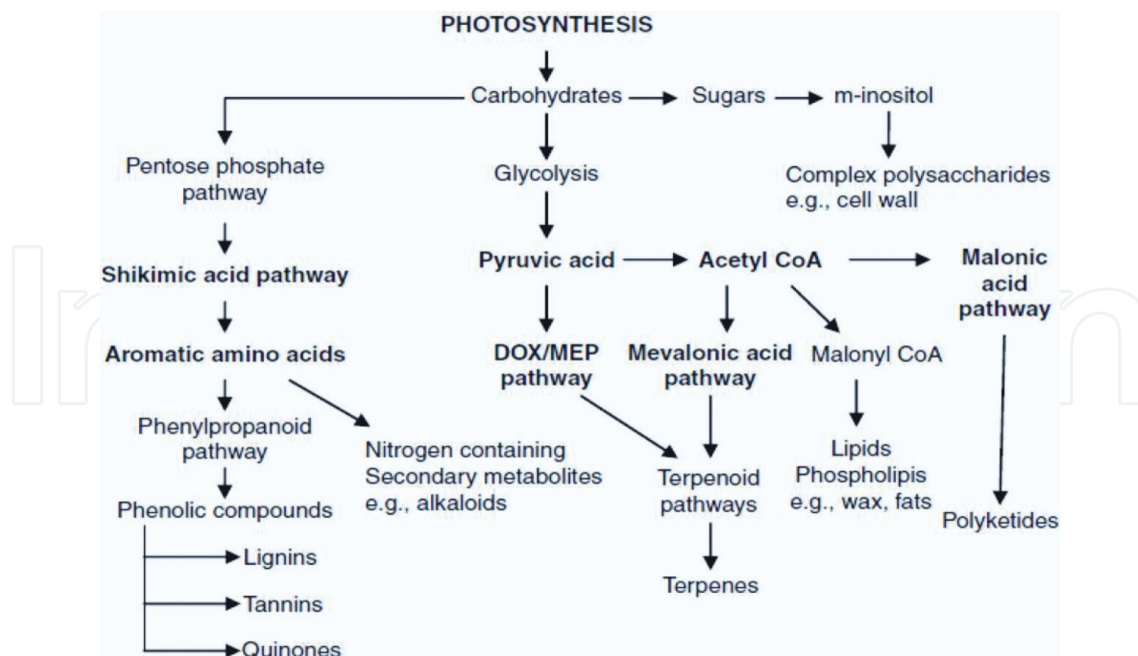


Figure 1. The metabolism of plants.

all living beings and a secondary metabolism that allows them to produce and accumulate compounds of diverse chemical nature (**Figure 1**).

Most of the carbon, nitrogen and energy ends up in common molecules to all the cells, which are necessary for their functioning and the organisms they belong [26]. These are amino acids, nucleotides, sugars and lipids, present in all plants and performing the same functions. They are called primary metabolites.

Plants allocate a significant amount of assimilated carbon and energy to the synthesis of a wide variety of organic molecules, that do not seem to have a direct function in photosynthetic, respiratory processes, nutrient assimilation, solute transport or protein synthesis, carbohydrates or lipids, and which are called secondary metabolites (also called by-products, natural products) [27].

Secondary metabolites are characteristic of superior plants. The essential characteristic of the superior plants is that they possess flower and, consequently, seeds. Its reproductive mechanism is different from that of the inferior ones. They are also called spermatophytes because their reproductive organs are visible and they are subdivided into gymnosperms and angiosperms.

Natural products have biological properties, and they are characterized by their different uses and applications as medicines, insecticides, herbicides, perfumes or dyes, among others. The biosynthesis of secondary metabolites is usually restricted to specific stages of plant development and periods of stress [25]. Some plant cells produce important secondary metabolites of the interactions of the plant with the environment (protection against predators, pathogens or environmental stress) or some related to the reproductive mechanism of the plant (attraction of insects for the promotion of pollination) (**Figure 2**).

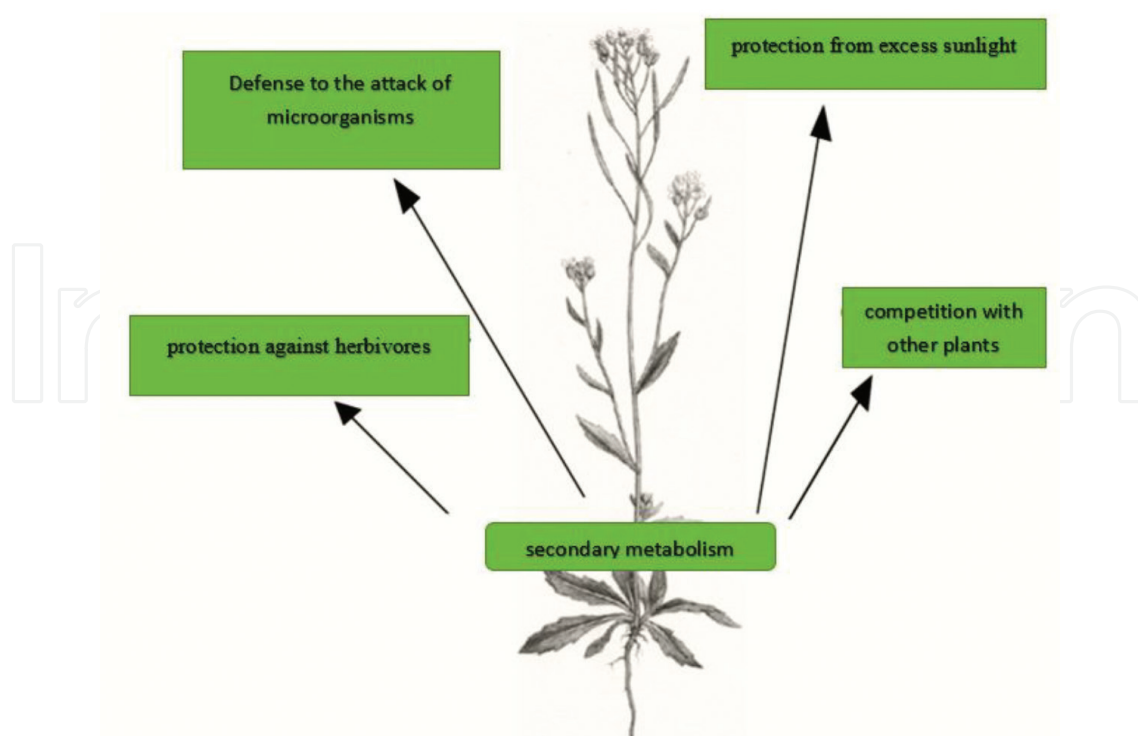


Figure 2. Production of secondary metabolites.

3. Phytochemistry

The discipline whose main objective is the study of the chemical constituents of plants is Phytochemistry. The study of such compounds includes: their chemical structures, metabolism (biosynthesis and degradation), natural distribution, biological function, extraction and qualitative-quantitative evaluation. Before starting, any phytochemical analysis is important to have an adequate preparation of our plant material. A practical and simple way of stabilization is by heat treatment, applied, for example, in an oven at a reference temperature of 60°C until the samples reach constant weight; this way, we will make sure that our compounds will be in the optimal conditions to be analyzed.

Phytochemical research of a plant includes several aspects:

- Extraction of the compounds to be analyzed from a sample or specimen.
- Separation and isolation of them.
- Identification and/or characterization of the isolated compounds.
- Investigation of the biosynthetic routes of a certain molecule.
- Determination or quantitative assessment.

