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New insights into the antifungal activity of *Taxus baccata* L.

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Abstract

Taxus baccata L., yew, is dioecious, small to medium-sized evergreen tree, native to Europe, Africa and Asia, but it is cultivated worldwide because of its ornamental features. It is long known that all parts of yew (except for aril) are toxic, due to the alkaloid taxine. Nevertheless, some parts of yew tree can be used to treat cancer and as antimicrobial agents. Unlike its antibacterial properties, antifungal activity of *T. baccata* is poorly investigated. In this research, antifungal activity of yew methanolic and aqueous extracts was tested against *Candida albicans* ATCC 10231, Aspergillus brasiliensis ATCC 16404, and *Ascosphaera apis* MUCL 30769, through the agar well diffusion method. Leaves, bark and reproductive structures extracts were prepared separately for male and female plants. Unlike the aqueous, methanolic extracts caused variable degree of fungal growth inhibition. The strongest inhibition was observed in the activity of the aril extract against *A. brasiliensis* and *C. albicans*, with the activity of the male bark extract against *A. apis* following close. Considering the emerging multidrug resistance in *C. albicans*, an interesting finding is the inhibition of this species by all tested methanolic extracts, which is significantly stronger in comparison to the inhibition by standard antimycotic solution. According to the available data, male reproductive structures of this species were not tested earlier for their antifungal activity, and our study showed high level of antifungal activity of methanolic microstrobili extract. The realized research indicates great antifungal potential of *T. baccata*, which should be further confirmed by more comprehensive studies.

Introduction

Genus *Taxus* L. (Taxaceae), yew, is widely distributed in Europe, North America, Eastern Asia and Asia Minor, and it comprises eight species and two hybrids worldwide (Farjon et al., 2019). Great attention is focused on the genus *Taxus*, due to the isolation of paclitaxel, a unique diterpene taxoid from the bark of the *T. brevifolia* Nutt. (Wani et al., 1971), that is widely used as anticancer agent (Erdemoğlu et al., 2003). *Taxus baccata* L. is a dioecious, evergreen and widespread shrub, commonly used for ornamental landscaping (Kucukboyaci & Şener, 2010). *T. baccata* is well

known for its poisonous properties. All plant parts with the exception of the aril, which envelopes seeds, contain toxic taxine alkaloids (Anadón et al., 2018). This is the main reason why this species has rarely been documented as a remedy in traditional medicine. Nevertheless, there are some reports that yew leaves have been used as abortifacient, antimalarian, antirheumatic and for bronchitis, while dried leaves and bark have been used against asthma (Küpeli et al., 2003). Recent studies show that lignans, specific secondary metabolites isolated from *T. baccata*, possess several biological activities: antiviral, antifungal, antibacterial, anticancer, antioxidant and anti-inflammatory (Kucukboyaci & Şener, 2010). Different parts of *T. baccata* are rich in bioflavonoids such as: sciadopitysin, ginkgetin, kayaflavone and amentoflavone (Krauze-Baranowska & Wiwart, 2003), and while dimeric flavones possess antiviral, antibacterial and antimycobacterial properties (Erdemoğlu et al., 2003), antifungal activity is still poorly investigated. Literature survey revealed some data regarding antifungal activity of *T. baccata* (Erdemoğlu & Şener, 2001; Krauze-Baranowska & Wiwart, 2003; Kucukboyaci & Şener, 2010), as well as of *T. cuspidata* Sieb. et Zucc. var. *nana* Rehder. (Tachibana et al., 2005) and *T. wallichiana* Zucc. (Nisar et al., 2008). Emergence of new, multidrug-resistant pathogens, as well as undesirable side effects of antimicrobial drugs have triggered tremendous interest in the search for new antimicrobial agents of plant origin (Bernaitis et al., 2013). The main goal of this investigation was to evaluate antifungal activity of methanolic and aqueous extracts of *T. baccata*, obtained from different vegetative and reproductive plant structures.

