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

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1 **Recovery of ergosterol and vitamin $D_2$ from mushroom waste - potential valorization by food**
2 **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

64 **1. Introduction**

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

72 Vitamin D is an essential vitamin for human health and plays a vital role in the regulation
73 and maintenance of calcium homeostasis. Vitamin D deficiencies have also been linked to an
74 increased risk of cancer, hypertension, autoimmune diseases, and diabetes (Jäpelt & Jakobsen,
75 2013). The main reasons for failing to meet vitamin D requirements are i) low dietary intake, since
76 only a few food products naturally contain vitamin D and ii) limited vitamin D synthesis due to
77 inadequate exposure to sunlight (Bailey et al., 2010; Jäpelt & Jakobsen, 2013).

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

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

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

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

102 The recovery of ergosterol and vitamin D is carried out by the application of either
103 conventional methods such as Soxhlet extraction or non-conventional such as ultrasound-assisted
104 extraction (UAE), microwave assisted extraction (MAE), deep eutectic solvents (DES),
105 supercritical fluid extraction (SFE), and pressurized liquid extraction (PLE) (Heleno, Diz, et al.,
106 2016; Heleno, Prieto, et al., 2016; Morales et al., 2017; Patil, Ghodke, Jain, & Dandekar, 2018).
107 Extraction method might have a significant impact on the recovery of both ergosterol and vitamin
108 $D_2$, therefore, the extraction conditions should be carefully selected and optimized. The application
109 of some non-conventional methods such as UAE and MAE may facilitate the avoidance of
110 saponification step reducing the total extraction time. Apart from the extraction, sample
111 preparation may impact the retention of vitamin D and ergosterol in samples (Gąsecka et al., 2019;
112 Tian, Zhao, Huang, Zeng, & Zheng, 2016).

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

116

117 ## 2. Ergosterol and vitamin $D_2$ in mushrooms

118 Vitamin D occurs in two main forms in nature: Vitamin $D_2$ (ergocalciferol) and Vitamin
119 $D_3$ (cholecalciferol). Vitamin $D_3$ is only found in animal sources such as fish liver oils, eggs and
120 milk products. Vitamin $D_2$ found in limited plant sources in far fewer amounts, putting in
121 vegetarians and vegans being at risk of having vitamin D deficient diets (Ho-Pham, Vu, Lai,
122 Nguyen, & Nguyen, 2012). Mushroom qualify for such a diet and while they are deficient in
123 vitamin D, widely available ones such as shiitake and button mushrooms, are rich sources of
124 ergosterol, a precursor of vitamin $D_2$. Ergosterol can be converted into vitamin $D_2$ by natural or
125 artificial UV irradiation which is a process that has been widely studied (Black, Lucas, Sherriff,
126 Bjorn, & Bornman, 2017; Taofiq, Fernandes, Barros, Barreiro, & Ferreira, 2017).

127 Mushrooms and other kinds of fungi have high concentrations of ergosterol in their cell
128 membranes where it performs a role similar to that of cholesterol in animals and sitosterol in plants,
129 i.e., strengthening and maintaining fluidity of cell membranes, protein functionality, and
130 modulating intracellular transport (Lingwood & Simons, 2010; Roncero-Ramos & Delgado-
131 Andrade, 2017). It is produced by a fungal specific pathway. Similar to animals, fungi convert
132 acetyl-CoA to lanosterol via the formation of squalene epoxide, however, fungi have a longer and
133 more energy-expensive conversion final process in which 11 oxygen atoms are added to lanosterol
134 to produce ergosterol whereas animals add 10 oxygen atoms in a more efficient process to produce
135 cholesterol (Dupont et al., 2012).
136 Figure 1 shows the process of conversion of ergosterol in fungal sources into vitamin $D_2$.
137 On exposure to UV radiation, ergosterol in exposed portions of mushrooms undergoes
138 photochemical cleavage in the B ring, leading to the formation of an intermediate called pre-
139 vitamin $D_2$. On subjection to heat, this intermediate undergoes thermal isomerization to form
140 ergocalciferol, i.e., vitamin $D_2$. The yield of the final product is heavily dependent on the balance
141 between thermal and photochemical reactions (Jasinghe, Perera, & Sablani, 2007), hence the
142 temperature of radiation is critical. Higher temperatures direct the reaction towards the formation
143 of by-products such as lumisterol and tachysterol, in addition to adversely affecting the texture and
144 colour of the mushrooms. Moisture content is also important since excessive dryness leads to an
145 increase in surface area and hence exposure to oxygen, which leads to oxidation of vitamin $D_2$. On
146 the other hand, excess moisture may have a dilution effect on ergosterol content, which might
147 result in a lower conversion rate to vitamin $D_2$ (Jasinghe et al., 2007). However, the total amount
148 of ergosterol produced per gram of mushroom might not be affected by the excess moisture
149 content. Hence, the vitamin $D_2$ fortification process in mushrooms should go hand in hand with
150 minimizing moisture loss and maintaining hardness. The optimal conditions vary with types of
151 mushrooms and there have been many studies to determine them (Ahn, 2018; Lee & Aan, 2016;
152 Won et al., 2018).
153