In the extraction and purification of organic compounds through the use of solvents, usually follows certain rules based on structural analogies between the substance to be extracted and the solvent that will be used for that purpose.

Chromatography	Thin-layer chromatography (TLC)
	Gas chromatography (GC)
	High-resolution liquid chromatography (HPLC)
	Capillary liquid chromatography (u-LC)
Electrophoresis	Thin-layer electrophoresis (TLE)
	Isotachopheresis (ITP) (electrophoresis at uniform speed)
	Capillary electrophoresis (CE)
Spectroscopic techniques	UV spectroscopy
	Infrared spectroscopy (IR)
	Near infrared spectroscopy (NIR)
	Nuclear magnetic resonance spectroscopy (NMR)
	Mass spectroscopy (MS)

Table 1. Separation and identification techniques.

The polarity of the compounds is another element to be taken into account, when considering the solubility of a solute in a given solvent. Thus, strongly polar solvents dissolve ionic or highly polar solutes, while low-polar solvents do not efficiently dissolve ionic solutes but do dissolve low-polarity solutes.

The extraction of the vegetal material is done consecutively using solvents, from a low polarity until reaching the water, which is the most polar solvent.

The obtained extracts can be clarified by filtration through celite with a vacuum pump and then concentrated under reduced pressure. This is generally carried out in a rotary evaporator, in which the solutions are concentrated until a volume reduction is achieved, at temperatures between 30 and 50°C. The concentrated extracts must be stored refrigerated.

In the separation and identification of natural products, different techniques for isolation and identification have been developed, in **Table 1** is a summary of the main techniques.

4. Families of phytochemicals

To establish an ordering, these compounds will be classified considering some characteristics like: their biosynthetic origin, the common structural characteristics and the solubility properties.

Some large groups of secondary metabolites are:

- Nitrogen and sulfur compounds, characterized by possessing nitrogen and/or sulfur in their structure, of solubility and diverse biosynthetic origin, but mostly derived from amino acids. Example of these compounds are the cyanogenic glycosides are nitrogen compounds, which are not toxic by themselves but degrade when the plant is crushed releasing toxic volatile substances such as hydrogen cyanide (HCN). An example is the amygdalin (**Figure 3**), found in the seeds of almond, apricot, cherry or peach.

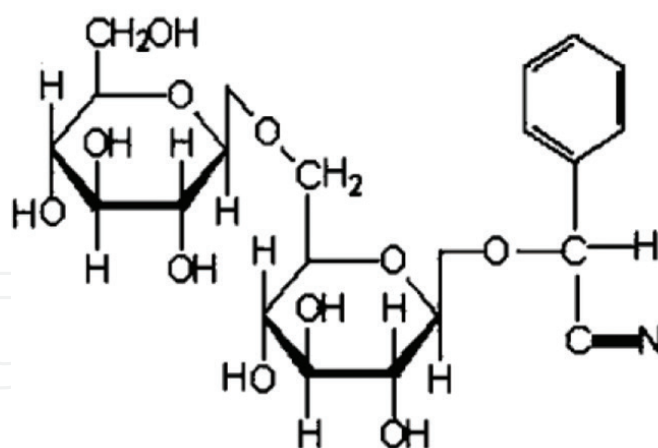


Figure 3. Structure of the amygdalin.

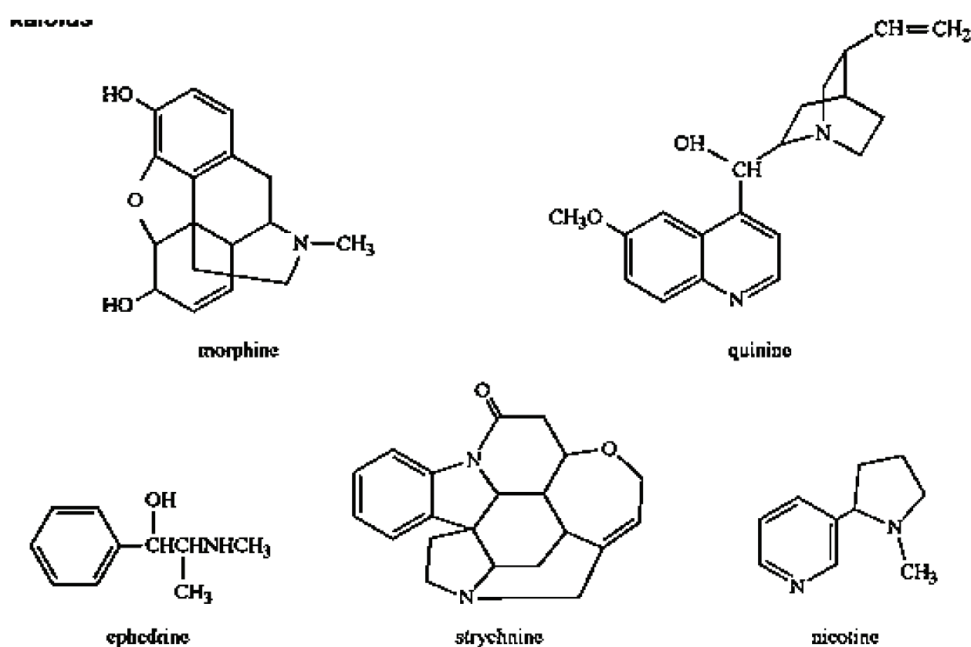


Figure 4. Alkaloid structures.

Other natural nitrogenated products with an important biological activity are alkaloids; some examples of alkaloids are in **Figure 4**. Alkaloids are a large family of more than 15,000 secondary metabolites that have these three characteristics in common: they are soluble in water, contain at least one nitrogen atom in the molecule and exhibit biological activity. The majority of them are heterocyclic although some are aliphatic (noncyclic) nitrogen compounds such as mescaline or colchicine, for example [28].

- Phenolic compounds, with at least one hydroxyl group attached to one or more aromatic rings in its chemical structure, most of which are water-soluble and biosynthetically derived from shikimic acid.
- Terpenoids, with the isoprene molecule as a structural unit, liposoluble, and biosynthetically associated to the mevalonic acid pathway or to the glyceraldehyde phosphate-pyruvic

Isoprene units <i>n</i>	Carbon atoms <i>n</i>	Name	Example
1	5	Hemi-terpenes	isoprene
2	10	Mono-terpenes	thymol
3	15	Sesqui-terpenes	δ -cadinene
4	20	Di-terpenes	taxol
6	30	Tri-terpenes	β -amyrin
8	40	Tetra-terpenes	β -carotene
9 – 30,000	> 40	Poly-terpenes	rubber

Table 2. Classification of terpenoids.