Materials and methods

Plant material

The plant material of female and male yew individuals was collected in Sarajevo, Bosnia and Herzegovina and determination was carried out in Laboratory for Plant Systematics, Department of Biology, Faculty of Science, University of Sarajevo. The plant material was separated into leaves, bark and reproductive structures (arils and microstrobili) and such separated material was dried in dark, in ventilated room at the ambient temperature.

Preparation of extracts

For this investigation of antifungal activity, methanolic and aqueous extracts were prepared from dried, finely ground plant material. For methanolic extracts 100 mg of dry plant material was soaked in 10 ml of 80% methanol, and the extraction lasted for 24 hours. Aqueous extracts were prepared by boiling the 100 mg of herbal powder in 10 ml of distilled water. The obtained extracts were dried, weighted and dissolved to the final concentration of 1 mg/mL for antifungal assays. All extracts were stored in dark, at +4 °C.

Investigation of antifungal activity

Antifungal activity of *T. baccata* methanolic and aqueous extracts was tested against three reference strains of fungi: *C. albicans* ATCC 10231, *A. brasiliensis* 16404 and *A. apis* MUCL 30769, using agar-well diffusion method (Balouiri et al., 2016). Fungal species were cultured at 37 °C on Sabouraud Dextrose Agar (HiMedia Laboratories Pvt.Ltd., India) for 24-48 hours. The inoculums harvested from agar plates were prepared in sterile saline solution and according to the National Committee for Clinical Laboratory Standards (2015), adjusted to 0.5 McFarland scale (~1.5 x 108 CFU/mL).
Prepared fungal inoculums (100 µl) were spread over the entire surface of plates with growth medium and left for 15 minutes at room temperature to achieve total absorption. Investigated extracts and control samples (50 µl) were then transferred into the wells of inoculated agar plates. Plates were incubated for 24-48 hours at 37 °C. Antifungal activity of the tested extracts was evaluated based on diameter (mm) of inhibition zones, which result from extract diffusion in the medium and inhibition of fungal growth. Antibiotic-antimycotic solution (10.000 units penicillin, 10 mg streptomycin and 25 µg amphotericin B per mL; Sigma-Aldrich) was used as positive control, while methanol and distilled water were used as negative controls. Positive control was prepared according to the manufacturer's recommendations. All the tests were performed in three replications and the mean values (±SD) were calculated.

Statistical analysis

All values are presented as mean ± standard deviation (SD). Descriptive statistical analyses were conducted using Microsoft Office 2013 Excel (Microsoft Corporation, Redmond, USA). Data were further analysed by using one-way ANOVA and post hoc LSD test (STATISTICA 10; StatSoft. Inc.), with significance level set at $p < 0.05$.

Results and Discussion

In this study, antifungal activities of methanolic and aqueous extracts obtained from leaves, bark and reproductive structures of *T. baccata* were tested against three fungal species. Antifungal activity of negative controls: 80% methanol and distilled water was not observed. Aqueous yew extracts did not inhibit fungal growth while methanolic extracts exhibited varying degree of antifungal activity (Table 1). It is known that methanol as a solvent gives better results in extraction of bioactive compounds, when compared to other solvents (Altemimi et al., 2017). Also, in antimicrobial surveys, methanolic extracts usually exhibit stronger antimicrobial activity then aqueous extracts (Ghosh et al., 2008, Mahesh & Satish, 2008).

The widest zones of inhibition were observed in *A. brasiliensis* (23.33±2.08 mm) and *C. albicans* (22.67±0.58 mm) treated with aril methanolic extract (Table 1.). Arils are soft, fleshy, bright red, berry-like structures that represent a specialized outgrowth of a seed, and are the only non-toxic parts of the yew plant.