154 ## 3. Extraction and determination of ergosterol and vitamin $D_2$ from mushrooms

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

158 quantification using HPLC or GC. The first step for the extraction of ergosterol and vitamin $D_2$ is
159 sample preparation. Although samples can be either fresh or dried, most of the studies have
160 determined sterols in lyophilized samples (Heleno et al., 2016a; Heleno et al., 2016b; Teichmann,
161 Dutta, Staffas, & Jägerstad, 2007; Wittig, Krings, & Berger, 2013). To date, not much work has
162 been done on the bulk recovery of ergosterol and vitamin $D_2$ from mushrooms but that there is
163 much to be learned from work done on its analytical determination.
164
165 **3.1 Sample preparation**
166 Sample preparation precedes extraction and determination steps. Studies have shown that
167 sample preparation may have a significant impact on the sterol content of different mushroom
168 species (Gąsecka et al., 2019; Nölle, Argyropoulos, Müller, & Biesalski, 2018). Recently, Gąsecka
169 et al. (2019) reported that the ergosterol content of two mushroom species (*Hericium erinaceus*
170 and *Leccinum scabrum*) was significantly affected by the processing method employed.
171 Specifically, the authors noted that the ergosterol content was higher in the fresh mushroom
172 samples while it declined as the drying temperature increased from 20 to 70 °C (Gąsecka et al.,
173 2019). Slawinska et al. (2016) investigated the effect of hot-air drying (the initial drying
174 temperature was 40 °C, and in the final stage of drying the temperature was raised to 60 °C) and
175 freeze-drying on ergosterol and vitamin $D_2$ contents of three different mushroom species (*Agaricus*
176 *bisporus*, *Pleurotus ostreatus* and *Lentinula edodes*). The authors reported that the vitamin $D_2$
177 content was higher in the mushrooms dried by freeze-drying than those dried by hot-air drying,
178 while drying treatment had a slight significant effect only on the ergosterol content of *A. bisporus*
179 (Slawinska et al., 2016). Similar results were reported by Bernas and Jaworska (2017) where
180 ergosterol content was higher in freeze-dried *A. bisporus* than its air-dried counterpart (30% loss).
181 Tian et al. (2016) examined the effect of different drying methods (microwave vacuum-drying
182 (microwave power density of 15 W/g, vacuum degree of −80 kPa, for 11 min), hot-air drying (60-
183 70 °C), microwave-drying (539 W for 18 min), vacuum-drying (−90 kPa at 60 °C for 15 h)) on
184 shiitake mushroom nutrient retention, including vitamin $D_2$. The authors noted that drying resulted
185 in the degradation of vitamin $D_2$, with the hot-air and microwave-drying resulting in greater
186 vitamin $D_2$ loss, compared to the other two drying techniques (Tian et al., 2016). These results
187 show the sensitivity of vitamin $D_2$ to degradation in the presence of oxygen. Nölle et al. (2018)
188 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)