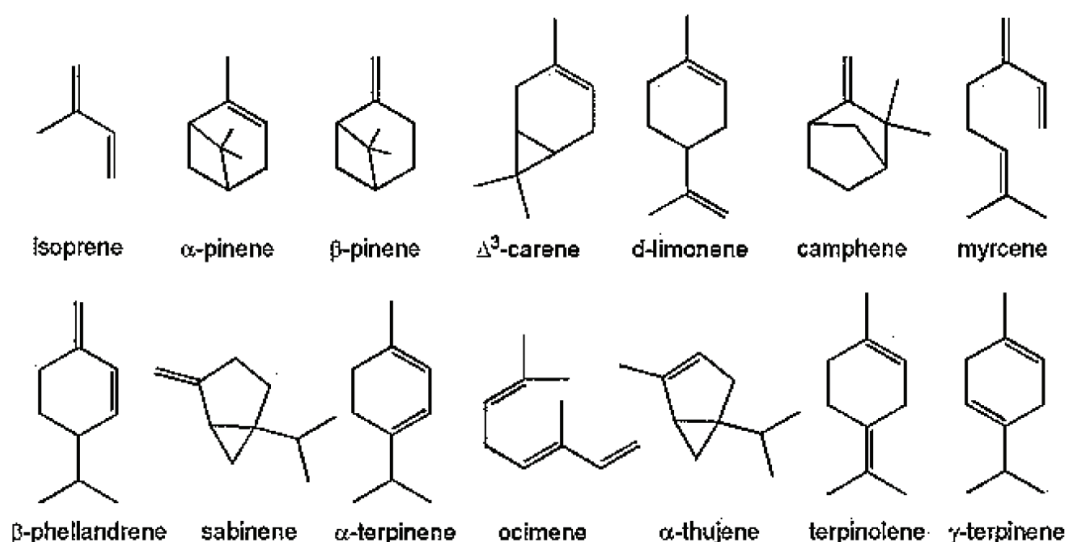


Figure 5. Examples of terpenoid structures.

acid pathway, depending on the class of terpenoids in question [29]. Table 2 shows the classification of the terpenoids, with respect to the number of isoprene units they contain, as well as an example of each type of terpenoid and Figure 5 shows some structures of terpenoids.

5. Case 1. Exploring new ways: Taxol

5.1. Taxol

Among the metabolites with greater interest are the toxoids, these are secondary metabolites that are synthesized by the *Taxus* spp. They are found in the foliage and bark of this tree. The main taxoid of pharmacological interest is Taxol; a polyoxygenated diterpene alkaloid approved by the Office of the Administration of Drugs and Foods. This taxoid is used in the

treatment of breast, ovarian, lung and Kaposi's sarcoma related to HIV. Hence, the importance of knowing everything related to the production of this powerful medicine.

5.2. Biosynthesis of Taxol

Several studies have been conducted on the biosynthesis of taxoids, especially Taxol. In **Figure 6**, it is one of the biosynthetic routes for the production of Taxol. Biosynthesis is a process that requires knowing the mainly enzymatic reactions that involve the construction of the skeleton and the addition of various oxygen and acyl functional groups. The central skeleton of the Taxol molecule is a taxane ring of isoprenoid nature and is derived from geranylgeranyl diphosphate (GGPP). What is the common precursor of 20 carbon atoms isoprenoids (diterpenes), among which compounds such as carotenoids can be found. The phytol chain of chlorophylls or gibberellins participates in the growth and development of plants. However, all of them are formed in the same way precursors, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) are formed. However, and despite the studies carried out, no biosynthetic route of Taxol has been reached. In **Figure 2**, there is one of the different proposed schemes for Taxol biosynthesis.

5.3. Research carried out

Several researches have been developed with the aim of finding new sources to obtain Taxol. But it has only been isolated from trees of the *Taxus* species. **Table 3** shows the percentage of yield obtained from the Taxol extractions of the different species of the *Taxus* spp., as well as the analysis of the different parts of the species.

Based on the previous table of contents, another taxonomic species was investigated such as *Taxodium mucronatum*. The *Taxodium mucronatum* belongs to the Cupressacea which is a gymnosperm. They are large trees over 25 m in height and 1.5 m in diameter from the trunk at chest height. Its leaves are small, elongated and grouped in twigs, in autumn the leaves turn reddish and fall.

The new shoots appear in spring. They can be distinguished at a distance by their dense foliage and their hanging branches, and they are always close to the water or in places with shallow water table (**Figure 7**).

They are distributed from Texas (USA) to Guatemala, but its presence is larger in Mexico. Best known as Ahuehuete tree, which comes from Nahuatl "atl" which means water and "huehuetl" which means old or grandfather, so the whole meaning is "old" of the water" [29–31]. The reason to choose this species, in addition to being abundant from North America to South America, this tree species has many similar characteristics to the species of *Taxus*, which makes it a potential source of Taxol.

The plant material from *Taxodium mucronatum* was collected by members of the Biotechnology laboratory of the Technological Institute of Celaya, from the community of Chamacuaro located on the outskirts of the municipality of Salvatierra in the state of Guanajuato, on the banks of the Lerma river. We proceeded to separate the plant material (branches, fruits and leaves), then dried under pressure and room temperature.

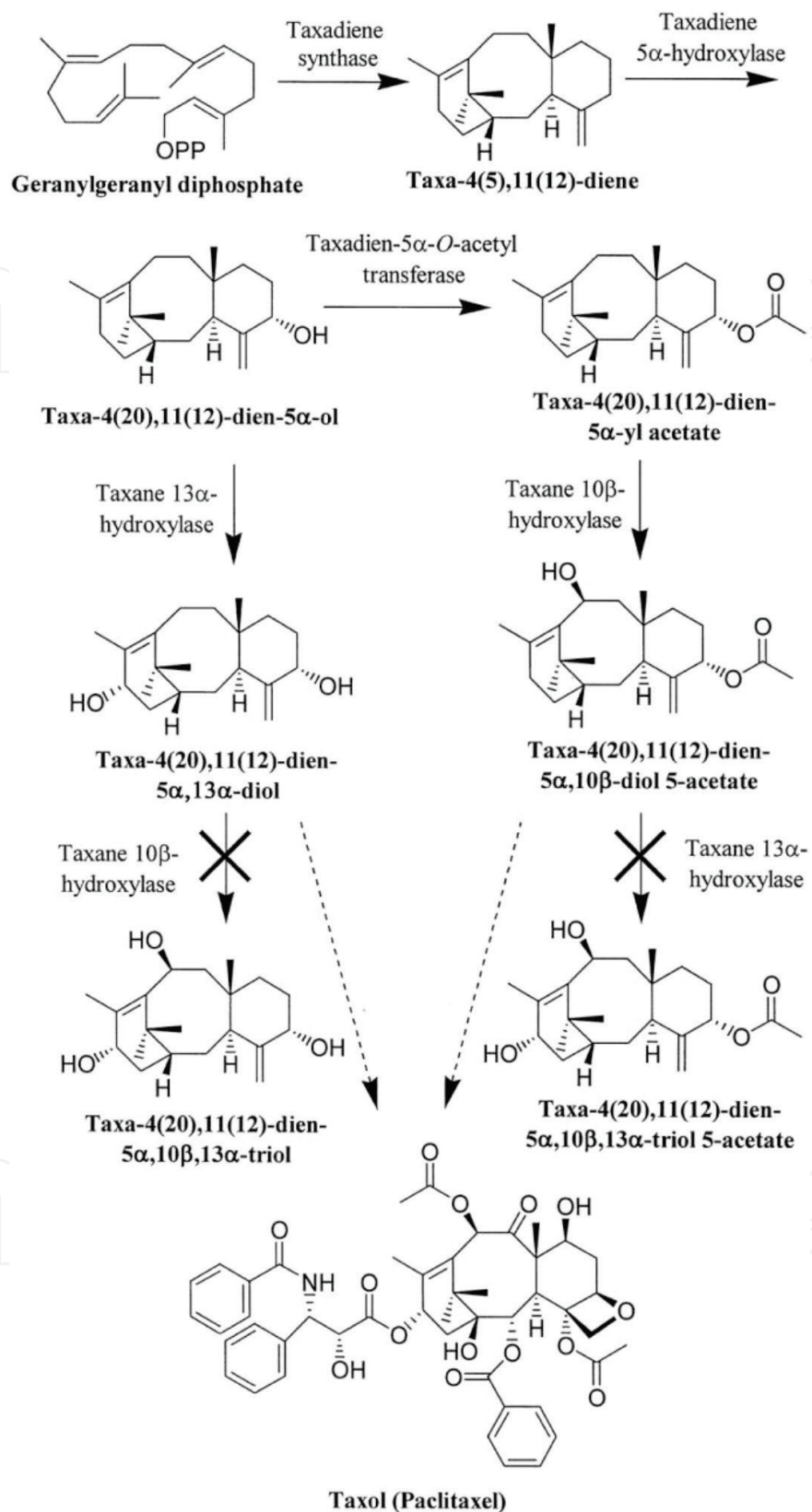


Figure 6. Taxol biosynthetic pathway.