Recent study (Siegle & Pietsch, 2018) reported that arils contain bioactive compounds: 3,5-dimethoxyphenol, 10-deacetylbaccatin III, baccatin III, cephalomannine, taxol A and taxinine M. Considering that these compounds are precursors of drugs derived from *T. baccata*, it is possible that they are also responsible for antifungal activity of aril methanolic extracts. Following an extensive literature survey, no data on the study of antifungal activity of *T. baccata* microstrobili extracts were found. Microstrobili are male cones of *T. baccata*, which are yellow, globose and pedunculate. Each male cone is composed of 6-14 peltate, radially extending microsporophylls, with 6-8 microsporangia i.e. pollen sacs (Predan & Toniuc, 2009). It has been proven that *T. baccata* pollen contains bioactive diterpenic alkaloids belonging to taxine and taxoids (Vanhaelen et al., 2002). Of all investigated fungi, the most sensitive species to the microstrobili methanolic extract was *A. brasiliensis*, with inhibition zones of 19.00±3.46 mm (Table 1.). In this research *C. albicans* was the most sensitive species in terms of yew extracts antifungal activity. All investigated extracts showed strong antifungal activity against *C. albicans* (Table 1.), with inhibition zones ranging from 14.67±1.15 mm (microstrobili) to 22.67±0.58 mm (aril). This result is particularly intriguing, since *C. albicans* is a multidrug-resistant pathogen (Arendrup & Patterson, 2017), and even in this study, positive control caused significantly narrower inhibition zones (Table 1.). In addition, the tested antibiotic-antimycotic solution showed no inhibitory effect

Table 1. Antifungal activity of investigated *Taxus baccata* extracts

Methanolic extracts	Tested fungal species		
	Aspergillus brasiliensis	*Candida albicans*	*Ascosphaera apis*
♀ Leaf	14.33±3.21 ^{b,c}	18.33±3.79 ^{b,e}	11.00±2.64 ^{b}
♀ Bark	20.67±2.08 ^{a,e}	18.33±2.31 ^{b,c,e}	11.66±2.08 ^{b}
Aril	23.33±2.08 ^{a}	22.67±0.58 ^{a}	NI ^{c}
♂ Leaf	10.33±0.58 ^{c}	18.67±1.53 ^{b,c,d}	19.67±4.93 ^{a}
♂ Bark	15.00±2.00 ^{b,d}	19.33±1.53 ^{a,b,c,d}	20.83±0.29 ^{a}
Microstrobili	19.00±3.46 ^{d,e}	14.67±1.15 ^{e}	18.00±0.50 ^{a}
Positive control	0.00±0.00 ^{f}	4.90±0.36 ^{f}	17.00±0.50 ^{a,b}

Results are mean diameter in mm ± SD (n=3). NI = No inhibition. 80% methanol = NI.
Values not sharing the same letters differ significantly at $p < 0.05$ after *post-hoc* LSD test.

