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Original Article

An *in silico* approach for evaluating the antitumor and epigenetic modulating potential of phenolic compounds occurring in edible and medicinal mushrooms

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Abstract

Introduction: As part of cancer research, mycotherapy is a relatively new and promissory source of agents with immunomodulating and antitumor properties. Ongoing research projects are aiming to provide mushrooms as a new generation of "biotherapeutics". In addition to high-molecular weight polysaccharides, efforts should be made to find new anticancer drugs using low-molecular weight secondary metabolites, e.g. phenolic compounds that can inhibit or trigger specific biochemical signals leading to cancer.

Methods: An *in silico* approach based on the structural similarity of low-molecular weight myco-compounds (phenolics) with respect to antitumor substances and molecules with modulatory effects on epigenetic events was used. For the screening of mushroom molecules with potential regulatory effects on epigenome (obtained on Web of Science, August 2015), the enzymes histone acetyltransferase (HAT), histone deacetylase (HDAC) and DNA methyltransferase (DNMT) were chosen as targets. Similarity analysis were performed with the software Saranea. Moreover, the determination of the chemical structural similitude between phenolic compounds of *Pleurotus ostreatus* (oyster mushroom) and antitumor reference compounds was carried out with the software Power MV 0.61. Tanimoto's coefficients (Tc) similar or higher to 0.90 were considered as significant.

Results: Seven mushroom compounds with high structural similarity to reference substances with modulatory activity on epigenetic events (Tc ≥0.90) were identified: 5 with a potential effect on histone acetylation/deacetylation, and 3 acting on the enzyme DNMT. Twenty antitumor reference compounds showed structural similarity to 3 phenols occurring in *P. ostreatus*, corresponding the largest number to protocatechuic acid and the flavonoids myricetin and naringin. According to its similarity to the antitumor compounds, they would act as DNA antimetabolites, antimitotic, and/or alkylating agents.

Conclusion: It seems feasible to harness the natural pool of mushrooms secondary metabolites and to predict by *in silico* approaches their potential modulatory effects on epigenetic events and antitumor activity, in special phenolics occurring in *P. ostreatus*. This is an exciting advance for developing nutraceuticals/ cosmeceuticals and innovative drugs.

Keywords: Antitumor, Epigenetic, *In silico,* Mushrooms, Phenolics, *Pleurotus ostreatus*

Introduction

Nowadays, cancer has been established as one of 2 leading causes of death worldwide (the other cause involves cardiovascular diseases). These top 2 causes accounted for 46.1% of all deaths in the United States in 2013. Unfortunately, this trend is projected to continue in the future, and according to the World Cancer Report, cancer rates are expected to grow about 50% to 15 million by 2020.[1]

Modern science is still not able to identify accurately the causes of cancer in each individual case, and the origins of most common cancers remain unclear. Recent breakthroughs in cancer genetics, genomics, proteomics and translational research have aided to develop a better understanding of the underlying mechanisms which are contributory in cancer development and progression.

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Increasingly it is being realized that genetic/ epigenetic mutations and loss of apoptosis also mandate a "multi-molecular" perspective for the development of therapies to treat cancer.[2] The extensive alterations of the epigenome including modification of histone proteins and chromatin remodeling complex are some of the vital epigenetic changes relevant to cancer phenotype.[3]

Traditional knowledge is gradually being implemented in medicine and we have witnessed landmark findings which have revolutionized treatment strategies for cancer. Natural products have been major molecular structural resources for drug discovery. Technological advances such as techniques for production of secondary plant metabolites, high-throughput screening and combinatorial synthesis have helped us to explore true potential of natural products in regulation of signaling pathways in different cancers. In particular, the abundance of mutations occurring in the epigenetic regulatory complexes and proteins provide a number of fundamental targets in the field of epigenetic drug discovery.[4,5]

A next challenge will be to determine which adverse epigenomic marks are reversible by specific diets, drugs or lifestyle changes. Numerous botanical species and plant parts contain a diverse array of phytochemicals, which exert health beneficial effects in man by its anti-inflammatory, antioxidant, cardioprotective and cancer preventive, by maintaining immune homeostasis.[6] Chemopreventive nutritional polyphenols (soy, genistein, resveratrol, catechin, curcumin) are currently evaluated for their ability to reverse adverse epigenetic marks in cancer (stem) cells to attenuate tumorigenesis-progression, prevent metastasis or sensitize for drug sensitivity.[7]