Species of <i>Taxus</i>	Trivial name	Part of the tree	Taxol (% of dry weight)	Year
<i>T. brevifolia</i>	Yew of the pacific	Cortex	0.0075–0.01	1986
<i>T. brevifolia</i>	Yew of the pacific	Leaves	0.0081	1992
<i>T. wallichiana</i>	Yew of the Himalayas	Cortex	0.0108	1981
<i>T. baccata</i>	European yew	Cortex	0.0165	1984
<i>T. baccata</i>	European yew	Leaves	0.0088	1992
<i>T. cuspidata</i>	Japanese shuffle	Leaves	0.0077	1992
<i>T. media</i>	Yew of Sumatra	Leaves	0.0056	1992
<i>T. floridiana</i>	Florida yew	Leaves	0.006	1992
<i>T. globosa</i>	Mexican Tejo	Cortex	0.0085	2000
<i>T. globosa</i>	Mexican Tejo	Leaves	0.013	2000
<i>T. globosa</i>	Mexican Tejo	Stem	0.0064	2000
<i>T. globosa</i>	Mexican Tejo	Sheet	0.0121	2003

Table 3. Obtaining Taxol from the different species of *Taxus* [6].

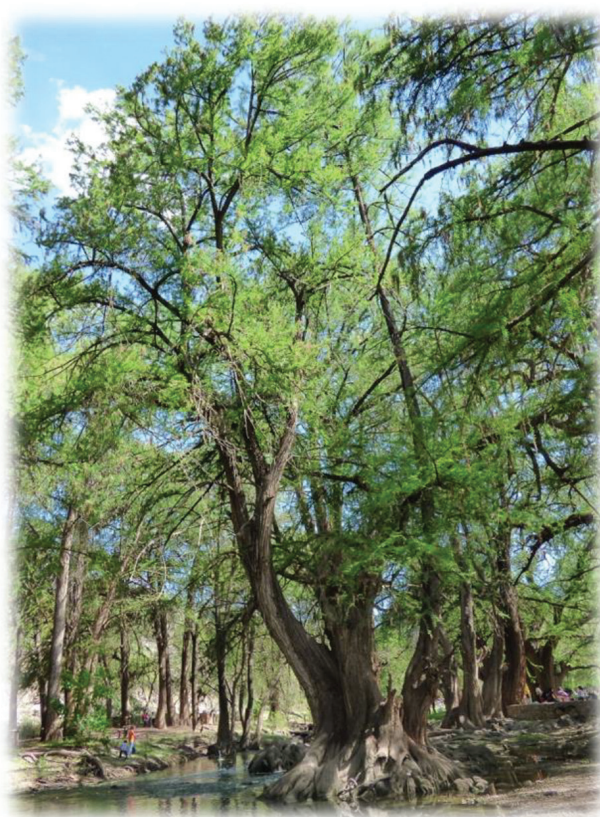


Figure 7. *Taxodium mucronatum*.



Figure 8. Fruits and leaves of *Taxodium mucronatum*.

They were used 300 g of dry leaves of *Taxodium mucronatum*. Milled with methanol, which were later packed for extraction in a glass column, where methanol was passed until the material was used up.

The study was also carried out with fruit, for which 467 g of fresh fruits of *Taxodium mucronatum* (**Figure 8**) were milled with methanol, which were subsequently packed for extraction in a glass column, where methanol was passed until the material was exhausted. The extractives were evaporated at reduced pressure in a rotary rotator at a temperature of 50°C.

5.4. Column chromatography

Column chromatography was used to separate the compounds. For this purpose, 1 g of sample was taken as a result of the rotations of the extracts and placed in a column (measures 20 cm long, 3 cm in diameter), prepacked with 30 g suspended in hexane. Silica gel was used to complete the separation of the components of the sample.

Three solutions were prepared at different proportions of hexane and ethyl acetate. The first solution containing 40 ml of hexane plus 10 ml of ethyl acetate, the second 20 ml of hexane plus 20 ml of ethyl acetate, and the third 10 ml of hexane plus 40 ml of ethyl acetate. To pack the column, 30 g silica gel were mixed with 100 ml of hexane, then poured into the column. Hexane is passed through the column to have a uniform packing. Then it was proceeded to repeat this operation several times to achieve a homogeneous packing. 3 ml of hexane was left on the surface of the silica, and then the sample was prepared. It is homogenized with 2 g of silica and rotavapor for a few minutes to evaporate solvent residues, and is added little by little to the column, leaving at least 1 ml of hexane remaining on the surface. Elute with the prepared solutions. Collect in a flask each fraction of the column (different color layers), evaporate each of the fractions in a rotavapor under reduced pressure and at a temperature of 50°C. Seven fractions of each of the columns were obtained for both the fruits and leaves. These were analyzed by thin-layer chromatography and HPLC.

5.5. Thin-layer chromatography

The technique of column chromatography helps us to know if the fractions recovered from the column chromatography are pure, otherwise it tells us how many components we

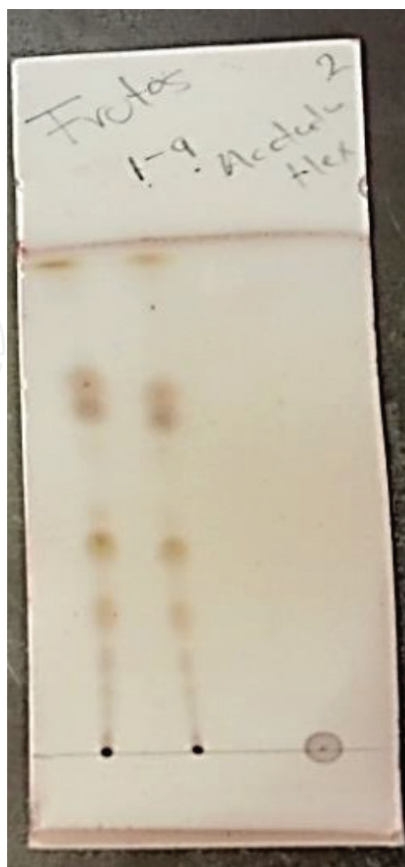


Figure 9. Chromatographic plates of the fruit extract.

have in these fractions. Column chromatography also helps us to know the polarity of our compounds present in the sample, and therefore it is necessary to make several different solvent systems, this is vitally important because the good or bad purification of our compounds will depend on it. For thin-layer chromatography, the following components were used: A stationary phase: Silica gel 60F 254 Merk 0.25 mm thick, with a ceric sulfate developer solution, and the following solvent systems were tested: chloroform-methanol (7:3) (9:1) (1:9) (5:5), hexane-ethyl acetate (9:1) (5:5) (1:9), methanol-acetone (4:6) (9:1) (3:9), chloroform-ethyl acetate (3:7), methanol-hexane (3:7), chloroform-acetone (9:1), and ternary systems were also tested: ethyl acetate-chloroform-methanol (2:7:1), acetone-chloroform-methanol (2:7:1).

