Recovery of ergosterol and vitamin D2 from mushroom waste - potential valorization by food and pharmaceutical industries

Recovery of ergosterol and vitamin D_2 from mushroom waste - potential valorization by food and pharmaceutical industries

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Abstract

Background. A large amount of mushroom waste is generated during mushroom production (account for up to 20% of total production) and is mainly composed of mushrooms that do not meet the specifications set by retailers because of misshapen caps and/or stalks. Mushrooms are notable for their ergosterol (a precursor of vitamin D_2) content which is converted to vitamin D_2 after exposure to natural or artificial ultraviolet (UV) irradiation. Therefore, mushroom waste could be used as a source for the recovery of both ergosterol and vitamin D_2 which could be valorized by both pharmaceutical and food industries.

Scope and approach. The current review presents a comprehensive summary of research performed regarding the extraction, purification and determination of ergosterol and vitamin D_2 (ergocalciferol) from mushroom matrices. Additionally, studies related to the impact of sample preparation and especially of drying methods on the retention of ergosterol and vitamin D_2 are presented. Finally, the potential valorization of mushroom waste sterols by food and pharmaceutical industries is discussed.

Key Findings and Conclusions. Ergosterol and vitamin D_2 contents vary among different mushroom species. Sample drying is a crucial step that precedes sterol extraction and has a significant impact on the retention of ergosterol and vitamin D_2. The extraction of sterols from mushroom can be conducted by either conventional (e.g., Soxhlet extraction) or non-conventional methods (e.g., ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), deep eutectic solvents (DES) extraction, supercritical fluid extraction (SFE), and pressurized liquid extraction (PLE)) or their combination. The application of non-conventional methods such as UAE and MAE facilitate in shorter extraction times than the conventional methods. The valorization of mushroom extracts enriched in ergosterol and vitamin D_2 by both pharmaceutical and food industries requires further work.

Keywords: ergocalciferol; sterols; sustainable extraction; sample preparation; saponification; ergosterol

1. Introduction

Mushrooms are a unique source of compounds such as polyphenols, amino acids (i.e. ergothioneine), polysaccharides (i.e. β-glucans), terpenoids, vitamins (i.e. vitamin D_2), and sterols (i.e. ergosterol) which have been linked to antioxidant, anticancer, antidiabetic, anti-inflammatory, hepatoprotective, antiallergic, antimicrobial, and antiviral activities (Kalač, 2016; Roncero-Ramos & Delgado-Andrade, 2017; Kalaras et al., 2017; Ramos et al., 2019). Because of their beneficial properties, mushrooms have become attractive as functional foods or as a source of compounds that can be extracted and incorporated into food products.

Vitamin D is an essential vitamin for human health and plays a vital role in the regulation and maintenance of calcium homeostasis. Vitamin D deficiencies have also been linked to an increased risk of cancer, hypertension, autoimmune diseases, and diabetes (Jäpelt & Jakobsen, 2013). The main reasons for failing to meet vitamin D requirements are i) low dietary intake, since only a few food products naturally contain vitamin D and ii) limited vitamin D synthesis due to inadequate exposure to sunlight (Bailey et al., 2010; Jäpelt & Jakobsen, 2013).

Vitamin D is a fat-soluble vitamin (non-polar compound) which is found in two major forms, namely D_2 and D_3 (Dawson-Hughes et al., 2010). The D_3 form is mainly contained in animal products such as egg, meat, and fish, while the D_2 form is mostly present in mushroom fruiting bodies. Mushrooms contain high levels of ergosterol, a precursor of vitamin D_2. The transformation of ergosterol to vitamin D_2 can be achieved by the application of artificial or natural ultraviolet (UV) irradiation (Wen et al., 2020).

Ergosterol, the precursor of vitamin D_2 is the most abundant sterol found in fungal cell membranes where it is critical for maintaining fluidity, permeability, carrying out various kinds of endocytosis and trafficking and for the cytoskeletal organization (Abe & Hiraki, 2009). In yeast mating, it is also linked with pheromone signalling and membrane fusion (Jin, McCaffery, & Grote, 2008). Recently, it has been suggested that ergosterol also has an immunoactivity role, as it is involved in triggering programmed cell death in host cells (Rodrigues, 2018).

High amounts of mushroom waste are generated during mushroom production (account for up to 20% of total production). Mushroom waste is mainly composed of mushrooms that their caps and/or stalks are misshapen and do not meet the specifications set by retailers (Aguiló-Aguayo, Walton, Viñas, & Tiwari, 2017). These mushroom by-products have high nutritional value. Moreover, their disposal is linked to managing costs and high environmental impact including

global warming, abiotic depletion, acidification, ozone layer depletion, eutrophication, human toxicity, eco-toxicity, depletion of natural resources, and energy consumption (Leiva, Saenz-Díez, Martínez, Jiménez, & Blanco, 2015). Therefore, new alternative and profitable solutions need to be explored (Royse, 2014). Mushroom waste could be utilized for the preparation of extracts enriched in vitamin D that could be used either by the pharmaceutical industry as nutritional supplements or by the food industry as an additional ingredient in foods, adding value to the agriculture sector.

The recovery of ergosterol and vitamin D is carried out by the application of either conventional methods such as Soxhlet extraction or non-conventional such as ultrasound-assisted extraction (UAE), microwave assisted extraction (MAE), deep eutectic solvents (DES), supercritical fluid extraction (SFE), and pressurized liquid extraction (PLE) (Heleno, Diz, et al., 2016; Heleno, Prieto, et al., 2016; Morales et al., 2017; Patil, Ghodke, Jain, & Dandekar, 2018). Extraction method might have a significant impact on the recovery of both ergosterol and vitamin D_2, therefore, the extraction conditions should be carefully selected and optimized. The application of some non-conventional methods such as UAE and MAE may facilitate the avoidance of saponification step reducing the total extraction time. Apart from the extraction, sample preparation may impact the retention of vitamin D and ergosterol in samples (Gąsecka et al., 2019; Tian, Zhao, Huang, Zeng, & Zheng, 2016).

The current review summarizes the steps from sample preparation until the detection of ergosterol and vitamin D from mushrooms, as well as their potential application in the food and pharmaceutical industries.

2. Ergosterol and vitamin D_2 in mushrooms

Vitamin D occurs in two main forms in nature: Vitamin D_2 (ergocalciferol) and Vitamin D_3 (cholecalciferol). Vitamin D_3 is only found in animal sources such as fish liver oils, eggs and milk products. Vitamin D_2 found in limited plant sources in far fewer amounts, putting in vegetarians and vegans being at risk of having vitamin D deficient diets (Ho-Pham, Vu, Lai, Nguyen, & Nguyen, 2012). Mushroom qualify for such a diet and while they are deficient in vitamin D, widely available ones such as shiitake and button mushrooms, are rich sources of ergosterol, a precursor of vitamin D_2. Ergosterol can be converted into vitamin D_2 by natural or artificial UV irradiation which is a process that has been widely studied (Black, Lucas, Sherriff, Bjorn, & Bornman, 2017; Taofiq, Fernandes, Barros, Barreiro, & Ferreira, 2017).

Mushrooms and other kinds of fungi have high concentrations of ergosterol in their cell membranes where it performs a role similar to that of cholesterol in animals and sitosterol in plants, i.e., strengthening and maintaining fluidity of cell membranes, protein functionality, and modulating intracellular transport (Lingwood & Simons, 2010; Roncero-Ramos & Delgado-Andrade, 2017). It is produced by a fungal specific pathway. Similar to animals, fungi convert acetyl-CoA to lanosterol via the formation of squalene epoxide, however, fungi have a longer and more energy-expensive conversion final process in which 11 oxygen atoms are added to lanosterol to produce ergosterol whereas animals add 10 oxygen atoms in a more efficient process to produce cholesterol (Dupont et al., 2012).