220 Saponification is a hydrolysis reaction where free hydroxide breaks the ester bonds
221 between the fatty acids and glycerol of a triglyceride, resulting in free fatty acids and glycerol
222 (Prabu, Suriya Prakash, & Thirumurugan, 2015). In ergosterol and vitamin $D_2$ extraction from
223 mushrooms, saponification can be conducted either on samples or on extracts derived from
224 different extraction methods. When hydrolysis is applied on mushroom samples, it breaks the
225 complex structure of mushrooms and improves the recovery of ergosterol and vitamin $D_2$ from the
226 surrounding matrix. Moreover, when conjugated sterols are hydrolyzed to free sterols, they have
227 similar polarity and can be extracted with a single solvent (Han & Zhou, 2015). The saponification
228 may take place at temperatures varying from 60 and 80 °C and times varying from 15 to 60 min
229 (Barreira et al., 2014; Gil-Ramirez et al., 2013; Heleno et al., 2016a). During the hydrolysis,
230 ascorbic acid solution is used to avoid any thermal degradation of sterols. Recent studies have
231 shown that saponification step can be avoided when some non-conventional extraction techniques
232 such as UAE and MAE are employed (Heleno et al., 2016a; Heleno et al., 2016b).
233
234 **3.2.2 Non-conventional extraction methods**
235 **3.2.2.1 Ultrasound-assisted extraction (UAE)**
236 UAE has been employed for the extraction of different classes of bioactive compounds
237 from mushrooms such as polyphenols, sugars, and vitamins from different mushrooms species
238 (Aguiló-Aguayo et al., 2017; Alzorqi, Sudheer, Lu, & Manickam, 2017; Heleno et al., 2016a; Xu
239 et al., 2016). Ultrasound devices use ultrasound waves above human hearing (>20 kHz) in which
240 pressure fluctuation leads to the cavitation with the resultant unstable bubbles imploding and
241 damaging cell membranes allowing for mass transfer out of the cell (Chemat et al., 2017). In sterol
242 extraction from mushroom, ultrasonic baths have been mainly used with a frequency ranging
243 between 20 to 65 kHz (Heleno et al., 2016a; Patil et al., 2018; Villares et al., 2012; Villares et al.,
244 2014). Several mechanisms are involved in ultrasound extraction such as fragmentation, erosion,
245 capillarity, detexturation, and sonoporation and have been described in the depth in the recent
246 review of Chemat et al. (2017) and summarized in Figure 3.
247 The extraction of sterols from mushrooms can be influenced by different parameters, such
248 as solvent type, extraction time and ultrasound power, while liquid-to-solid ratio seems to have no
249 significant effects (Heleno et al., 2016a). Patil et al. (2018) employed UAE (ultrasonic bath) in
250 conjunction with deep eutectic solvents (DES) for the extraction of vitamin $D_2$ from *A. bisporus*.

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

257 Heleno et al. (2016a) optimized the UAE of ergosterol from *Agaricus bisporus* using
258 response surface methodology (RSM). In this study the effects of different parameters were
259 investigated including the type of extraction solvent, liquid-to-solid ratio, extraction time, and
260 ultrasound power. Among the different solvents investigated, ethanol resulted in the highest
261 ergosterol yields followed by limonene and n-hexane. Both ergosterol and vitamin $D_2$ are
262 amphiphilic molecules with small hydroxyl polar heads which allow them to be extracted by a
263 variety of polar and non-polar solvents (Heleno et al., 2016a; Hsueh et al., 2007). The authors
264 noted that at the optimal UAE conditions (extraction time of 15 min and ultrasound power of
265 375W) ergosterol yields were higher than those obtained by a time consuming Soxhlet extraction
266 method in significantly shorter times (from 4h to 15 min). The authors also claimed that the
267 saponification step can be avoided when the UAE is employed (Heleno et al., 2016a). However,
268 the efficiency of the UAE on purifying the extracts is influenced by the solvent used. For instance,
269 the authors showed that the purity of the extracts obtained by n-hexane and limonene was similar
270 to the extracts obtained after saponification, while ethanol during UAE resulted in less pure
271 extracts. This was attributed to the higher polarity of ethanol than the other two solvents which
272 may lead to the extraction of various compounds (polar and non-polar). In summary, UAE can be
273 used for the extraction of sterols from mushrooms facilitating shorter extraction times.
274