In this context, mushrooms are emerging as a vital components of the human diet and have become attractive as a functional food and as a source of drugs and nutraceuticals.[8-10] Fruiting bodies as well as mushroom mycelia have a broad range of bioactive properties. Mushrooms are thought to exert approximately 130 pharmacological functions such as antitumor, immunomodulatory, antigenotoxic, antioxidant, anti-inflammatory, hypocholesterolemic, antihypertensive, antiplatelet-aggregating, antihyperglycemic, antimicrobial, and antiviral activities.[11,12] These pharmacological effects have been demonstrated for many traditionally used mushrooms, including species from genera *Ganoderma, Lentinus (Lentinula), Agaricus, Auricularia, Flammulina, Grifola, Hericium, Pleurotus, Trametes (Coriolus), Schizophyllum, Lactarius, Phellinus, Cordyceps, Inonotus, Inocybe, Tremella,* and *Russula*.[13,14] Many controlled studies have investigated this long list of medicinal actions, thus upgrading mushrooms to today's world of evidence-based medicine.[15]

As part of cancer research, mycotherapy is a relatively new and promissory field as a source of agents with immunomodulating and antitumor properties.[16,17] The bioactive molecules comprise high-molecular weight polysaccharides, and low-molecular weight secondary metabolites.[18] Given that only 10% of mushroom biodiversity has been studied so far, and few of them have been characterized with regard to health benefits, it is likely that new active compounds will be discovered in the future. Particularly in tropical areas, 22%-55% (in some cases up to 73%) of mushroom species have not yet been described.[19]

Though *Pleurotus* genus (oyster mushroom) is the second important mushroom of culinary value, there has been an upsurge in *Pleurotus* research activities in the last 2 decades in view of its biopotentialities. Recent studies on various *Pleurotus* species have shown a number of the pharmacological activities mentioned above.[20,21] In particular, *Pleurotus* spp. is distinguished as important natural resources for immunotherapy, in view of the content of several bioactive compounds able to augment or complement a desired immune response defined as ·host defense potentiators" (HDPs).[22-24] On the other hand, *Pleurotus* mushrooms packed with a wide array of bioactive components are excellent antioxidants and anti-inflammatory agents which may help to prevent the occurrence and aid the treatment of chronic diseases including heart disease and various cancers.[25] Particularly, phenolic compounds were detected in 5 extracts obtained from fruit bodies of *Pleurotus* sp., obtained with solvents of different polarity; however, the highest levels were found in polar extracts (water and ethanol).[26]

The main objective of this study was to evaluate through an *in silico* approach the antitumor and epigenetic modulating potential of phenolic compounds occurring in edible and medicinal mushrooms. The results would provide a framework for further exploration of the chemopreventive potential effects against cancer of mushrooms, in special, *Pleurotus* spp.

Materials and Methods

Screening of Phenolic Compounds with Potential Regulatory Effects on Epigenome

A control group of known epigenome modulating compounds (Set A) was made from a literature review (Web of Science, August 2015) and the Sigma-Aldrich web site http://www.sigmaaldrich.com/life-science/epigenetics/bioactive-small-molecules.html.[27]

The molecules were classified according to the epigenetic event that modulate: (i) DNA methylation by acting on the enzyme DNA methyltransferase (DNMT), and (ii) histones modification with the enzymes histone acetyltransferase (HAT) and histone deacetylase (HDAC) as targets.

Low-molecular weight substances from mushrooms with unknown effects on epigenome –but biologically active- (set B) were also obtained from the past decade findings.