Figure 1 shows the process of conversion of ergosterol in fungal sources into vitamin D_2. On exposure to UV radiation, ergosterol in exposed portions of mushrooms undergoes photochemical cleavage in the B ring, leading to the formation of an intermediate called pre-vitamin D_2. On subjection to heat, this intermediate undergoes thermal isomerization to form ergocalciferol, i.e., vitamin D_2. The yield of the final product is heavily dependent on the balance between thermal and photochemical reactions (Jasinghe, Perera, & Sablani, 2007), hence the temperature of radiation is critical. Higher temperatures direct the reaction towards the formation of by-products such as lumisterol and tachysterol, in addition to adversely affecting the texture and colour of the mushrooms. Moisture content is also important since excessive dryness leads to an increase in surface area and hence exposure to oxygen, which leads to oxidation of vitamin D_2. On the other hand, excess moisture may have a dilution effect on ergosterol content, which might result in a lower conversion rate to vitamin D_2 (Jasinghe et al., 2007). However, the total amount of ergosterol produced per gram of mushroom might not be affected by the excess moisture content. Hence, the vitamin D_2 fortification process in mushrooms should go hand in hand in hand with minimizing moisture loss and maintaining hardness. The optimal conditions vary with types of mushrooms and there have been many studies to determine them (Ahn, 2018; Lee & Aan, 2016; Won et al., 2018).

3. Extraction and determination of ergosterol and vitamin D_2 from mushrooms

Ergosterol and vitamin D_2 extraction requires long determination times. Several steps are involved in the extraction and determination of ergosterol and vitamin D_2 from mushrooms including sample preparation, saponification, extraction, cleaning, as well as detection and

quantification using HPLC or GC. The first step for the extraction of ergosterol and vitamin D_2 is sample preparation. Although samples can be either fresh or dried, most of the studies have determined sterols in lyophilized samples (Heleno et al., 2016a; Heleno et al., 2016b; Teichmann, Dutta, Staffas, & Jägerstad, 2007; Wittig, Krings, & Berger, 2013). To date, not much work has been done on the bulk recovery of ergosterol and vitamin D_2 from mushrooms but that there is much to be learned from work done on its analytical determination.

3.1 Sample preparation

Sample preparation precedes extraction and determination steps. Studies have shown that sample preparation may have a significant impact on the sterol content of different mushroom species (Gąsecka et al., 2019; Nölle, Argyropoulos, Müller, & Biesalski, 2018). Recently, Gąsecka et al. (2019) reported that the ergosterol content of two mushroom species (*Hericium erinaceus* and *Leccinum scabrum*) was significantly affected by the processing method employed. Specifically, the authors noted that the ergosterol content was higher in the fresh mushroom samples while it declined as the drying temperature increased from 20 to 70 °C (Gąsecka et al., 2019). Slawinska et al. (2016) investigated the effect of hot-air drying (the initial drying temperature was 40 °C, and in the final stage of drying the temperature was raised to 60 °C) and freeze-drying on ergosterol and vitamin D_2 contents of three different mushroom species (*Agaricus bisporus*, *Pleurotus ostreatus* and *Lentinula edodes*). The authors reported that the vitamin D_2 content was higher in the mushrooms dried by freeze-drying than those dried by hot-air drying, while drying treatment had a slight significant effect only on the ergosterol content of *A. bisporus* (Slawinska et al., 2016). Similar results were reported by Bernas and Jaworska (2017) where ergosterol content was higher in freeze-dried *A. bisporus* than its air-dried counterpart (30% loss). Tian et al. (2016) examined the effect of different drying methods (microwave vacuum-drying (microwave power density of 15 W/g, vacuum degree of −80 kPa, for 11 min), hot-air drying (60-70 °C), microwave-drying (539 W for 18 min), vacuum-drying (−90 kPa at 60 °C for 15 h)) on shiitake mushroom nutrient retention, including vitamin D_2. The authors noted that drying resulted in the degradation of vitamin D_2, with the hot-air and microwave-drying resulting in greater vitamin D_2 loss, compared to the other two drying techniques (Tian et al., 2016). These results show the sensitivity of vitamin D_2 to degradation in the presence of oxygen. Nölle et al. (2018) investigated the effect of a high precision drying (temperatures: 40, 60, and 80 °C, specific

humidity: 10 g/kg, and air velocity: 0.6 m/s) and freezing-drying on the retention of vitamin D_2 of different mushroom species, including shiitake (*L. edodes*), oyster (*P. ostreatus*), as well as white and brown button mushrooms (*A. bisporus*) treated with UV-B light (1.5 J/cm^2). In the case of oyster and white button mushrooms, the highest vitamin D_2 content was found in the freeze-dried samples, while for shiitake and brown mushrooms there was insignificant difference between the freeze-dried and hot-air dried samples. Therefore, it can be concluded that the drying process should be carefully selected according to the mushroom species.

3.2 Extraction of ergosterol and vitamin D_2

Conventional (e.g., Soxhlet extraction) and non-conventional (e.g., microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), deep eutectic solvents (DES), supercritical fluid extraction (SFE)) methods have been investigated and developed for the recovery of ergosterol and vitamin D_2 from mushrooms (Heleno et al., 2016a; Heleno et al., 2016b; Patil et al., 2018) (Figure 2). The current literature portrays different definitions for conventional extraction. For instance, according to Barba et al. (2017) conventional extraction should be considered as the extraction protocol using conventional solvents which are toxic to the environment and human health. According to Heleno et al. (2016b) conventional extraction is a method that is time-consuming and requires large quantities of hazardous solvents. On the other hand, as a non-conventional is considered the method that requires a lower temperature and shorter treatment times compared to conventional methods (Roselló-Soto et al., 2016). Non-conventional methods have been employed by studies for the recovery of sterols using organic solvents such as methanol. In this case, even though a non-conventional method is employed the whole process cannot be considered as environmentally and human friendly since the principles of 'green extraction' are not satisfied (Chemat, Vian, & Cravotto, 2012). According to Chemat et al. (2012) 'green extraction' is an extraction process that involves: i) reduced energy consumption, ii) the use of alternative non-toxic solvents and renewable natural products, and iii) results in a safe and high-quality extracts. Table 1 presents the summary of studies conducted to date extracting vitamin D_2 and ergosterol from mushroom matrices. The extraction methods are divided considering the technology used for the extraction rather than the solvent.

3.2.1 Saponification (hydrolysis)

Saponification is a hydrolysis reaction where free hydroxide breaks the ester bonds between the fatty acids and glycerol of a triglyceride, resulting in free fatty acids and glycerol (Prabu, Suriya Prakash, & Thirumurugan, 2015). In ergosterol and vitamin D_2 extraction from mushrooms, saponification can be conducted either on samples or on extracts derived from different extraction methods. When hydrolysis is applied on mushroom samples, it breaks the complex structure of mushrooms and improves the recovery of ergosterol and vitamin D_2 from the surrounding matrix. Moreover, when conjugated sterols are hydrolyzed to free sterols, they have similar polarity and can be extracted with a single solvent (Han & Zhou, 2015). The saponification may take place at temperatures varying from 60 and 80 °C and times varying from 15 to 60 min (Barreira et al., 2014; Gil-Ramirez et al., 2013; Heleno et al., 2016a). During the hydrolysis, ascorbic acid solution is used to avoid any thermal degradation of sterols. Recent studies have shown that saponification step can be avoided when some non-conventional extraction techniques such as UAE and MAE are employed (Heleno et al., 2016a; Heleno et al., 2016b).

3.2.2 Non-conventional extraction methods

3.2.2.1 Ultrasound-assisted extraction (UAE)

UAE has been employed for the extraction of different classes of bioactive compounds from mushrooms such as polyphenols, sugars, and vitamins from different mushrooms species (Aguiló-Aguayo et al., 2017; Alzorqi, Sudheer, Lu, & Manickam, 2017; Heleno et al., 2016a; Xu et al., 2016). Ultrasound devices use ultrasound waves above human hearing (>20 kHz) in which pressure fluctuation leads to the cavitation with the resultant unstable bubbles imploding and damaging cell membranes allowing for mass transfer out of the cell (Chemat et al., 2017). In sterol extraction from mushroom, ultrasonic baths have been mainly used with a frequency ranging between 20 to 65 kHz (Heleno et al., 2016a; Patil et al., 2018; Villares et al., 2012; Villares et al., 2014). Several mechanisms are involved in ultrasound extraction such as fragmentation, erosion, capillarity, detexturation, and sonoporation and have been described in the depth in the recent review of Chemat et al. (2017) and summarized in Figure 3.