275 **3.2.2.2 Supercritical fluid extraction (SFE) and pressurized liquid extraction (PLE)**

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

282 al., 2017). Morales et al. (2017) employed SFE for the production of extracts enriched in vitamin
283 $D_2$. Specifically, SFE was employed for the extraction of ergosterol from shiitake mushrooms and
284 subsequently, extracts were exposed to UV light for the conversion of ergosterol to vitamin $D_2$. In
285 this study, two extraction parameters were examined including extraction temperature (°C) and
286 pressure (bar). Both extraction parameters had a significant effect on the recovery of ergosterol.
287 The highest ergosterol yields (180 mg/g dw) were obtained at a pressure of 350 bar and a
288 temperature of 70 °C (Morales et al., 2017). Gil-Ramirez et al. (2013) optimized and compared
289 the extraction conditions of SFE and PLE for the recovery of sterols from *Agaricus bisporus*
290 fruiting bodies. In SFE, extraction pressure and use of co-solvent (10% ethanol) were investigated.
291 Lower sterol yields were obtained when a co-solvent was used, while there was no significant
292 effect of the extraction pressure. Fractions containing 60% of sterols were obtained at 40 °C and
293 30 MPa. In the case of PLE, several parameters were investigated including extraction time, the
294 number of cycles, ratio mushroom powder/sand and temperature. The optimal PLE conditions
295 using ethanol as the pressurized solvent were a pressure of 10.7 MPa, a temperature of 50 °C, 5
296 cycles of 5 min and mushroom/sand ratio of 1:4 (Gil-Ramirez et al., 2013). Between SFE and PLE,
297 PLE found to extract all the sterols from mushrooms, however, SFE was found to be more
298 selective. SFE has the potential of being upscaled for the extraction of both ergosterol and vitamin
299 $D_2$ since it has been upscaled for the extraction of other compounds (e.g., caffeine to make
300 decaffeinated products) whereas PLE remains a small-scale batch technique with mostly analytical
301 applications.
302

303 **3.2.2.3 Microwave-assisted extraction (MAE)**

304 MAE uses microwaves which are electromagnetic irradiation ranging in frequency from
305 300 MHz to 300 GHz. MAE has been employed for the extraction of different classes of bioactive
306 compounds, such as polyphenols, polysaccharides, lipids, and fatty acids (Kumar, Sivacumar, &
307 Ruckmani, 2016; Maeng, Muhammad Shahbaz, Ameer, Jo, & Kwon, 2017; Sinanoglou et al.,
308 2015). However, only few studies to date have employed and optimized the MAE of ergosterol
309 from mushrooms while there is no study optimizing the MAE of vitamin $D_2$ from mushrooms
310 (Young, 1995; Heleno et al., 2016b; Taofiq et al., 2019). Heleno et al. (2016b) investigated and
311 optimized three parameters named extraction time, extraction temperature and solid-to-liquid ratio
312 using RSM for the recovery of ergosterol from *A. bisporus*. The optimal MAE conditions were