In **Figure 9**, the separation of components of the fruit extract is observed, where at least six components are distinguished, the solvent mixture was hexane/ethyl acetate in a 9:1 ratio. **Figure 10** shows the results for the leaf extract, where at least three main components are identified.

In **Figure 11**, other plates are shown chromatography's with a different mixture of solvents, without success in the separation of components. Observing the results of the chromatography, we know that most of our compounds have an intermediate polarity, this based on our solvent system that is the best for the separation of compounds. Another interesting fact about our results is that we have several compounds with very similar characteristics, this is deducible because although you can see spots individually (each corresponding to a different compound) they are too close together.

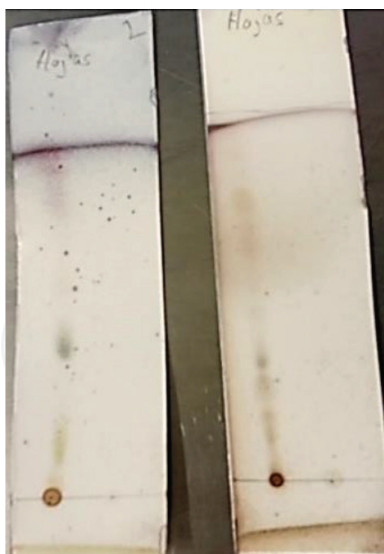


Figure 10. Chromatographic plates of the leaf extract.



Figure 11. Chromatographic plates with other solvent mixtures.

5.6. High-resolution liquid chromatography

The identification of compounds is a task for liquid chromatography of high resolution, as long as our sample meets the necessary characteristics. The extracts obtained by means of column chromatography were analyzed by means of HPLC, in a Varian chromatograph with a C18 column an isocratic development acetonitrile-water was made 70:30 at a rate of 1 ml/min, and the injection volume was 20 μ l. The identification of Taxol, in this case our metabolite of interest, was carried out by means of an external standard from the *Taxus brevifolia* (**Figure 12**), a retention time of 4.65 min is observed.

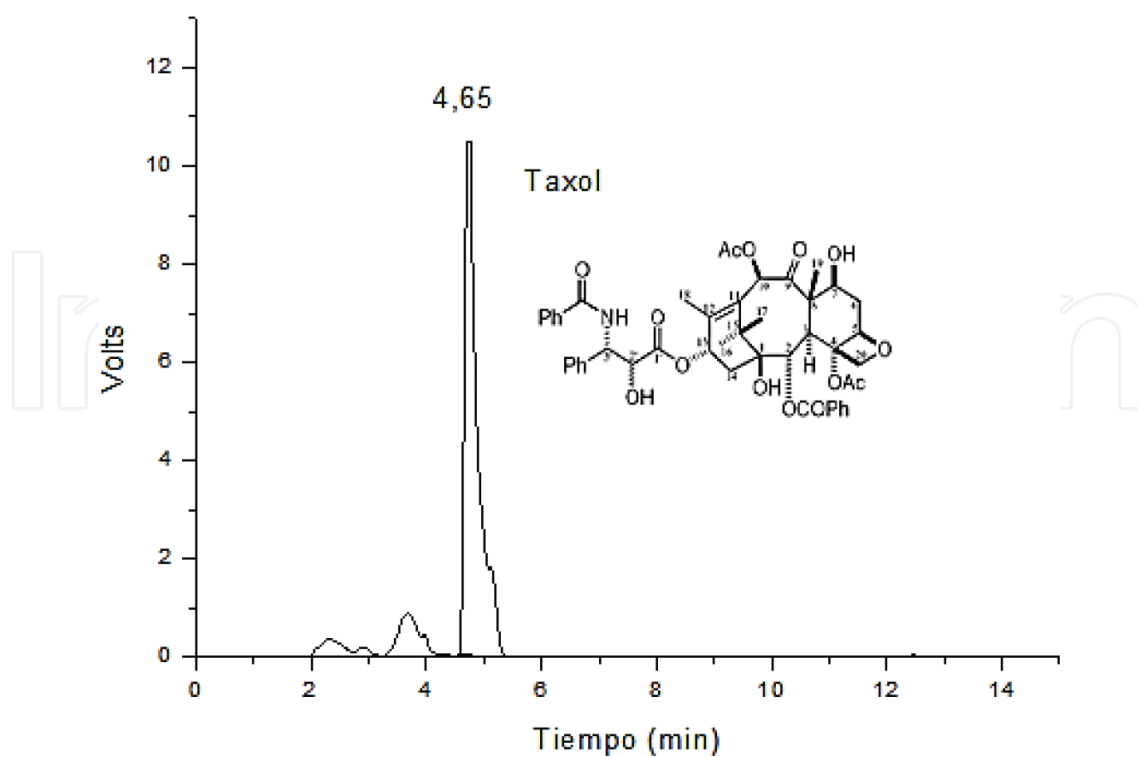


Figure 12. Taxol standard.

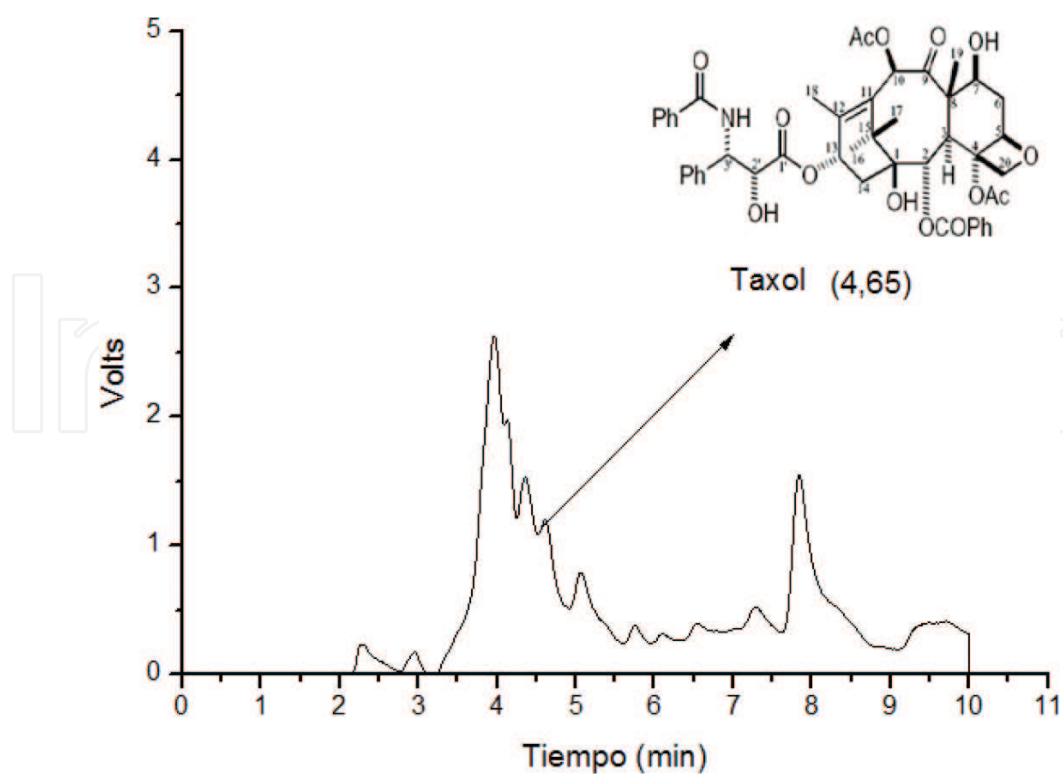


Figure 13. Fraction 4 of the preparative column of silica with leaf extract of *Taxodium mucronatum*.