The extraction of sterols from mushrooms can be influenced by different parameters, such as solvent type, extraction time and ultrasound power, while liquid-to-solid ratio seems to have no significant effects (Heleno et al., 2016a). Patil et al. (2018) employed UAE (ultrasonic bath) in conjunction with deep eutectic solvents (DES) for the extraction of vitamin D_2 from *A. bisporus*.

The vitamin D synthesis was achieved by exposing the extracts to UV light. Villares et al. (2012) used UAE (ultrasonic bath) for the recovery of ergosterol from *Tuber melanosporum* and *T. aestivum* using chloroform/methanol mixture (2:1, v/v). After UAE, a clean-up stage of the extracts was conducted with Oasis MAX column preconditioned with 8 mL of hexane. Sterol separation from other lipids was conducted by passing chloroform. The solvent in the final extracts was evaporated under a nitrogen stream and redissolved in 2 mL of chloroform.

Heleno et al. (2016a) optimized the UAE of ergosterol from *Agaricus bisporus* using response surface methodology (RSM). In this study the effects of different parameters were investigated including the type of extraction solvent, liquid-to-solid ratio, extraction time, and ultrasound power. Among the different solvents investigated, ethanol resulted in the highest ergosterol yields followed by limonene and n-hexane. Both ergosterol and vitamin D_2 are amphiphilic molecules with small hydroxyl polar heads which allow them to be extracted by a variety of polar and non-polar solvents (Heleno et al., 2016a; Hsueh et al., 2007). The authors noted that at the optimal UAE conditions (extraction time of 15 min and ultrasound power of 375W) ergosterol yields were higher than those obtained by a time consuming Soxhlet extraction method in significantly shorter times (from 4h to 15 min). The authors also claimed that the saponification step can be avoided when the UAE is employed (Heleno et al., 2016a). However, the efficiency of the UAE on purifying the extracts is influenced by the solvent used. For instance, the authors showed that the purity of the extracts obtained by n-hexane and limonene was similar to the extracts obtained after saponification, while ethanol during UAE resulted in less pure extracts. This was attributed to the higher polarity of ethanol than the other two solvents which may lead to the extraction of various compounds (polar and non-polar). In summary, UAE can be used for the extraction of sterols from mushrooms facilitating shorter extraction times.

3.2.2.2 Supercritical fluid extraction (SFE) and pressurized liquid extraction (PLE)

SFE and PLE are sustainable extraction techniques that have been used for the recovery of various bioactive compounds, including polyphenols, pigments, oils, and sterols from different materials (Aladić et al., 2016; Valadez-Carmona, Ortiz-Moreno, Ceballos-Reyes, Mendiola, & Ibáñez, 2018). In SFE the use of supercritical CO_2 (non-polar) facilitates the recovery of non-polar and mid-polar compounds (Gallego, Bueno, & Herrero, 2019). SFE has been employed for the recovery of both ergosterol and vitamin D_2 from mushrooms (Gil-Ramirez et al., 2013; Morales et

al., 2017). Morales et al. (2017) employed SFE for the production of extracts enriched in vitamin D_2. Specifically, SFE was employed for the extraction of ergosterol from shiitake mushrooms and subsequently, extracts were exposed to UV light for the conversion of ergosterol to vitamin D_2. In this study, two extraction parameters were examined including extraction temperature (°C) and pressure (bar). Both extraction parameters had a significant effect on the recovery of ergosterol. The highest ergosterol yields (180 mg/g dw) were obtained at a pressure of 350 bar and a temperature of 70 °C (Morales et al., 2017). Gil-Ramirez et al. (2013) optimized and compared the extraction conditions of SFE and PLE for the recovery of sterols from *Agaricus bisporus* fruiting bodies. In SFE, extraction pressure and use of co-solvent (10% ethanol) were investigated. Lower sterol yields were obtained when a co-solvent was used, while there was no significant effect of the extraction pressure. Fractions containing 60% of sterols were obtained at 40 °C and 30 MPa. In the case of PLE, several parameters were investigated including extraction time, the number of cycles, ratio mushroom powder/sand and temperature. The optimal PLE conditions using ethanol as the pressurized solvent were a pressure of 10.7 MPa, a temperature of 50 °C, 5 cycles of 5 min and mushroom/sand ratio of 1:4 (Gil-Ramirez et al., 2013). Between SFE and PLE, PLE found to extract all the sterols from mushrooms, however, SFE was found to be more selective. SFE has the potential of being upscaled for the extraction of both ergosterol and vitamin D_2 since it has been upscaled for the extraction of other compounds (e.g., caffeine to make decaffeinated products) whereas PLE remains a small-scale batch technique with mostly analytical applications.

3.2.2.3 Microwave-assisted extraction (MAE)

MAE uses microwaves which are electromagnetic irradiation ranging in frequency from 300 MHz to 300 GHz. MAE has been employed for the extraction of different classes of bioactive compounds, such as polyphenols, polysaccharides, lipids, and fatty acids (Kumar, Sivacumar, & Ruckmani, 2016; Maeng, Muhammad Shahbaz, Ameer, Jo, & Kwon, 2017; Sinanoglou et al., 2015). However, only few studies to date have employed and optimized the MAE of ergosterol from mushrooms while there is no study optimizing the MAE of vitamin D_2 from mushrooms (Young, 1995; Heleno et al., 2016b; Taofiq et al., 2019). Heleno et al. (2016b) investigated and optimized three parameters named extraction time, extraction temperature and solid-to-liquid ratio using RSM for the recovery of ergosterol from *A. bisporus*. The optimal MAE conditions were

extraction time of 19.4 ± 2.9 min, extraction temperature of 132.8 ± 12.4 °C and the solid-to-liquid ratio of 1.6 ± 0.5 g/L, yielding 5.56 ± 0.26 mg of ergosterol per g of mushroom by-products. The authors noted that the extraction yields increased as the extraction time and extraction temperature increased, while extraction yields decreased as the solid-to-liquid ratio increased. The authors also reported that the purity of extracts in ergosterol reduced by increasing temperature. This could be either due to the extraction of other compounds than ergosterol as the extraction temperature increased, or ergosterol was degraded as the temperature increased (Heleno et al., 2016b). Recently, Taofiq et al. (2019) optimized the MAE (extraction time (2-25 min) and temperature (60-150 °C)) of ergosterol (dependent variables examined: extraction yields, purity and total extraction yields) from *A. blazei* and compared the dependent variables with those obtained at the optimal UAE and heat-assisted extraction (HAE). Authors noted that among the different extraction methods MAE was the most efficient in terms of extraction yields (25.44 ± 5.1 mg/ 100g dw) compared to the UAE and HAE 21.49 ± 0.9 and 18.84 ± 2.3 mg/ 100g dw, respectively. However, UAE resulted in extracts with higher ergosterol purity which could be attributed to the combined mechanisms such as fragmentation, erosion and sonoporation that take place through UAE. MAE is a promising extraction technique that can significantly reduce the extraction time of ergosterol recovery from mushrooms. Future studies are encouraged to investigate and optimize the MAE of vitamin D_2 (extraction yields and purity) from different mushroom varieties.

3.2.3 Conventional extraction methods

Currently, the extraction of ergosterol and vitamin D_2 from mushrooms is mainly conducted using conventional extraction methods (Table 1). Soxhlet extraction is used for the extraction of both sterols, followed by saponification and purification. Heleno et al. (2016a) employed Soxhlet extraction for the recovery of ergosterol from *A. bisporus*. Specifically, 4.5 g of samples were extracted with 150 mL of solvent (the effect of n-hexane, ethanol and limonene was examined) during 4 h Soxhlet extraction. After the extraction the solvent was rotary evaporated, and the extracts were saponified using 2 M KOH and 0.1 M ascorbic acid solution at 60 °C for 45 min. Subsequently, the samples were mixed with NaCl solution and n-hexane. Sterols due to their lipophilic nature were transferred from the aqueous to n-hexane phase. The remained aqueous layer was reextracted again using n-hexane. Finally, the n-hexane fraction was dried and the residue was dissolved in methanol (1 mL) before HPLC analysis (Heleno et al., 2016a).