The SMILES codes of the compounds -a fingerprint of their chemical structures- were obtained from the

PubChem database (http://www.ncbi.nlm.nih.gov/pubmed) of the National Center for Biotechnology Information (NCBI, USA).[28]

For the analysis of the structure-function relationship, compounds data were organized for SMILES code, molecular fingerprint, identifier, potency (according to the target). The SMILES codes of the compounds were introduced into the program ChemDraw 7.0.1 (CambridgeSoft Corporation, USA), for obtaining the 2D chemical structures and to save them in the structural format MDLMol. Then, the chemical structures were introduced in the program OpenBabel version 2.3.2 (http://openbabel.org/wiki/Windows-GUI) prior the structural similarity comparison made with the bioinformatic software Saranea v 1.0 (http://www.gnu.org/licenses/gpl-2.0.txt). Tanimoto´s coefficients (Tc) similar or higher to 0.90 were considered as significant. Tanimoto's coefficient is directly proportional to the reliability of the prediction of the antitumor activity based on the chemical similitude.[29]

Screening of the Potential Antitumor Activity of Pleurotus ostreatus Phenolic Compounds

This *in silico* study was encouraged by previous findings of our group related with the *in vitro* antitumor activity of aqueous extracts from both mycelia and fruiting bodies of *Pleurotus ostreatus* on human acute promyelocytic leukemia (NB4) cells,[30] and in human colorectal carcinoma (Caco2), human hepatocellular carcinoma (HepG2) and murine neuroblastoma (N2A).[31] The presence of phenolic compounds have been demonstrated in these extracts.[25,26]

A selection of common phenolic compounds found in the chemical composition of *P. ostreatus* was used for the screening of its potential antitumor activity, comprising one phenolic acid: the protocatechuic acid (3,4-dihydroxybenzoic acid); and the flavonoids: myricetin (3,5,7-trihydroxy-2-(3,4,5-trihydroxyphenyl) chromen-4-one) and naringin ((2S)-7-[(2S,3R,4S,5S,6R)-4,5-dihydroxy-6-(hydroxymethyl)-3-[(2S,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyloxan-2 yl] oxyoxan-2-yl] oxy-5-hydroxy-2-(4-hydroxyphenyl)-2,3-dihydrochromen-4-one).

Reference compounds were taken from Keskin et al,[32] in which 122 compounds with antitumor activity and mechanisms of action are well characterized. SMILES codes of all the compounds were obtained from the PubChem database, and then introduced into the program ChemDraw, for obtaining the chemical structures in MDL SDF format.

The determination of the structural similarity between the antitumor reference compounds and the phenols was carried on with the software PowerMV 0.61 (National Institute of Statistical Sciences, http://www.niss.org/PowerMV). The 2D chemical structures of the 4 phenolic compounds were introduced into the program, in order to select the ten antitumor reference compounds of higher structural similitude to each mushroom phenolic acid and/or flavonoid. The antitumor reference compounds were classified into six classes considering their mechanisms of action: alkylating agents, inhibitors of topoisomerase I, inhibitors of topoisomerase II, RNA/DNA antimetabolites, DNA antimetabolites and antimitotics.

Results and Discussion

It is likely that understanding and manipulating the epigenome by prevention and therapy with individualized tailoring of optimal epigenetic diets or supplements is conceivable. Medical benefits of dietary compounds as epigenetic modulators in chemoprevention or stabilization of various cancers and inflammatory disorders, especially with respect to their chronic use as nutraceutical agents, will rely on our further understanding of their epigenetic effects.[4,33]

Because epigenetic changes are reversible, developing drugs that control epigenetic regulation now attract substantial research investment, including the development of functional foods or supplements as nutrition based epigenetic modulators.[34] We should move beyond the traditional approach to newer avenues to achieve maximum benefit in terms of research and development productivity. This principle is also valid for mushroom-derived preparations.

Screening of Phenolic Compounds with Potential Regulatory Effects on Epigenome

Histone acetylation/ deacetylation. With respect to the activity of the enzyme HAT, only 5 of the 28 mushroom low-molecular weight compounds identified in the bibliographic search (set B) were structurally related with reference compounds with a known activity on epigenome (set A). The hesperetin proved to be a curcumin analogue, (Tc= 0.91), and probably it causes a decrease of the activity of the HAT (Figure 1). The hesperetin is a cancer-preventing flavonoid[35], contained in the medicinal mushroom *Ganoderma lucidum*in at a concentration of 30 mg/g.[36] Four compounds showed a high similarity to the anacardic acid (Tc= 0.90): the caffeic acid, the *p*-coumaric acid, the homogentisic acid, and the 5-sulfosalicylic acid. The caffeic acid is an organic compound classified as a hydroxycinnamic acid, similar to the *p*-coumaric acid. It possesses antioxidant and anti-mutagenic activity, and also prevents cardiovascular diseases[37]; this compound is present in the edible mushroom *Flammulina velutipes* in at levels of 17 mg/g.[36] The *p*-coumaric acid can be found in edible plants like peanuts, tomatoes, carrots and garlic; it shows a dual mechanism of antibacterial activity: (i) destroys cell membranes, and (ii) binding to the bacterial DNA, thus inhibiting cell functions and leading to cell death.[38] On the other hand, homogentisic acid has anti-plasmodical activity, inhibiting the activity of the protein kinase Pfnek-1.[39]