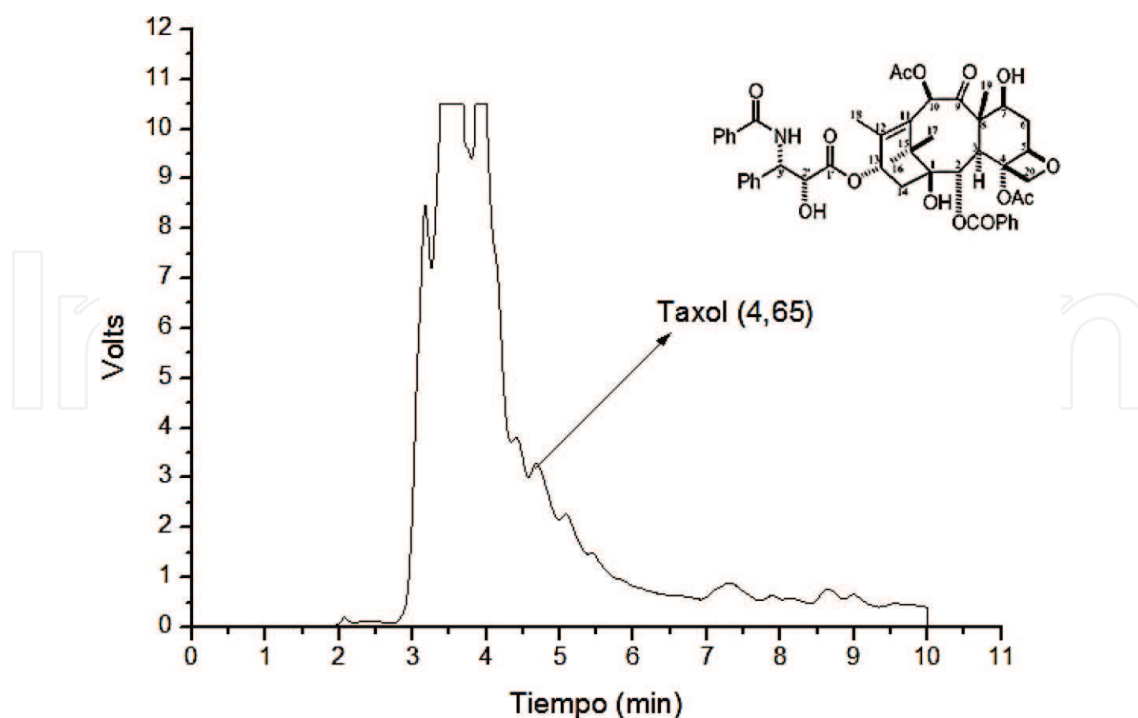


Figure 14. Fraction 5 of the silica preparative column with leaf extract of *Taxodium mucronatum*.

In **Figure 13**, it is the chromatogram of the analysis of fraction 4 of the column of silica gel of 1 g of the leaf extract of *Taxodium mucronatum*, in it a peak is observed with the same retention time with the standard which indicates us Taxol inside the leaves. Observing the distance that exists between the signals that are close to the retention time characteristic of Taxol, one of these signals could also be about a mixture of taxoids. **Figure 14**, corresponding to fraction 5 of the extract of the leaves shows a characteristic retention time of Taxol, but as in fraction 4, it also presents signals with times very close to the retention time of Taxol, which is why we can also deduce that there is presence of other taxoids.

6. Case study 2: alternative for obtaining natural products

Secondary metabolites can also be produced by endophytic fungi, and this feature has opened the door to new research. Some of the most important advantages of this find are that no plant species will be threatened. The process for the production of natural products can be industrialized. Therefore, the following research is about the production of Taxol by means of endophytic fungi of *Taxodium mucronatum*.

The main source of obtaining Taxol until now is the extraction of trees of the genus *Taxus* (Tejo); however, it is estimated that the amount of purified Taxol required to treat only 500 patients with cancer is 1 kg, equivalent to the performance of near of 10 tons of bark or the felling of 700 trees. Therefore, the next step for science is to find new ways to obtain this drug. Some of the sources to obtain this medicine, are the semi-synthesis, from other Taxanes being the most used the 10-Deacetylbaconitina III obtained from the leaves of *Taxus*, the disadvantage that this method has, is the low yield and the high cost; by total synthesis of plant cell cultures of *Taxus* and cultures of microorganisms such as fungi and bacteria (**Table 4**). The production

Isolation source	Fungus	Concentration (µg/L)	Year
<i>T. brevifolia</i>	<i>T. andrene</i>	0.024–0.025	1993
<i>T. wallaiciana</i>	<i>Pestalotiopsis. Microespora</i>	60–70	1996
<i>T. baccata</i>	<i>Monochaetia</i> sp.	0.102	1996
<i>T. baccata</i>	<i>Fusarium lateritium</i>	0.13	1996
<i>T. cuspidata</i>	<i>Alternaria</i> sp.	0.157	1996
<i>T. cuspidata</i>	<i>Pestalotiopsis. Microespora</i>	0.268	1996
<i>T. wallaiciana</i>	<i>Pestalotiopsis. Microespora</i>	0.5	1996
<i>T. Sumatrana</i>	<i>Phitomyces</i> sp.	0.095	1996
<i>T. baccata</i>	<i>Pestalotia bicilia</i>	1.081	1996
<i>Wollemia nobilis</i>	<i>P. guepinii</i>	0.481	1997
	<i>Pestalotiopsis. Microespora</i>	1.487	1998

Table 4. Production of Taxol by endophytic fungi [32].

of Taxol, by means of microorganisms, represents a potential source of Taxol; due to its multiple advantages among them that no plant species is affected, the process is reproducible and controllable, which is important for its industrial scaling.

For this reason, the main purpose of this project is to isolate and select strains of endophyte microorganisms capable of producing Taxol and also develop a biotechnological process that allows the production.

The proceeding of isolating the fungi associated with *Taxodium mucronatum* was made by getting a collection of samples of microorganisms than was carried out in test tubes with nutritious broth, at room temperature. A short and deep cut was made in the bark of the selected tree; in the cut with an applicator, three samples were taken. Later in the laboratory, the preparation of five culture media was made: Czapeck medium, Sabourod, PDA, YPD, Agar Plate count; microorganisms were seeded from each in tubes in five different culture media. This was realized with the purpose of observing in which agar these microorganisms grow better and make a cellular differentiation.

Then proceeded to cultivate the fungi; using 2 ml of saline containing the contents of a fungal Petri dish in 250 ml of PDA were inoculated and incubated in a shaker at 250 rpm and 27°C for 7 days. After this time, the culture broths were filtered to remove the biomass, and extractions of each Erlenmeyer flask were carried out with ethyl acetate. The organic phase was separated and dried with anhydrous sodium sulfate and filtered; then the organic phase was evaporated in a rotatory evaporator until the solvent was removed at 50°C and in vacuum. The extract was resuspended in 1 ml of acetonitrile HPLC grade with 0.01% acetic acid to avoid esterification of Taxol and was placed in Bakelite tubes and kept in refrigeration at 4°C for future analysis.