313 extraction time of 19.4 ± 2.9 min, extraction temperature of 132.8 ± 12.4 °C and the solid-to-liquid
314 ratio of 1.6 ± 0.5 g/L, yielding 5.56 ± 0.26 mg of ergosterol per g of mushroom by-products. The
315 authors noted that the extraction yields increased as the extraction time and extraction temperature
316 increased, while extraction yields decreased as the solid-to-liquid ratio increased. The authors also
317 reported that the purity of extracts in ergosterol reduced by increasing temperature. This could be
318 either due to the extraction of other compounds than ergosterol as the extraction temperature
319 increased, or ergosterol was degraded as the temperature increased (Heleno et al., 2016b).
320 Recently, Taofiq et al. (2019) optimized the MAE (extraction time (2-25 min) and temperature
321 (60-150 °C)) of ergosterol (dependent variables examined: extraction yields, purity and total
322 extraction yields) from *A. blazei* and compared the dependent variables with those obtained at the
323 optimal UAE and heat-assisted extraction (HAE). Authors noted that among the different
324 extraction methods MAE was the most efficient in terms of extraction yields (25.44 ± 5.1 mg/
325 100g dw) compared to the UAE and HAE 21.49 ± 0.9 and 18.84 ± 2.3 mg/ 100g dw, respectively.
326 However, UAE resulted in extracts with higher ergosterol purity which could be attributed to the
327 combined mechanisms such as fragmentation, erosion and sonoporation that take place through
328 UAE. MAE is a promising extraction technique that can significantly reduce the extraction time
329 of ergosterol recovery from mushrooms. Future studies are encouraged to investigate and optimize
330 the MAE of vitamin $D_2$ (extraction yields and purity) from different mushroom varieties.
331

332 ### 3.2.3 Conventional extraction methods

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

344 In most of the studies that have been conducted so far, saponification of mushroom samples
345 precedes extraction and purification. A protocol that is usually reported in the literature is
346 saponification of mushroom samples for 30 min to 1 h at 80 or 85 °C, followed by liquid-liquid
347 extraction using non-polar solvents (i.e., n-pentane). Subsequently, the non-polar phase is washed
348 with potassium hydroxide 3% (w/v) in 5% (v/v) ethanol and neutralized using deionized water.
349 Finally, the organic phase fraction is rotary evaporated to dryness and then it is dissolved either in
350 ethanol or methanol (Heleno et al., 2016a; Jasinghe & Perera, 2005, 2006; Jasinghe et al., 2007;
351 Ko, Lee, Lee, & Park, 2008). In some studies before saponification an internal standard is added
352 (Roberts et al., 2008).
353

354 ## 3.3 Determination of ergosterol and vitamin $D_2$

355 ### 3.3.1 High-performance liquid chromatography (HPLC)

356 HPLC is the most commonly employed technique for the identification and quantification
357 of vitamin $D_2$ and ergosterol from mushrooms (Table 2). Before injection, samples are filtered
358 using disposable filter discs to remove small particles and thus protect HPLC columns. For the
359 detection of vitamin $D_2$ and ergosterol, reverse phases are usually employed containing carbon
360 chains of 18 carbon atoms, while there is one study used a column with carbon chains of 30 carbon
361 atoms (Table 2). The temperature of the stationary phase (column) is a critical parameter that may
362 affect the retention times of bioactive compounds. In the case of ergosterol and vitamin $D_2$
363 detection, the temperatures of the stationary phase may vary between 25 and 50 °C. For instance,
364 in the studies of Yuan, Wang, Liu, Kuang, and Zhao (2007) the stationary phase was maintained
365 at ambient temperature, while in the study of Morales et al. (2017) the stationary phase was
366 maintained at 50 °C. For HPLC analysis an injection volume of 10 or 20 μL is usually used,
367 however, when UHPLC (Ultra-high performance liquid chromatography) is employed, an
368 injection volume of 1 μL can be used (Slawinska et al., 2016). Either isocratic or gradient elution
369 can be implemented in HPLC analysis. The combination of methanol and acetonitrile is mainly
370 used as a mobile phase in isocratic elution while in gradient elution two or three mobile phases
371 can be used (Ahlborn et al., 2018). The flow rate that is usually employed is 1 mL/min (Ahlborn
372 et al., 2018; Heleno et al., 2016a; Heleno et al., 2016b; Huang, Lin, & Tsai, 2015; Morales et al.,
373 2017). UV-Vis and photodiode array detectors have been employed for the detection and the
374 wavelengths that are usually used for the detection of both ergosterol and vitamin D are 264 and