Some mushroom compounds (set B) were structurally related with compounds of known activity on the enzyme

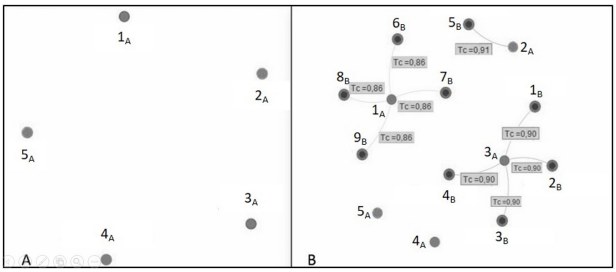

Figure 1. Neighbor Similarity Graph (NSG) of the Structural Relationship of Set A and B Compounds**. A.** Set A -reference compounds with activity on the enzyme histone acetyltransferase (HAT) - nodes: 1: genistein, 2: curcumin, 3: anacardic acid, 4: ursodeoxycholic acid, 5: garcinol. **B.** Set B -phenols derived from medicinal mushrooms- nodes: 1: crysin, 2: myricetin, 3: kaempferol, 4: quercetin, 5: hesperetin, 6: caffeic acid, 7: *p*-coumaric acid, 8: 5-sulfosalicylic acid, 9: homogentisic acid.

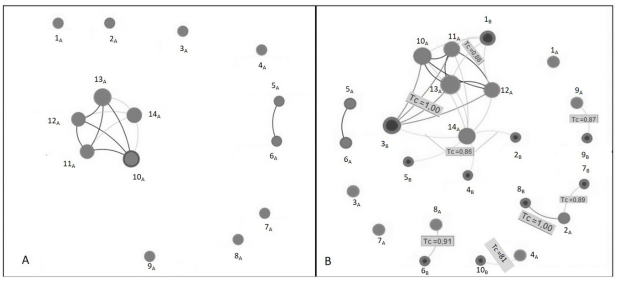

Figure 3. Neighbor Similarity Graph (NSG) of the Structural Relationship of Set A and B Compounds. **A.** Set A – reference compounds with activity on the enzyme DNA methyltransferase (DNMT)- nodes: 1. p-XSC, 2: mahanine, 3: sulforaphane, 4: mithramicina A, 5: phenylethyl isothiocyanate, 6: 6-phenylhexyl isothiocyanate, 7: parthenolide, 8: curcumin, 9: rosmarinic acid, 10: nordihydroguaiaretic acid, 11: ellagic acid, 12: baicalein, 13: fisetin, 14: genistein. **B.** Set B -phenols derived from medicinal mushrooms- nodes: 1: pyrogallol, 2: crysin, 3: myricetin, 4: quercetin, 5: kaempferol, 6: hesperetin, 7: formononetin, 8: biochanin A, 9: chlorogenic acid, 10: naringin.

HDAC, but only one had a structural similarity within the established range (Figure 2). The hesperetin, proved to be similar to the curcumin with Tc=0.91, and thus presumably is able to inhibit HDAC activity.

The FDA has approved HDAC inhibitors which are used clinically without reports of any genetic alteration occurring in this enzyme.[2]

The predictive study showed a significant structural relationship between the mushroom compounds and those with a reported modulatory activity on DNA methylation (Figure 3). The myricetin exhibited a Tc=1 when comparing to fisetin, nordihydroguaiaretic acid, baicalein and the ellagic acid. Therefore, myricetin was an analogue of compounds which inhibit or decrease the DNMT activity. Myricetin is a flavonol that exhibits antioxidant and liver protection activities.[40] The hesperetin showed a similitude to the curcumin with Tc=0.91, and would be involved in decreasing the global methylation of DNA by inhibiting the DNMT activity, in a similar way to curcumin. The hesperetin inhibited the methylation of DNA at concentrations of 20 and 50 mmol/L.[41] In the case of biochanin A, its chemical structure was similar to mahanine (Tc =1), that in association with the gene