In most of the studies that have been conducted so far, saponification of mushroom samples precedes extraction and purification. A protocol that is usually reported in the literature is saponification of mushroom samples for 30 min to 1 h at 80 or 85 °C, followed by liquid-liquid extraction using non-polar solvents (i.e., n-pentane). Subsequently, the non-polar phase is washed with potassium hydroxide 3% (w/v) in 5% (v/v) ethanol and neutralized using deionized water. Finally, the organic phase fraction is rotary evaporated to dryness and then it is dissolved either in ethanol or methanol (Heleno et al., 2016a; Jasinghe & Perera, 2005, 2006; Jasinghe et al., 2007; Ko, Lee, Lee, & Park, 2008). In some studies before saponification an internal standard is added (Roberts et al., 2008).

3.3 Determination of ergosterol and vitamin D_2

3.3.1 High-performance liquid chromatography (HPLC)

HPLC is the most commonly employed technique for the identification and quantification of vitamin D_2 and ergosterol from mushrooms (Table 2). Before injection, samples are filtered using disposable filter discs to remove small particles and thus protect HPLC columns. For the detection of vitamin D_2 and ergosterol, reverse phases are usually employed containing carbon chains of 18 carbon atoms, while there is one study used a column with carbon chains of 30 carbon atoms (Table 2). The temperature of the stationary phase (column) is a critical parameter that may affect the retention times of bioactive compounds. In the case of ergosterol and vitamin D_2 detection, the temperatures of the stationary phase may vary between 25 and 50 °C. For instance, in the studies of Yuan, Wang, Liu, Kuang, and Zhao (2007) the stationary phase was maintained at ambient temperature, while in the study of Morales et al. (2017) the stationary phase was maintained at 50 °C. For HPLC analysis an injection volume of 10 or 20 μL is usually used, however, when UHPLC (Ultra-high performance liquid chromatography) is employed, an injection volume of 1 μL can be used (Slawinska et al., 2016). Either isocratic or gradient elution can be implemented in HPLC analysis. The combination of methanol and acetonitrile is mainly used as a mobile phase in isocratic elution while in gradient elution two or three mobile phases can be used (Ahlborn et al., 2018). The flow rate that is usually employed is 1 mL/min (Ahlborn et al., 2018; Heleno et al., 2016a; Heleno et al., 2016b; Huang, Lin, & Tsai, 2015; Morales et al., 2017). UV-Vis and photodiode array detectors have been employed for the detection and the wavelengths that are usually used for the detection of both ergosterol and vitamin D are 264 and

280 (Table 2). Mass spectrometers (MS) directly or coupled with a PDA detector have also been used for detecting sterols, including vitamin D_2, and ergosterol in mushrooms.

3.3.2 Gas-chromatography (GC)

In addition to HPLC, the determination of ergosterol and vitamin D_2 has been conducted using GC coupled with MS and/or flame ionization detector (FID) (Gil-Ramirez et al., 2013; Morales et al., 2017; Teichmann et al., 2007). In the study of Gil-Ramirez et al. (2013), prior to the injection into the GC system, dried extracts were dissolved in chloromethane: methanol ($ClCH_3$: MeOH) (2:1 v/v). A capillary column connected to a gas chromatograph equipped with electronic pressure control, auto injector, a triple-axis mass spectrometer detector, and GC–MS solution software. Helium (He) was used as carrier gas with a flow rate of 29.4 mL/ min and an inlet pressure of 0.19 MPa. The injector was set at 260 °C, the detector at 350 °C and the oven temperature program was 60 °C for 1 min, at a rate of 40 °C/min, to a final temperature of 310 °C, and held for 30 min. Apart from ergosterol (ergosta-5,7,22-trien3β-ol) which was the major sterol (83% of total sterols) and detected at 13.3 min, other sterols such as ergosta-7,22-dienol, ergosta-5,7dienol, ergosta-7-enol (fungisterol), ergosta-4,7,2-trien-3-ona and ergosta-4,6,8(14),22-tetraen-3-ona were also detected at 13.6, 14.0, 14.3, 14.9, and 15.7 min, respectively.

4. Potential application in the food industry

Efforts have been made to add value to mushroom waste through its use as animal feed, fuel or to extract valuable compounds that can be used as food safe ingredients, such as chitin, chitosan, β-glucan, sterols and prebiotic polysaccharides (Chou, Sheih, & Fang, 2013; Gil-Ramírez, Ruiz-Rodríguez, Marín, Reglero, & Soler-Rivas, 2014; Mahfuz, Song, Miao, & Liu, 2019; Vasylenko, Loewe, & Wegener, 2008; Wu, Zivanovic, Draughon, & Sams, 2004). Furthermore, mushroom-derived β-glucans, lentinan and polysaccharide-K have been used for thousands of years to improve general health as a part of traditional medicine especially in Asian countries including China, Japan and Korea (Lemieszek & Rzeski, 2012; Ina, Kataoka, & Ando, 2013).

It is known that mushrooms have the potential to be the only food source of vitamin D (non-animal and unfortified), while providing a substantial amount of vitamin D_2 in a single serving (Cardwell, Bornman, James, & Black, 2018). Fresh mushrooms sold in UK retailers

provide 3-5 μg (60-100 % of the RI) per 100 g, which equals to 4-5 chestnut mushrooms or 1-2 portobello mushrooms (Food Manufacture, 2016).

Sun or hot-air dried mushrooms have about 15% of the original weight of fresh mushrooms and will retain about 5% of water, while freeze dried mushrooms will have close to zero moisture and 8-10% of the weight of the original mushroom. While dried mushrooms are cheaper to transport and might represent a cheaper source of vitamin D_2, several variables (time of exposure, temperature, and exposure to UV-B radiation) are known to influence their vitamin D_2 production (Cardwell et al., 2018).

The potential application of waste mushroom extracts (such as vitamin D_2 extracts) in new foods could have regulatory implications. In the European Union, mushroom extracts might fall under the Novel Food Regulation, which relates to foods not widely consumed by people in the EU before May 1997. Mushroom extracts could fall under the category "food consisting of isolated from or produced from plants or their parts" (EFSA, 2016). Only UV treated mushrooms (*A. bisporus*) and an aqueous extract from shiitake mushroom with the glucan lentinan (*L. edodes*) have so far been authorized as novel foods through the lengthy authorization process.

Practical applications of vitamin D_2 from mushroom waste are limited. The concept of extracting chitin and chitosan from mushroom waste is not new (Wu et al., 2004). Bilbao-Sainz et al. (2017) developed this concept further, creating vitamin D-fortified chitosan films from mushroom waste. The authors treated mushroom stalk bases with UV-B light, obtaining about 90 μg of vitamin D_2 per gram on a dry weight basis (4.5 to 6 times the RDA/g). They then prepared fungal chitosan films with characteristics similar to animal derived chitosan. Correa et al., (2018) obtained and extract rich in ergosterol from commercially discarded *A. Blazei* fruits and used it as a fortifier ingredient in yogurts. The ergosterol extract in yogurts had antioxidant properties and did not alter the nutritional profile of the yogurt.

The use of vitamin D extracts from mushroom waste in new foods could benefit the food industry, as several nutritional and health claims could be made on food packaging. According to the European Food Safety Authority (EFSA, 2006), vitamin D can be added to foods as cholecalciferol (D_3) or ergocalciferol (D_2). The daily Reference Intake (RI) value for vitamin D is 5 μg, previously known as Recommended Daily Allowances (RDAs), and this is the reference value used on food labels (EU Regulation, 2011). According to the EFSA register of nutrition and health claim (EFSA, 2012), there are several health claims that can be made on foods that are a

"source of" or "high" in vitamin D. "Source of" and "high" refer to foods that contain respectively at least 15% or 30% of the vitamin D RDA (15%=0.8 μg, 30%=0.16 μg) per 100 g of solid food. The allowed health claims on vitamin D adapted from the EFSA register of nutrition and health claims are shown in Table 3.