375 280 (Table 2). Mass spectrometers (MS) directly or coupled with a PDA detector have also been
376 used for detecting sterols, including vitamin $D_2$, and ergosterol in mushrooms.
377
378 ### 3.3.2 Gas-chromatography (GC)
379 In addition to HPLC, the determination of ergosterol and vitamin $D_2$ has been conducted
380 using GC coupled with MS and/or flame ionization detector (FID) (Gil-Ramirez et al., 2013;
381 Morales et al., 2017; Teichmann et al., 2007). In the study of Gil-Ramirez et al. (2013), prior to
382 the injection into the GC system, dried extracts were dissolved in chloromethane: methanol
383 ($ClCH_3$: MeOH) (2:1 v/v). A capillary column connected to a gas chromatograph equipped with
384 electronic pressure control, auto injector, a triple-axis mass spectrometer detector, and GC–MS
385 solution software. Helium (He) was used as carrier gas with a flow rate of 29.4 mL/ min and an
386 inlet pressure of 0.19 MPa. The injector was set at 260 °C, the detector at 350 °C and the oven
387 temperature program was 60 °C for 1 min, at a rate of 40 °C/min, to a final temperature of 310 °C,
388 and held for 30 min. Apart from ergosterol (ergosta-5,7,22-trien3β-ol) which was the major sterol
389 (83% of total sterols) and detected at 13.3 min, other sterols such as ergosta-7,22-dienol, ergosta-
390 5,7dienol, ergosta-7-enol (fungisterol), ergosta-4,7,2-trien-3-ona and ergosta-4,6,8(14),22-tetraen-
391 3-ona were also detected at 13.6, 14.0, 14.3, 14.9, and 15.7 min, respectively.
392
393 ## 4. Potential application in the food industry
394 Efforts have been made to add value to mushroom waste through its use as animal feed,
395 fuel or to extract valuable compounds that can be used as food safe ingredients, such as chitin,
396 chitosan, β-glucan, sterols and prebiotic polysaccharides (Chou, Sheih, & Fang, 2013; Gil-
397 Ramírez, Ruiz-Rodríguez, Marín, Reglero, & Soler-Rivas, 2014; Mahfuz, Song, Miao, & Liu,
398 2019; Vasylenko, Loewe, & Wegener, 2008; Wu, Zivanovic, Draughon, & Sams, 2004).
399 Furthermore, mushroom-derived β-glucans, lentinan and polysaccharide-K have been used for
400 thousands of years to improve general health as a part of traditional medicine especially in Asian
401 countries including China, Japan and Korea (Lemieszek & Rzeski, 2012; Ina, Kataoka, & Ando,
402 2013).
403 It is known that mushrooms have the potential to be the only food source of vitamin D
404 (non-animal and unfortified), while providing a substantial amount of vitamin $D_2$ in a single
405 serving (Cardwell, Bornman, James, & Black, 2018). Fresh mushrooms sold in UK retailers

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

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

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

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

430 The use of vitamin D extracts from mushroom waste in new foods could benefit the food
431 industry, as several nutritional and health claims could be made on food packaging. According to
432 the European Food Safety Authority (EFSA, 2006), vitamin D can be added to foods as
433 cholecalciferol ($D_3$) or ergocalciferol ($D_2$). The daily Reference Intake (RI) value for vitamin D is
434 5 μg, previously known as Recommended Daily Allowances (RDAs), and this is the reference
435 value used on food labels (EU Regulation, 2011). According to the EFSA register of nutrition and
436 health claim (EFSA, 2012), there are several health claims that can be made on foods that are a