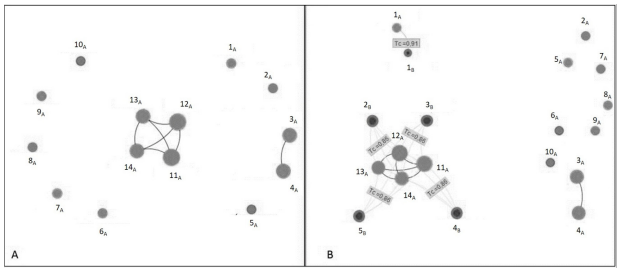

Figure 2. Neighbor Similarity Graph (NSG) of the Structural Relationship of Set A and B Compounds. **A.** Set A -reference compounds with activity on the enzyme histone deacetylase (HDAC)- nodes: 1: curcumin, 2: 4-dihydrocoumarin, 3: 6- phenylhexyl isothiocyanate, 4: phenylethyl isothiocyanate, 5: retinoic acid, 6: allyl mercaptan, 7: sulforaphane, 8: curcumin, 9: butyrate, 10: apicidin, 11: resveratrol, 12: daidzein, 13: cambinol**. B.** Set B -phenols derived from medicinal mushrooms- nodes: 1: hesperetin, 2: crysin, 3: kaempferol, 4: quercetin, 5: myricetin.

RASSF1 regulates cell proliferation and apoptosis in prostate cancer, presumably due to an inhibitory effect on the activity of DNMT.[41] Because of the high chemical structural similarity of biochanin A to mahanine, presumably this also would have similar biologic activity.

In sum, various nutritional natural compounds (including epigallocatechingallate, resveratrol, genistein, curcumin, isothiocyanates, etc) have been characterized to interfere with enzymatic activity of DNMT, Class I, II, IV HDAC, HAT and Class III HDAC sirtuins (SIRT), all of which modulate inflammatory responses and immunological senescence.[2]

These naturally occurring compounds, especially polyphenols have gained much attention for their DNMT inhibitory activity.[7] Studies have shown that flavonoids like quercetin, myricetin and fisetin inhibited DNMT1 enzyme in a dose dependent manner.[42] Moreover flavones and flavanones like apigenin and hesperetin are also been proved to inhibit DNMT enzyme in human cancer cell lines.[41]

As judged by the significant structural similarity with known molecules active on epigenome, we can hypothesize that some mushroom compounds included in this work would modulate certain epigenetic events, such as HAT activity (hesperetin, caffeic acid, *p*-coumaric acid, homogentisic acid, and 5-sulfosalicylic acid), HDAC activity (hesperetin), and DNMT activity (myricetin, hesperetin and biochanin A).

In this context, experimental research with 3 fractions obtained from *Phellinus linteus* mushroom – PL-1(crude extract), PL-II (water extracted) and PL-III (ethanol-extracted) – showed anticancer effects on urothelial cell carcinoma (UCC) cells, although PL-III appears to be the most potent. Additionally, HDAC activity was significantly (>60%) lost, while both histones H3 and H4 were highly acetylated, indicating epigenetic modifications in the chromatin structure, ultimately leading to apoptotic cell death. Therefore, PL-fractions

may have clinical implications in a safer and improved therapeutic modality for urothelial cell carcinoma.[43] In addition, apoptosis induction was demonstrated in both *in vitro* and *in vivo* studies with *Ganoderma applanatum* secondary metabolites,[44] and on colorectal cancer cell lines treated with *Pleurotus sajor-caju* (Fr.) Singer extracts.[45]

Screening of the Potential Antitumor Activity of *Pleurotus ostreatus* Phenolic Compounds

This study was focused to predict putative mechanisms of action of phenolic compounds occurring in *P. ostreatus*, those with significant Tanimoto's coefficient, since it would correspond to structurally similar molecules that present high probability to show a similitude in their biological activities. Twenty antitumor reference compounds showed structural similarity to 3 phenols occurring in *P. ostreatus*: protocatechuic acid, and the flavonoids myricetin and naringin.