against *A. brasiliensis*, while all investigated methanolic extracts of *T. baccata* successfully inhibited growth of this fungal species (Table 1.). Antifungal drugs in general can be divided into four groups: polyenes (1), nucleic acid synthesis inhibitors (2), ergosterol biosynthesis inhibitors (3) and echinocandins (4), but clinical resistance has been observed for all classes of antifungals (Marie & White, 2009). Microbiological resistance of a fungi to an antifungal agent can be primary (intrinsic) or secondary (acquired). While primary resistance is found naturally among certain species without prior exposure to antifungal agents, secondary resistance develops among previously susceptible strains after exposure to antifungal agent, and is usually dependent on altered gene expression (Kanafani & Perfect, 2008). Mechanisms of antifungal drug resistance include resistance genes, transcriptional regulation of drug resistance and some alternative mechanisms such as: overexpression of PDR16 gene (Liu et al., 2007; Znaidi et al., 2007), aneuploidy (as a way of increasing gene copy number by whole chromosome duplication or other genomic rearrangements) which serves as an adaptive response when cells are stressed (Selmecki et al., 2008), and formation of biofilms (Nett et al., 2007; Seneviratne et al., 2008). The resistance to antifungal agents is mainly the result of specific gene mutations, and many of them have so far been identified. Among the most investigated are point mutations in Erg11p that result in amino acid substitutions in lanosterol demethylase (Mellado et al., 2007); several mutations in the glucan synthase gene FKS1 (Cleary et al., 2008; Garcia-Effron et al., 2008); and mutation of the genes involved in 5-FC toxicity (Papon et al., 2007). When discussing transcriptional regulation of resistance, mechanisms that should be mentioned are overexpression of ERG11 and other ergosterol biosynthetic genes due to the Upc2p - the major regulator of ergosterol biosynthesis (White & Silver, 2005). Furthermore, fungal efflux pumps could mediate drug resistance through different inducible and constitutive pathways (Sanglard & White, 2006). In general, *A. apis* was the most resistant fungal species in this investigation, which could be partly explained by its morphology. *A. apis* is spore-forming, filamentous fungus, with mature spores tightly packed inside the spherical spore balls. More than ten spore balls could be wrapped and formed spherical, nearly hyaline spore cyst. The spore wall is two-layered, and chitin is the major component of the spore wall. Furthermore, in addition to the typical fungal spore organelles, an unknown structure positioned close to the inner spore wall, and covering almost the entire wall area, was recently discovered in this fungus (Li et al., 2018). *A. apis* was the most susceptible to the male bark extract (20.83±0.29 mm), while the aril extract showed no antifungal activity (Table 1.). *T. baccata* methanolic leaves extracts were efficient against all three investigated fungal species, especially in case of the *A. apis* inhibition (19.67±4.93 mm) by the male leaves extract (Table 1.). Investigation of Krauze-Baranowska & Wiwart (2003) showed that methanolic extracts made from *T. baccata* leaves contain biflavones: bilobetin, amentoflavone, 4-O methyl amentoflavone, 7-O methyl amentoflavone, sciadopitysin and ginkgetin. These compounds significantly inhibit the growth of fungal sporae and germ tubes. Furthermore, Patel et al. (2009) reported lignans and flavonoids in the yew leaves extract, which are the potential antimicrobial agents. Extracts made from leaves, bark and heartwood of *T. baccata* successfully inhibited some other fungal species such as: *Trichophyton longifusus, Micosporum canis,* and *Fusarium solani*, but failed to inhibit of the growth of investigated *Candida* and *Aspergillus* species (Nisar et al., 2008). Contrary to these findings, the realized study accomplished significant inhibition of *C. albicans* and *A. brasiliensis* by all investigated yew extracts. Erdemoğlu & Şener (2001) reported successful inhibition of *Nigrospora oryzae*, *Epidermophyton floceasum*, *Curvularia lunata* and *Pleuralus astreatus* with *T. baccata* extracts, which is attributed to the presence of the lignan derivative taxiresinol and 3'-demethylisolariciresinol (Kucukboyaci & Şener, 2010). Overall results of this investigation showed that methanolic extracts obtained from female yew plant exhibited greater antifungal potential when compared with the extracts made from male individuals (except in *A. apis*). It is known that the sex of yew trees may have a significant impact on the content of bioactive compounds taxanes. Females of dioecious plants usually have greater reproductive effort in

comparison to males, and this phenomenon was found in the case of yew. The greater reproductive effort by females results in the intensification of gas exchange and consequently significantly higher concentrations of carbon-based secondary metabolites, including taxanes (Iszkuło et al., 2013).

Conclusions

Taxus baccata is well known plant, mainly for its poisonous and ornamental features. Although it is rarely mentioned in traditional (folk) medicine, some studies do confirm its bioactive properties. However, antifungal effects of yew are poorly investigated, and this study highlighted significant antifungal activity of *T. baccata* methanolic extracts, obtained from leaves and bark of female and male individuals, as well as from reproductive structures: arils and microstrobili. The most sensitive fungal species was *C. albicans*, while the most resistant was *A. apis*. Realized investigation represents an original approach in obtaining extracts of diecious plants with potential antimicrobial activity. Also, to the best of our knowledge, this is the first time that *T. baccata* was investigated in a way of separating extracts per plant part and sex. Considering the fact that tested fungal species exhibit multidrug-resistance, successful inhibition by obtained yew extracts is noteworthy. With regard to the poisonous activity of *T. baccata*, these results should be further confirmed by more comprehensive studies, especially in terms of toxicity and genotoxicity. Also, wider use of *T. baccata* extracts inevitably implies determination of adequate therapeutic concentrations.

Conflict of interest

Authors declare no conflict of interest.

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