6.1. High-resolution liquid chromatography

All the extracts were analyzed in HPLC, in a Varian chromatograph 8090 mod. (USA) with a C18 column under isocratic conditions and in an 80:20 acetonitrile-water mixture at a flow rate of 1 ml/min, the injection volume was 20 µl. The identification of Taxol was carried out

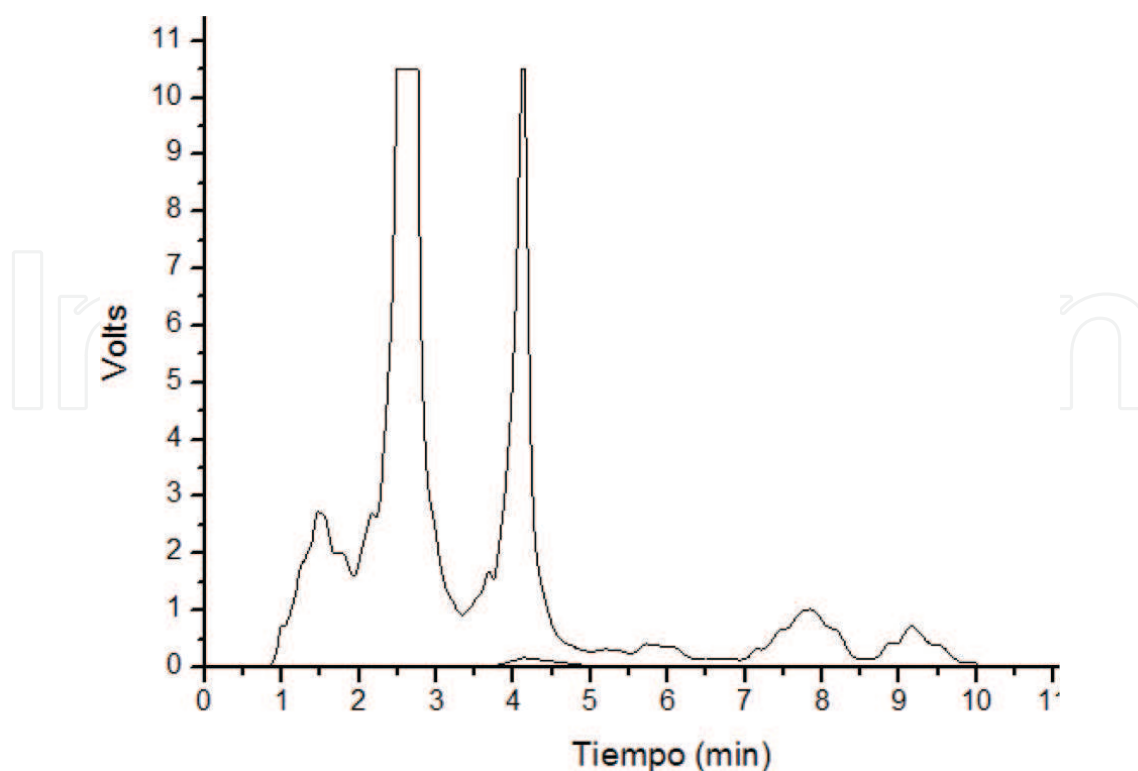


Figure 15. Chromatogram of the culture extract of strain 1.

by means of a Taxol standard of sigma Aldrich (Toluca, México) from *Taxus brevifolia* which presented a retention time of 4.65 min.

Figure 12 shows the chromatogram corresponding to the Taxol standard of Sigma Aldrich from *Taxus brevifolia*. **Figure 15** is the chromatogram corresponding to strain 1, and this strain is matter of interest because it produces very few natural products, although it does not show any time characteristic of Taxol. Therefore, the purification process would be easier and identify these products. **Figure 16** shows the chromatogram corresponding to strain 17, in which we observe a signal with the same retention time as the characteristic signal of Taxol.

Based on our chromatogram of the extract of strain 17 and identifying Taxol in our extract, we conducted another experiment in which we enriched our culture medium using brown sugar, which contains different types of salts as well as different carbon sources (sucrose, glucose, etc.). In the chromatogram of **Figure 17**, we observe the effect of enrichment of the medium, our corresponding to Taxol increases its area and volume; however, the rest of our compounds also increase significantly. The result of Taxol production was confirmed by adding 0.5 ml of standard as internal control To 0.5 ml of sample **Figure 18**.

In the identification of the Taxol-producing endophytic fungus, lacto phenol blue staining was performed for its microscopic morphological structure. **Figure 19** shows the endophytic fungus with Taxol production capacity, in the image, the growth in petri box and its microscopic view is appreciated.

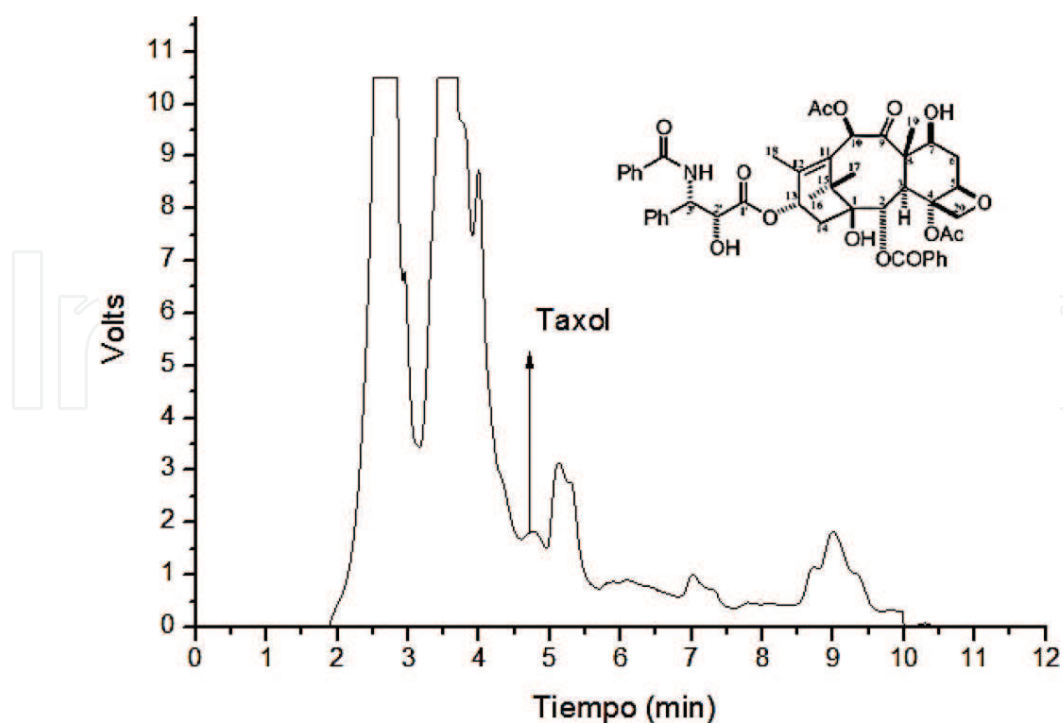


Figure 16. Chromatogram of the culture extract of strain 17 in potato broth.