The ten compounds with greater structural similitude to the protocatechuic acid were: the DNA antimetabolites aphidicolin glycinate, hydroxycarbamide, pyrazoloimidazole and Ara-C; the RNA/DNA antimetabolites 5,6 dihydroxiazacitidin, antifol, and L-alanosine; and alkylating agents: Mitomycin C, mustard of uracil nitrogen and Yoshi 864 (Figure 4). All of them showed high values in the Tanimoto's coefficients (94-98 %). The elevated values of the Tanimoto's coefficient between the reference antitumor compounds and the protocatechuic acid allowed to predict the potential mechanisms of the antitumor action of this phenolic acid. This compound demonstrated high structural analogy with RNA/DNA antimetabolites and DNA antimetabolites. Therefore, it could interfere with DNA and RNA synthesis. Antimetabolite agents induce cell death during the S-phase of the cellular cycle when bind to RNA or DNA molecules, or when inhibit the activity of enzymes necessary for nucleic acids synthesis.[46]

The flavonoid myricetin was highly similar to the RNA/DNA antimetabolites: aphidicolin glycinate, hydroxycarbamide, imidazole piridazol and Ara-C; to the alkylating agents: fluoropan, mitomycin C, mustard of uracil nitrogen, asaley and triethylenemelamine; and to the Topoisomerase I inhibitors derived of the camptothecin 6 (Figure 5). The high values of Tanimoto's coefficient (0.92-0.96) when comparing the myricetin and the reference compounds allow to suggest putative mechanisms of

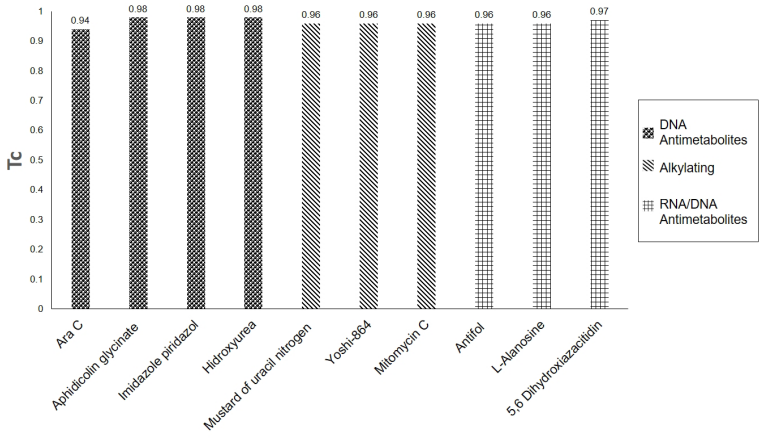

Figure 4. Structural Analogy of 10 Antitumor Reference Compounds with Known Mechanism of Action to Protocatechuic Acid According to the Tanimoto's Coefficient (Tc).

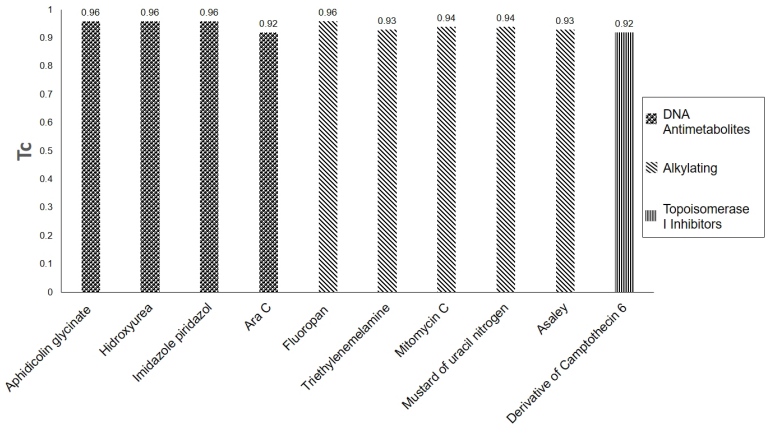

Figure 5. Structural Analogy of 10 Antitumor Reference Compounds with Known Mechanism of Action to Myricetin According to the Tanimoto´s Coefficient (Tc).