5. Potential application in the pharmaceutical industry

Ergosterol, a vital component of the fungal and protozoal cell membranes plays a fundamental role in membrane fluidity and integrity while acting as a drug target of several antifungal agents (Rodrigues, 2018). Interestingly, ergosterol and its derivatives isolated from mushroom and other natural sources have been reported to have several therapeutic properties. A review by (Picotto, Liaudat, Bohl, & Talamoni, 2012) emphasized the importance of vitamin D (ergocalciferol and cholecalciferol) in anticancer research. Table 4 represents the major preclinical studies portrayed in the current literature underlining the bioactivity of ergosterol and its natural, semi-synthetic and synthetic derivatives. In particular, ergosterol peroxide, a natural ergosterol derivative has been studied more extensively and shown to confer a greater anti-proliferative and cytotoxic potential compared to ergosterol against breast, colorectal, ovarian and renal cancer cell lines (He, Shi, Liu, Zhao, & Zhang, 2018; Kang et al., 2015; L. M. Kuo et al., 2005; Martínez-Montemayor et al., 2019; Russo et al., 2010; Tan et al., 2017).

Mechanistically, ergosterol in its pure or derivative forms were reported to induce caspase-mediated intrinsic apoptosis, arrest cell cycle and inhibit migration and invasion of cancer cells while regulating several signalling pathways including the STAT3 and the IGFR/IRS-1/2-MEK-ras-ERK1/2 (W. J. Chen et al., 2008; Li et al., 2015; Martínez-Montemayor et al., 2019; Tan et al., 2017). Downregulation of β-catenin has also been identified as one of the key molecular mechanisms of action against colorectal and renal cancer cells (He et al., 2018; Kang et al., 2015). β-Catenin is associated with cadherin-mediated intercellular adhesion and the Wnt signalling pathway (Sineva & Pospelov, 2014).

The AK3/STAT3/NF-κB pathway was found to play an important role in mediating the anti-inflammatory activity of ergosterol and ergosterol peroxide in cellular and animal models (Huan, Tianzhu, Yu, & Shumin, 2017; Kobori, Yoshida, Ohnishi-Kameyama, & Shinmoto, 2007; C.-F. Kuo, Hsieh, & Lin, 2011; Zhang, Xu, Li, & Wang, 2015). The anti-inflammatory activity of ergosterol has also been attributed to the suppression of the proinflammatory cytokines including

TNF-α, IL-6 and IL-1β (Huan et al., 2017). Although these reports demonstrated the potential therapeutic benefits of ergosterol and its derivatives against cancer and inflammatory diseases, further *in vivo* mechanistic studies to evaluate their bioavailability and potential toxicity are warranted to lay the foundation for future clinical trials. Moreover, preclinical studies investigating the interactions of ergosterol (and its derivatives) with common anticancer and anti-inflammatory drugs could also open up exciting new avenues for prospective combination therapies especially for rare cancers and inflammatory diseases with limited therapeutic options.

6. Future directions

Drying may precede extraction and may affect the retention of both ergosterol and vitamin D_2. Due to the controversial results and limited knowledge regarding ergosterol and vitamin D_2 stability during drying, further studies are required in order to elucidate the mechanism of vitamin D_2 formation at different drying temperatures. -Omics technologies, including metabolomics, genomics, and proteomics may facilitate in better understanding of sterol retention in mushrooms after the application of different drying techniques. Apart from the conventional drying technologies that have been applied to date, future studies should be conducted investigating the effect of ohmic heating (also known as Joule heating) on dried mushroom bioactive compounds. Ohmic heating is a process of heating the food by passing electric current and may result in faster dehydration of food, avoiding any color deterioration and nutritional value degradation (Kaur & Singh, 2016).

The extraction of sterols from mushrooms requires long extraction times when conventional extraction protocols are applied. The application of non-conventional extraction technologies such as UAE and MAE may result in pure extracts without saponification decreasing the extraction times. Future studies should focus on the development of sustainable extraction protocols on a commercial scale for the recovery of ergosterol and vitamin D_2 from mushrooms. This could be achieved by the combination of two or more different non-conventional extraction techniques. Pulsed electrical fields (PEF) which is a non-thermal technique has the potential of being employed for the preparation of pure extracts enriched in vitamin D_2 and ergosterol. PEF may result in an increase mass transfer by electroporation which induces cell membrane permeabilization (Barbosa-Pereira, Guglielmetti, & Zeppa, 2018).

The use of vitamin D extracts from mushroom waste in new foods could benefit the food industry, yet, the practical applications of vitamin D_2 from mushroom by-products are limited. Therefore, potential food applications of mushroom extracts enriched in ergosterol and vitamin D_2 is a field that requires further work. Nonetheless, the legislation should be considered when using vitamin D_2 extracts from mushroom waste in new foods. Similarly, despite its potential especially in anticancer and anti-inflammatory drug development, the pharmaceutical application of ergosterol and vitamin D_2 from mushroom needs further in vivo mechanistic studies for future clinical trials. As novel drug development entails large resources and time, the approach of combining pre-existing drugs with natural product-based adjuvants to increase their efficiency is promising and economical. Therefore, studies investigating the potential synergistic interactions of ergosterol and vitamin D_2 with pre-existing anticancer and anti-inflammatory drugs can provide interesting insights for their future pharmaceutical applications.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Figures

Figure 1. Process of conversion of ergosterol into vitamin D_2 in fungal sources.

Figure 2. Extraction and determination of ergosterol and vitamin D_2 by (a) conventional and (b) non-conventional extraction methods.

Figure 3. Mechanisms involved in ultrasound-assisted extraction (UAE).

877 **Table 1.** Summary of studies conducted to date extracting vitamin D_2 and ergosterol from mushrooms.

Mushroom species	Extraction method	Solvent used	Compound extracted	Extraction yields (mg/ 100g dw)	References
Non-conventional extraction techniques					
A. bisporus	MAE	Ethanol	Ergosterol	555.3 ± 23.6	(Heleno et al., 2016b)
A. bisporus	UAE	Ethanol	Ergosterol	671.5 ± 0.5	(Heleno et al., 2016a)
A. bisporus	DES/UAE	Glycerol: Choline chloride	Vitamin D_2	1.2 ± 0.3	(Patil et al., 2018)
A. bisporus	DES/MAE	Glycerol: Choline chloride	Vitamin D_2	0.31 ± 0.04	(Patil et al., 2018)
A. bisporus	PLE	Ethanol	Ergosterol/total sterols	N.M.	(Gil-Ramirez et al., 2013)
	SFE	CO_2/Ethanol as co-solvent	Ergosterol/total sterols	N.M.	(Gil-Ramirez et al., 2013)
A. bisporus	SFE	CO_2	Ergosterol	39.5 ± 2.8	(Young, 1995)
T. melanosporum, T. aestivum, T. indicum	UAE	Chloroform: Methanol	Ergosterol	190 ± 13, 186 ± 25 and 137 ± 12, respectively	(Villares, Garcia-Lafuente, Guillamon, & Ramos, 2012)
Different species of edible mushrooms	UAE	Chloroform: Methanol	Ergosterol	Ranged from 23 ± 1 to 681 ± 72	(Villares, Mateo-Vivaracho, Garcia-Lafuente, & Guillamon, 2014)
L. edodes	SFE	CO_2	Ergosterol, Vitamin D_2	N.M.	(Morales et al., 2017)
A. bisporus	MAE	Methanol	Ergosterol	687 ± 0.4	(Young, 1995)
A. blazei	MAE	Ethanol	Ergosterol	25.44 ± 5.1	(Taofiq et al., 2019)
A. blazei	UAE	Ethanol	Ergosterol	21.49 ± 0.9	(Taofiq et al., 2019)
Conventional extraction techniques					
A. bisporus	SE/saponification	Ethanol	Ergosterol	676 ± 3	(Heleno et al., 2016a)
Different species of cultivated and wild mushrooms	SE/saponification	Hexane	Ergosterol	Ranged from 77.0 ± 1.0 to 352.0 ± 1.0, respectively	(Barreira, Oliveira, & Ferreira, 2014)
Different species of cultivated mushrooms	Saponification/ LLE	Ethanol/Pentane	Vitamin D_2, Ergosterol	Vitamin D_2 ranged from 1.25 ± 0.03 to 4.51 ± 0.31; Ergosterol ranged from 68 ± 14 to 780 ± 35	(Jasinghe & Perera, 2005)
Different species of cultivated mushrooms	Saponification/ LLE	Ethanol/Pentane	Vitamin D_2	Vitamin D_2 ranged from 2.29 ± 0.27 to 18.4 ± 0.57	(Jasinghe & Perera, 2006)
A. bisporus	Saponification/ LLE	Ethanol/Heptane	Vitamin D_2	0.38 ± 0.02	(Roberts, Teichert, & McHugh, 2008)