437 “source of” or “high” in vitamin D. “Source of” and “high” refer to foods that contain respectively
438 at least 15% or 30% of the vitamin D RDA (15%=0.8 μg, 30%=0.16 μg) per 100 g of solid food.
439 The allowed health claims on vitamin D adapted from the EFSA register of nutrition and health
440 claims are shown in Table 3.
441
442 **5. Potential application in the pharmaceutical industry**
443 Ergosterol, a vital component of the fungal and protozoal cell membranes plays a
444 fundamental role in membrane fluidity and integrity while acting as a drug target of several
445 antifungal agents (Rodrigues, 2018). Interestingly, ergosterol and its derivatives isolated from
446 mushroom and other natural sources have been reported to have several therapeutic properties. A
447 review by (Picotto, Liaudat, Bohl, & Talamoni, 2012) emphasized the importance of vitamin D
448 (ergocalciferol and cholecalciferol) in anticancer research. Table 4 represents the major preclinical
449 studies portrayed in the current literature underlining the bioactivity of ergosterol and its natural,
450 semi-synthetic and synthetic derivatives. In particular, ergosterol peroxide, a natural ergosterol
451 derivative has been studied more extensively and shown to confer a greater anti-proliferative and
452 cytotoxic potential compared to ergosterol against breast, colorectal, ovarian and renal cancer cell
453 lines (He, Shi, Liu, Zhao, & Zhang, 2018; Kang et al., 2015; L. M. Kuo et al., 2005; Martínez-
454 Montemayor et al., 2019; Russo et al., 2010; Tan et al., 2017).
455 Mechanistically, ergosterol in its pure or derivative forms were reported to induce caspase-
456 mediated intrinsic apoptosis, arrest cell cycle and inhibit migration and invasion of cancer cells
457 while regulating several signalling pathways including the STAT3 and the IGFR/IRS-1/2-MEK-
458 ras-ERK1/2 (W. J. Chen et al., 2008; Li et al., 2015; Martínez-Montemayor et al., 2019; Tan et al.,
459 2017). Downregulation of β-catenin has also been identified as one of the key molecular
460 mechanisms of action against colorectal and renal cancer cells (He et al., 2018; Kang et al., 2015).
461 β-Catenin is associated with cadherin-mediated intercellular adhesion and the Wnt signalling
462 pathway (Sineva & Pospelov, 2014).
463 The AK3/STAT3/NF-$\kappa$B pathway was found to play an important role in mediating the
464 anti-inflammatory activity of ergosterol and ergosterol peroxide in cellular and animal models
465 (Huan, Tianzhu, Yu, & Shumin, 2017; Kobori, Yoshida, Ohnishi-Kameyama, & Shinmoto, 2007;
466 C.-F. Kuo, Hsieh, & Lin, 2011; Zhang, Xu, Li, & Wang, 2015). The anti-inflammatory activity of
467 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).

498 The use of vitamin D extracts from mushroom waste in new foods could benefit the food
499 industry, yet, the practical applications of vitamin $D_2$ from mushroom by-products are limited.
500 Therefore, potential food applications of mushroom extracts enriched in ergosterol and vitamin $D_2$
501 is a field that requires further work. Nonetheless, the legislation should be considered when using
502 vitamin $D_2$ extracts from mushroom waste in new foods. Similarly, despite its potential especially
503 in anticancer and anti-inflammatory drug development, the pharmaceutical application of
504 ergosterol and vitamin $D_2$ from mushroom needs further in vivo mechanistic studies for future
505 clinical trials. As novel drug development entails large resources and time, the approach of
506 combining pre-existing drugs with natural product-based adjuvants to increase their efficiency is
507 promising and economical. Therefore, studies investigating the potential synergistic interactions
508 of ergosterol and vitamin $D_2$ with pre-existing anticancer and anti-inflammatory drugs can provide
509 interesting insights for their future pharmaceutical applications.
510

511 **Declaration of competing interest**
512 The authors declare that they have no conflict of interest.
513

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518

519 **References**

520 Abe, F., & Hiraki, T. (2009). Mechanistic role of ergosterol in membrane rigidity and
521 cycloheximide resistance in Saccharomyces cerevisiae. *Biochimica et Biophysica Acta*
522 *(BBA)-Biomembranes, 1788*, 743-752.
523 Aguiló-Aguayo, I., Walton, J., Viñas, I., & Tiwari, B. K. (2017). Ultrasound assisted extraction of
524 polysaccharides from mushroom by-products. *LWT Food Science and Technology, 77*, 92-
525 99.
526 Ahlborn, J., Calzolari, N., Spielmeyer, A., Avci, S. S., Zimmer, M., & Rühl, M. (2018).
527 Enrichment of vitamin D2 in mycelium from submerged cultures of the agaric mushroom
528 Pleurotus sapidus. *Journal of Food Science and Technology, 55*(9), 3833-3839.
529 Ahn, B.-Y. (2018). Optimization of Vitamin $D_2$ Production using enoki mushroom powder and the
530 preparation of doenjang using the powder. *The Journal of the East Asian Society of Dietary*
531 *Life, 28*(1), 88-100.