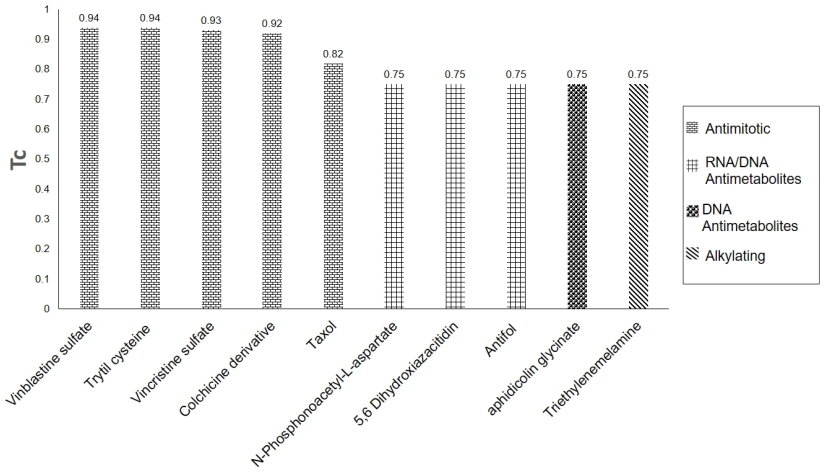

Figure 6. Structural Analogy of 10 Antitumor Reference Compounds with Known Mechanism of Action to Naringin According to the Tanimoto´s Coefficient (Tc).

the antitumor action for this flavonoid. The anticancer compounds more similar to myricetin influence cell crucial processes acting as DNA antimetabolites, alkylating agents as well as inhibitors of Topoisomerase I.[46] It has been suggested that the antitumor activity of this flavonoid could be also attributed to its efficiency in the inhibition of the topoisomerases I and II.[47]

On the other hand, 4 similar antitumor reference compounds were identified with a high similitude to the flavonoid naringin with values of Tanimoto's coefficients between 0.92 and 0.94, which possess antimitotic activity: vinblastine sulfate, trytil cysteine, vincristine sulfate and a colchicine derivative (Figure 6).

Our results suggest a group of phenolic compounds isolated from edible and medicinal mushrooms which would exert chemopreventive and therapeutic potential effects on cancer treatment and/or with potentialities for epigenetic therapy. As for low molecular weight mushroom compounds, only a minute fraction of the newly discovered substances have proceeded to a higher level of evaluation. Hispolon, an active phenolic compound extracted from *Phellinus* spp., is known to possess potent antineoplastic properties and to potentiate the cytotoxicity of chemotherapeutic agents. Hispolon induces epidermoid and gastric cancer cell apoptosis and, regardless of p53 status, it inhibited breast and bladder cancer cell growth. A crucial role of hispolon in ubiquitination and downregulation of MDM2 (the proto-oncogene inhibiting the tumor suppressor function of p53) was reported, suggesting this phenolic compound as an attractive therapeutic strategy in breast, gastric, and bladder cancers.[48]

The recent finding of the grow inhibitory effect on HCT116 colon cancer cells and anti-inflammatory function of polyphenols-rich extract from *Pleurotus eryngii*[49] highlights the contribution of our work to get new insights about the mechanisms of action of individual molecules.

In sum, the *in silico* approach used in this research represent a natural framework for not only testing biological hypotheses and generating new ones but also optimizing experimental protocols. Many of the tools have been developed, what is now needed is to translate the information into validated models that are specialized for particular tumors and drugs, with the power to generate both qualitative and quantitative predictions. The increasing number of *in silico* papers on antitumor and other biological activities appearing in the literature indicates that the transition is starting to happen.[50-52]

Conclusion

"Let food be your epigenetic medicine" could represent a novel interpretation of what Hippocrates said already 25 centuries ago.[34] As such, it will be a challenge for future preventive cancer research to identify novel epigenetic targets which allow selective modulation of cancer signaling. It seems feasible to harness the natural pool of mushrooms secondary metabolites and to predict by *in silico* approaches their potential modulatory effects on epigenetic events and antitumor activity, in special phenolics occurring in *P. ostreatus*. Of special note, these *in silico* tools should be moved into an integrated platform with *in vitro* and *in vivo* studies. This is an exciting advance for developing nutraceuticals/ cosmeceuticals and innovative drugs.

Conflict of Interests

The authors declare no conflicts of interest.

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