A. blazei	SE	Ethanol	Ergosterol	58.53 ± 1.72	(Correa et al., 2019)
A. bisporus	Saponification/ LLE	Methanol/ Pentane	Ergosterol	409 ± 1.1	(Young, 1995)
A. blazei	HAE	Ethanol	Ergosterol	18.84 ± 2.3	(Taofiq et al., 2019)

A. Bisporus: *Agaricus bisporus*
DES: Deep eutectic solvents
Dw: Dry weight
HAE: Heat-assisted extraction
L. edodes: *Lentinula edodes*
LLE: Liquid-liquid extraction
MAE: Microwave-assisted extraction
PLE: Pressurized liquid extraction
SE: Soxhlet extraction
SFE: Supercritical fluid extraction
T. melanosporum: *Tuber melanosporum*
T. aestivum: *Tuber aestivum*
T. indicum: *Tuber indicum*
UAE: Ultrasound-assisted extraction

911 **Table 2.** Summary of high-performance liquid chromatography (HPLC) conditions been for the detection
912 of ergosterol and vitamin D_2 in mushrooms.

Column/ temperature/ injection volume	Sterol identified	Mobile phases/Flow rate	Detector/ Wavelength	Reference
RP-C_{18} column/ N.M./ N.M.	Ergosterol/ Vitamin D_2	Methanol/H_2O (95:5, v/v)/ Flow rate of 1.0 mL/min/Isocratic elution	UV-Vis/ 254 nm	(Huang et al., 2015)
RP-C_{18} column/ 40 °C/ 10 µL	Ergosterol/ Vitamin D_2	Three mobile phases: Methanol (A), acetonitrile (B) and 0.05% (v/v) formic acid (C)/ Flow rate of 1.0 mL/min/ Gradient elution	DAD/ 265 nm	(Ahlborn et al., 2018)
RP-C_{18}/ 35 °C/ 20 µL	Ergosterol	Acetonitrile/methanol (70:30, v/v)/ Flow rate of 1 mL/min/Isocratic elution	UV-Vis/ 280 nm	(Heleno et al., 2016b)
RP C_{18} column/ 25 °C/ 10 µL	Ergosterol	Acetonitrile/methanol (75:25, v/v)/ Flow rate of 1.5 mL/min/Isocratic elution	PAD/ 280 nm	(Villares et al., 2014)
RP C_{18} column/ 25 °C/ 20 µL	Ergosterol	Acetonitrile/methanol (75:25, v/v)/ Flow rate of 1.5 mL/min/Isocratic elution	PAD/ 280 nm	(Villares et al., 2012)
RP-C30 column/ 50 °C/ 20 µL	Ergosterol/ Vitamin D_2	Methanol 85% (v/v) (A), ethanol (B)/ Flow rate of 1.0 mL/min/ Gradient elution	DAD/ 265 nm, PDA coupled with MS	(Morales et al., 2017)
RP-C_{18} column/ 30 °C/ 20 µL	Ergosterol/ Vitamin D_2	Two mobile phases: Methanol/water 80:20 (v/v) (A), methanol/dichloromethane, 75:25 (v/v) (B)/ Flow rate of 1.0 mL/min/ Gradient elution	PAD/ 280 nm for ergosterol and 266 nm for vitamin D_2	(Yuan, Kuang, Wang, & Liu, 2008)
RP-C_{18} column/ room temperature/ 20 µL	Ergosterol	Two mobile phases: Methanol/water 80:20 (v/v) (A), methanol/dichloromethane, 75:25 (v/v) (B)/ Flow rate of 1.0 mL/min/ Gradient elution	PDA/ 280 nm	(Yuan et al., 2007)
RP-C_{18} column/N.M./100 µL	Ergosterol/ Vitamin D_2	Methanol/acetonitrile 25:75 (v/v)/ Flow rate of 0.2 mL/min/Isocratic elution	MS/ MS	(Koyyalamudi, Jeong, Song, Cho, & Pang, 2009)

RP-C_{18} column/N.M./ 20 µL	Ergosterol/ Vitamin D_2	Acetonitrile/methanol 75:25 (v/v) / Flow rate of 2.3 mL/min/ Isocratic elution	UV-Vis/ 282 nm	(Jasinghe & Perera, 2006; Jasinghe et al., 2007)
RP-C_{18} column/35°C/N.M.	Ergosterol	Acetonitrile/methanol 70:30 (v/v) / Flow rate of 1.0 mL/min/ Isocratic elution	UV-Vis/ 280 nm	(Taofiq et al., 2019)
UHPLC; RP-C_{18} column /30°C/1µL	Ergosterol/ Vitamin D_2	Methanol/ Flow rate 0.3 mL/min/Isocratic elution	MS/MS	(Slawinska et al., 2016)

RP: Reverse phase
UHPLC: Ultra-high-performance liquid chromatography
N.M.: Not mentioned
UV: Ultraviolet
DAD: Diode-array-detector
PAD: Photodiode array detector
MS: Mass spectrometer
d.w.: dry weight
d.s.: dry solids

937 **Table 3.** Summary of health claims allowed on foods that are at least a "source of" or "high" in vitamin D.

Claim type	Claim	Health relationship
Art.13(1) - Function Health Claims' relating to the growth, development and functions of the body	Vitamin D contributes to normal absorption/utilization of calcium and phosphorus	Absorption and utilization of calcium and phosphorus and maintenance of normal blood calcium concentrations
	Vitamin D contributes to normal blood calcium levels	Absorption and utilization of calcium and phosphorus and maintenance of normal blood calcium concentrations
	Vitamin D contributes to the maintenance of normal bones	Maintenance of bones and teeth
	Vitamin D contributes to the maintenance of normal muscle function	Normal muscle function
	Vitamin D contributes to the maintenance of normal teeth	Maintenance of bones and teeth
	Vitamin D contributes to the normal function of the immune system	Normal function of immune system and inflammation response
	Vitamin D has a role in the process of cell division	Cell division
Art.14(1)(b) - Claims referring to children's development	Vitamin D is needed for normal growth and development of bone in children.	
	Vitamin D contributes to the normal function of the immune system in children	

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Table 4. Preclinical studies underlining the bioactivity of ergosterol and its derivatives and possible molecular mechanisms of action.