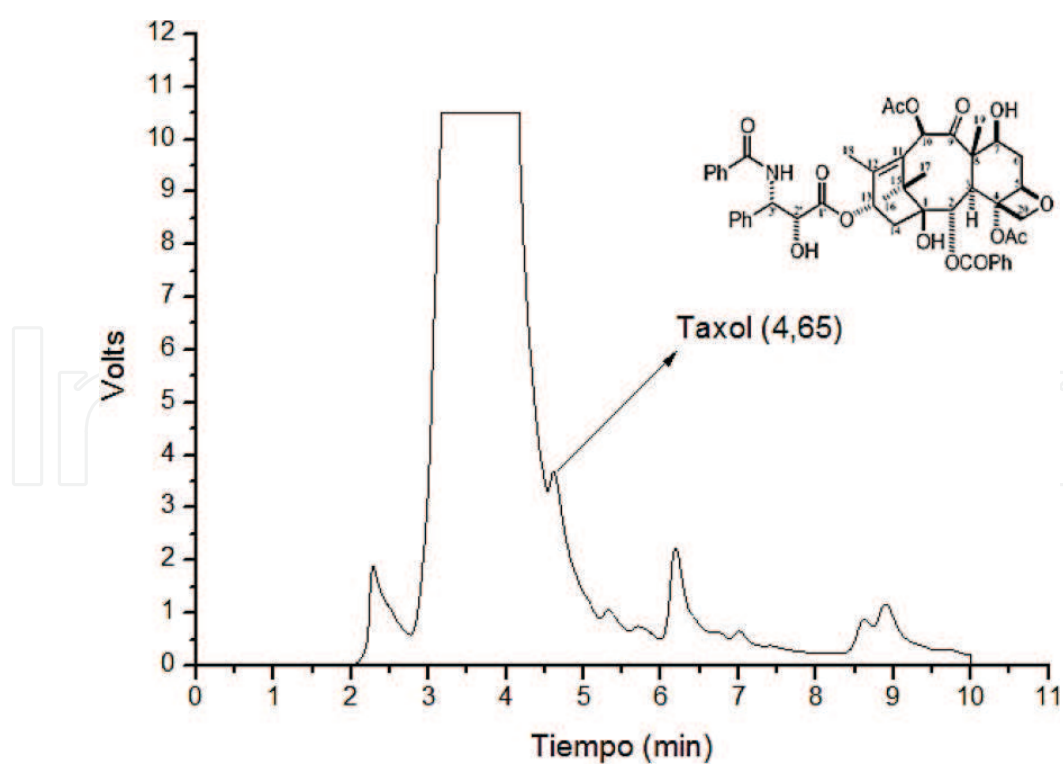


Figure 17. Chromatogram of extract of strain 17.

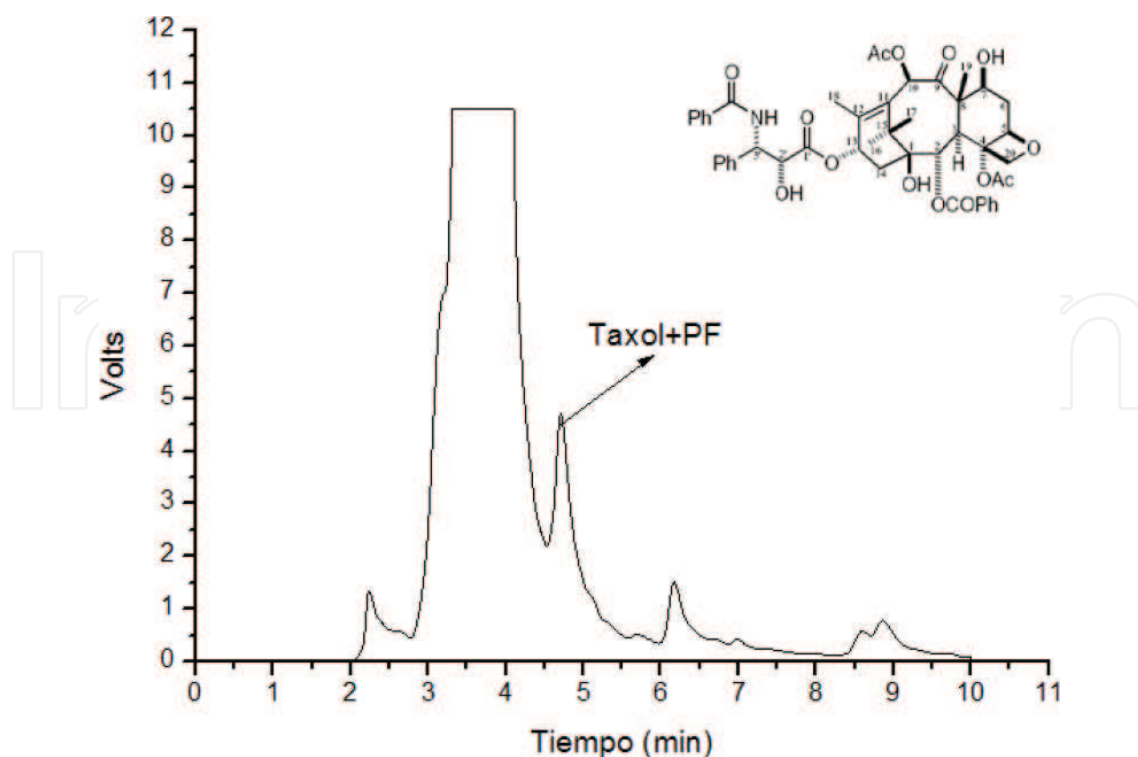


Figure 18. Chromatography of the extract of strain 17 with the addition of 0.5 ml of standard.

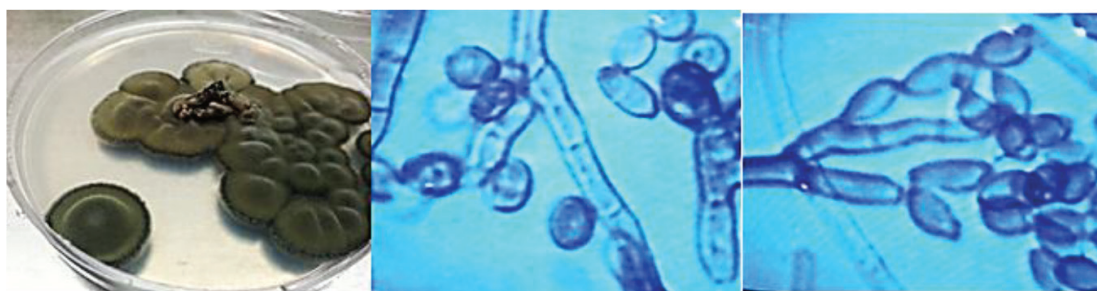


Figure 19. Image of the Taxol producing endophytic fungus.

7. Conclusion

Natural products (secondary metabolites) are important sources for the cure against many of the diseases that humans are currently fighting. However, it is necessary to conduct research whose objective is the production of natural products through biotechnological routes, protecting plant species. One of the clear examples is Taxol, which can be obtained through its endophytic fungi; the production of Taxol was achieved by the submerged fermentation of one of the native strains of *Taxodium mucronatum*, identifying it by means of high-resolution chromatography. In the production of Taxol by fermentation it is possible that the processes must be sought by which other secondary metabolites can be obtained for conservation of plant species. As is the case of Taxol, there are other substances that can be obtained by fermentative routes, always protecting our plant species.

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