Anticancer activity

Type of compound and derivatives	Source	Study types, cell lines and animal models	Activity	Molecular mechanisms of action	Reference
Ergosterol, 5,6-dehydroergosterol, ergosterol peroxide and ergosterol peroxide sulfonamide	*Ganoderma lucidum*	*In vitro* MDA-MB-231 and MCF7 breast cancer, SUM-149, and SUM-190 inflammatory breast cancer (IBC), KOPN8, BCR-ABL, UoCB-1, SUP-B15, and NALM06 human and murine leukemia cells, BJ normal human skin fibroblasts and MCF10A noncancerous mammary epithelial cells.	Ergosterol peroxide reduced the viability of the SUM-149 cells in a time- and dose-dependent manner ($p < 0.05$) with EC_{50} values of 34 and 20 µM at 24 and 72 h, respectively. It also reduced the viability of the MDA-MB-231 and SUM-190 IBC cells dose-dependently with EC_{50} of 19 and 43 µM, respectively. Presence of vacuoles and altered cell morphology was observed in SUM-49 cells after the treatment with ergosterol peroxide. In other cancer cells, ergosterol peroxide reduced viability with EC_{50} values 7 - 22 µM with no cytotoxicity against the normal cells.	Ergosterol peroxide generated reactive oxygen species (ROS), arrested cell cycle at G1 phase, induced apoptosis mediated via caspase-3/7 activation, and cleavage of PARP. It also decreased the migration and invasion of cancer cells while preventing the expression of total AKT1, AKT2, BCL-XL, Cyclin D1 and c-Myc in the tested IBC cells. Ergosterol peroxide sulfonamide, a derivative of ergosterol improved the activity against IBC cells with a therapeutic index of > 10 compared to normal cells.	(Martínez-Montemayor et al., 2019)
Ergosterol peroxide	*Paecilomyces cicadae*	*In vitro* Human renal cell carcinoma (RCC) cells	Significantly inhibited the growth of RCC cells with an IC_{50} value of 30 µM and significantly decreased the colony number compared with the control ($p < 0.01$) *in vitro*.	Dose-dependently induced apoptosis (caspase-3), inhibited the migration and invasion and arrested the cell cycle of RCC cells at G_0/G_1 phase with downregulation of β-catenin expression.	(He et al., 2018)

Ergosterol peroxide	Chemical standard	*In vitro* OVCAR-3, CAOV3, OC-7 and MPSC-1 ovarian cancer and normal OSE cells	Significantly inhibited the growth of cancer cells with no apparent cytotoxicity against the normal OSE cells.	Downregulated β-catenin (reduced cyclin D1 and c-Myc expression) and modulated STAT3 signaling (activation of SHP2 phosphatase and inhibition of Src kinase) pathways.	(Tan et al., 2017)
5α,8α-Ergosterol Peroxide 3-Carbamate Derivatives	Chemical synthesis	*In vitro* HepG2, SK-Hep1human hepatocellular carcinoma, and MCF7, MDA-MB231 human breast cancer cells	Significant antiproliferative activity by 5α,8α-Epidioxyergosta-3-yl-(piperazine-1) carbamate and 5α,8α-epidioxyergosta-3-yl-(piperidin-4-ethylamine)carbamate and their hydrochloride salts in vitro with IC_{50} values 0.85 - 4.62 μM.	5α,8α-Epidioxyergosta-3-yl-(piperazine-1) carbamate showed mitochondrial localization.	(Bu et al., 2017)
Ergosterol derivatives	*Ganoderma lucidum*	*In vitro* MDA-MB-231, HepG2, and A549 cancer, human umbilical vein endothelial (HUVECs)and normal NIH/3T3 fibroblast cells	(3*β*, 5*α*, 6*β*, 22*E*)-ergosta-7, 9 (11), 22-triene-3, 5, 6-triol exhibited selective toxicity against MDA-MB-231 and HepG2 cells with IC_{50} values 56.3 - 62.5 μM. (3*β*, 5*α*, 6*β*, 9*α*, 22*E*)-ergosta-7, 22-diene-3, 5, 6, 9-tetrol showed selective toxicity against HUVECs. Compounds did not show cytotoxicity against the normal cells.	Not reported	(Chen et al., 2017)
Ergosterol peroxide	*Inonotus Obliquus*	*In vitro* HCT116, HT-29, SW620 and DLD-1 human colorectal cancer cells *In vivo* Colitis-induced (AOM/DSS-treated) adenocarcinoma C57BL/6 female mice	Dose-dependent reduction of cellular viability of the tested cancer cell lines. Time-dependent reduction of anchorage-dependent colony formation. Suppressed tumour growth in AOM/DSS-treated mice.	Induced caspase-3 mediated apoptosis, PARP cleavage and arrested all CRC cells at $subG_1$ stage. Suppressed the nuclear levels of β-catenin leading to reduced transcription of c-Myc, cyclin D1, and CDK-8.	(Kang et al., 2015)

Ergosterol	*Amauroderma rude*	*In vitro* MDA-MB-231, SK-BR-3, MDA-MB-468, MCF7, and 4T1 breast cancer and NIH3T3 non cancer fibroblast cells.	Time and dose-dependent reduction of cellular viability of the tested cancer cell lines with no cytotoxicity against the NIH3T3 cells. At 75 µM, it inhibited the colony formation in both the sizes and number of the colonies of MDA-MB-231 cells. Prolonged the survival of B16-induced tumour-bearing Balb/c mice.	Induced apoptosis, inhibited cell migration and invasion with upregulation of tumour suppressors-Foxo3, Bim, Fas, and Fas L in MDA-MB-231 cells	(Li et al., 2015)
Ergosterol and ergosterol peroxide	*Inonotus obliquus*	*In vitro* PC3 human prostatic carcinoma and MDA-MB-231 breast carcinoma cells	Ergosterol (9.82- >100 µM) and ergosterol peroxide (30.23- 38.19 µM) exhibited cytotoxicity against the PC3 and MB-231 cells.	Not reported	(Ma, Chen, Dong, & Lu, 2013)
MT19c, a heterocyclic vitamin-D derivative	Chemical synthesis	*In vitro* SKOV-3 human ovarian epithelial adenocarcinoma, BxPC-3 human pancreatic adenocarcinoma, PC-3 and LNCaP human prostate adenocarcinoma, SH- SY5Y, SMS-KCNR and SK-N-SH human neuroblastoma cells	Displayed significant cytotoxicity against SKOV-3 and PC-3 cancer cells (with the IC_{50} values in the range of 100-300 nM) but not against the other tested cell lines. Significantly greater cytotoxicity was observed against SKOV-3 cells compared to cisplatin, Iressa and the vitamin-D receptor (VDR) ligands- calcitriol and EB1089. The cytotoxicity was comparable to Taxol.	Caspase dependent apoptosis, DNA fragmentation and cell cycle abrogation in SKOV-3 cells. Downregulated the IGFR/IRS-1/2-MEK-ras-ERK1/2-pathway via activated TNFα-receptor/SAPK/JNK component without altering VDR.	(Brard et al., 2011)
Ergosterol, ergosterol peroxide, (22E)-ergosta-7,22-dien-5α-hydroxy-3,6-dione	*Dyctionema glabratum, Leioderma pycnophorum* and chemical synthesis	*In vitro* LNCaP and DU-145 human prostate cancer cells	Ergosterol peroxide and (22E)-ergosta-7,22-dien-5α-hydroxy-3,6-dione exhibited significant cell growth inhibition in the MTT assay with the IC_{50} values in the range of 6.12 – 24.03 µM/mL against both cell lines. Ergosterol was reported to have an IC_{50} value of > 100 µM/mL against both cell lines.	Ergosterol peroxide and (22E)-ergosta-7,22-dien-5α-hydroxy-3,6-dione induced apoptosis with a significant increase in caspase-3 activity, marker of membrane breakdown and DNA fragmentation.	(Russo et al., 2010)
Ergosterol and ergocalciferol	Chemical standard	*In vitro* HL-60 human leukemia cells	Dose-dependent cell growth inhibition was exhibited by ergocalciferol and ergosterol. Ergocalciferol displayed better activity than ergosterol.	Induced DNA fragmentation and increased sub-G_1 DNA contents in HL-60 cells. Modulated mitochondrial ROS production, GSH depletion and Fas induction. Induced caspase (2, -3, -	(Chen et al., 2008)

				6, and -9) dependent apoptosis as indicated by the cleavage of downstream caspase-3 targets, DNA fragmentation factor (DFF-45), and PARP. Reduced Mcl-1 to Bax ratio indicating mitochondrial decay.	
Ergosterol peroxide	*Penicillium oxalicum*	*In vitro* COLO-205 human colon tumour, KB human oral epithelium carcinoma, Hepa-3B hepatoma and Hela cervical carcinoma cells	Displayed cytotoxicity against the COLO-205 cells with an ED_{50} of 8.56 μg/mL. No activity against the other cell lines.	Inhibited the relaxation of supercoiled DNA (pBR322) induced by DNA topoisomerase I.	(Kuo et al., 2005)
Ergosterol peroxide	*Sarcodon aspratus*	*In vitro* HL-60 human leukemia cells	Cell growth inhibition at a dose of >10 μM.	Nucleosomal DNA fragmentation.	(Takei, Yoshida, Ohnishi-Kameyama, & Kobori, 2005)
Ergosterol peroxide	*Paecilomyces tenuipes*	*In vitro* SNU-1 human gastric tumour, SNU-354 human hepatoma, SNU-C4 human colorectal tumour and murine sarcoma-180 cells	The IC_{50} values were 18.7, 158.2, 84.6 and 74.1 μM against the SNU-1, SNU-354, SNU-C4 and murine sarcoma-180 cells lines, respectively.	Not reported	(Nam, Jo, Kim, Hyun, & Kim, 2001)

Anti-inflammatory activity

Type of compound and derivatives	**Source**	**Study types, cell lines and animal models**	**Activity**	**Molecular mechanisms of action**	**Reference**
Ergosterol	Chemical standard	*In vivo* Cigarette smoke (CS)-induced chronic obstructive pulmonary disease (COPD) in male ICR mice	Inhibited CS-induced pathological injury in lung tissue.	Restored superoxide dismutase activity in the serum and the lung and catalase activity in serum. Decreases the content of Malondialdehyde in the serum and the lung. Inhibited pro-inflammatory cytokines including tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β) in serum and the lung. Protective effect on CS-induced COPD by inhibiting	(Huan et al., 2017)

				inflammatory response via the AK3/STAT3/NF-κB pathway.	
Ergosterol	*Scleroderma Polyrhizum*	*In vivo* Female BALB/c mice	Ergosterol pretreatment at 25 and 50 mg/kg decreased lipopolysaccharide (LPS)-induced lung histopathological changes and lung wet-to-dry weight ratio	Suppressed inflammatory cells and proinflammatory cytokines including TNF-α and IL-6. Blocked the activation of NF-*κ*B, COX-2 and iNOS pathways.	(Zhang et al., 2015)
Ergosterol	*Cordyceps militaris* and chemical standard	*In vitro* BV2 microglia cells	Dose-dependent reduction of NO production ranging from 13% to 48% at 1 μg/mL – 10 μg/mL with no cytotoxicity at 0.1-10 μg/mL. Commercial ergosterol was not effective.	Not reported	(Nallathamby et al., 2015)
Ergosterol and ergosterol peroxide	*Inonotus obliquus*	*In vitro* RAW 264.7 murine macrophage cells	Inhibited the production of nitric oxide (NO).	Inhibited NF-*κ*B luciferase activity.	(Ma et al., 2013)
Ergosterol	Chemical standard	*In vitro* RAW 264.7 murine macrophage cells	Dose-dependent cytotoxicity with IC_{50} of 24.5 μg/mL	Downregulated NF-*κ*B cascade-associated proteins, inhibited TNF- α production and COX-2 expression and modulated Rho family GTPase and cytoskeleton-related proteins at 10 μg/mL.	(Kuo et al., 2011)
Ergosterol	Saireito	*In vitro* Mucosal-type murine bone marrow-derived mast cells (mBMMCs)	The degranulation of mBMMCs was significantly suppressed at ≥32 μM in a dose-dependent manner.	Not reported.	(Kageyama-Yahara, Wang, Wang, Yamamoto, & Kadowaki, 2010)
Ergosterol peroxide	*Heritiera littoralis bark*	*In vitro* RAW 264.7 murine macrophage cells	Inhibited NO and PGE2 release with the IC_{50} values of 2.5 μM and 28.7 μM, respectively.	Dose-dependent downregulation of mRNA expressions of iNOS and COX-2.	(Tewtrakul, Tansakul, Daengrot, Ponglimanont, & Karalai, 2010)
Ergosterol, ergosterol peroxide, 5a,8a-epidioxy-(24S)-	*Lactarius hatsudake*	-	Ergosterol peroxide and 5a,8a-epidioxy-(24S)-ergosta-6-en-3b-ol selective inhibitory activity against *Crotalus adamenteus* venom	Not reported	(Gao et al., 2007)

ergosta-6-en-3b-ol and cerevisterol			phospholipase A2 (PLA2) enzyme with an ED_{50} value of 100 µg/mL		
Ergosterol peroxide	*Sarcodon aspratus*	*In vitro* RAW264.7 mouse macrophage and HT29 human colorectal adenocarcinoma cells	Ergosterol peroxide suppressed LPS-induced TNF-α secretion and IL-*1α/β* expression in RAW264.7 cells	Suppressed NF-*k*B and C/EBPβ transcriptional activity, and phosphorylation of MAPKs. Inhibited cell growth and STAT1 mediated inflammatory responses by modulating the redox state in HT29 cells.	(Kobori et al., 2007)
Ergosterol peroxide	*Cordyceps cicadae*	*In vitro* Primary human T lymphocytes	Inhibited T-cell proliferation for about 24 h after stimulation with phytohemagglutinin (PHA).	Suppressed the expression of cyclin E, IFN-γ, IL-2, and IL-4, and by arrested cell cycle progression from the G1 to the S phase in T lymphocytes. Downregulated AP-1 proteins including c-Fos and c-Jun in activated T lymphocytes.	(Kuo, Weng, Chou, Chang, & Tsai, 2003)
Ergosterol and ergosterol peroxide	*Naematoloma fasciculare*	Normal human serum	Ergosterol and ergosterol peroxide displayed anti-complimentary activity on the classical pathway with IC_{50} values of 5 and 1 µM, respectively.	Not reported	(Kim et al., 1997)
Other bioactivity					
Type of compound and derivatives	**Source**	**Study types, cell lines and animal models**	**Activity**	**Molecular mechanisms of action**	**Reference**
Ergosterol	*Pleurotus salmoneostramineus*	*In vitro* Trypanosoma cruzi maintained in LLC-MK2 cells, peritoneal macrophages from the peritoneal cavity of female BALB/c mice	Exhibited antiparasitic activity against trypomastigotes, with an IC_{50} value of 51.3µg/mL with no cytotoxicity against mammalian peritoneal macrophages even at 200 µg/mL.	Permeabilization of the plasma membrane and depolarization of mitochondrial membrane Potential. No ROS generation.	(Alexandre et al., 2017)
Ergosterol peroxide	*Cordyceps cicadae*	*In vitro* Transforming growth factor-β1 (TGF-β1) treated NRK-49F normal rat kidney fibroblast cells	Mitigated the proliferation of renal fibroblast cells in dose (6.25-25 µM) and time-dependent manner.	Downregulated the expression of cytoskeleton protein and CTGF and ECM production. Inhibited TGF-β1-stimulated phosphorylation of ERK1/2, p38 and JNK pathway.	(Zhu, Zheng, Deng, Chen, & Zhang, 2014)
Ergosterol	*Cordyceps sinensis*	*In vitro* LX-2 human hepatic stellate and HL-7702 human hepatic cell lines	Ergosterol inhibited activated LX-2 and HL-7702 cells in a dose-dependent manner. It protected the	Upregulated expressions of permeability of the lysosomal membrane and downregulated the levels of EdU, F-actin, and *α*-SMA.	(Peng et al., 2014)

	In vivo Male C57BL/6 mice	liver against CCl4-induced hepatic fibrosis in vivo at a 50 mg/kg dose.	Improved- serum liver function and expression of α-SMA in vivo. Reduced- liver/BW or spleen/BW, hepatic inflammation and collagen deposition in vivo.