532 Aladić, K., Vidović, S., Vladić, J., Balić, D., Jukić, H., & Jokić, S. (2016). Effect of supercritical
533 $CO_2$ extraction process parameters on oil yield and pigment content from by-product hemp
534 cake. *International Journal of Food Science & Technology, 51*(4), 885-893.
535 Alexandre, T. R., Lima, M. L., Galuppo, M. K., Mesquita, J. T., do Nascimento, M. A., Dos Santos,
536 A. L., Sartorelli, P., Pimenta, D. C., & Tempone, A. G. (2017). Ergosterol isolated from
537 the basidiomycete Pleurotus salmoneostramineus affects Trypanosoma cruzi plasma
538 membrane and mitochondria. *The Journal of Venomous Animals and Toxins including*
539 *Tropical Diseases, 23*, 30-30.
540 Alzorqi, I., Sudheer, S., Lu, T. J., & Manickam, S. (2017). Ultrasonically extracted β-D-glucan
541 from artificially cultivated mushroom, characteristic properties and antioxidant activity.
542 *Ultrasonics Sonochemistry, 35*, 531-540.
543 Bailey, R. L., Dodd, K. W., Goldman, J. A., Gahche, J. J., Dwyer, J. T., Moshfegh, A. J., Sempos,
544 C. T., Picciano, M. F. (2010). Estimation of total usual calcium and vitamin D intakes in
545 the United States. *The Journal of Nutrition, 140*, 817-822.
546 Barba, F. J., Putnik, P., Kovacevic, D. B., Poojary, M. M., Roohinejad, S., Lorenzo, J. M., &
547 Koubaa, M. (2017). Impact of conventional and non-conventional processing on prickly
548 pear (Opuntia spp.) and their derived products: From preservation of beverages to
549 valorization of by-products. *Trends in Food Science & Technology, 67*, 260-270.
550 Barbosa-Pereira, L., Guglielmetti, A., & Zeppa, G. (2018). Pulsed electric field assisted extraction
551 of bioactive compounds from cocoa bean shell and coffee silverskin. *Food and Bioprocess*
552 *Technology, 11*, 818-835.
553 Barreira, J. C. M., Oliveira, M. B. P. P., & Ferreira, I. C. F. R. (2014). Development of a novel
554 methodology for the analysis of ergosterol in mushrooms. *Food Analytical Methods, 7*,
555 217-223. doi:10.1007/s12161-013-9621-9
556 Bernas, E., & Jaworska, G. (2017). Culinary-Medicinal Mushroom Products as a Potential Source
557 of Vitamin D. *International Journal of Medicinal Mushrooms, 19*, 925-935.
558 Bilbao-Sainz, C., Chiou, B.-S., Williams, T., Wood, D., Du, W.-X., Sedej, I., Ban, Z., Rodov, V.,
559 Poverenov, E., Vinokur, Y., &McHugn, T. (2017). Vitamin D-fortified chitosan films from
560 mushroom waste. *Carbohydrate Polymers, 167*, 97-104.
561 Black, L. J., Lucas, R. M., Sherriff, J. L., Bjorn, L. O., & Bornman, J. F. (2017). In Pursuit of
562 Vitamin D in Plants. *Nutrients, 9*, 136.
563 Brard, L., Lange, T. S., Robison, K., Kim, K. K., Ara, T., McCallum, M. M., Arnold, L. A., Moore, R. G.,
564 & Singh, R. K. (2011). Evaluation of the first Ergocalciferol-derived, non hypercalcemic anti-
565 cancer agent MT19c in ovarian cancer SKOV-3 cell lines. *Gynecologic Oncology, 123*, 370-378.
566 Bu, M., Cao, T., Li, H., Guo, M., Yang, B. B., Zeng, C., & Hu, L. (2017). Synthesis of
567 5alpha,8alpha-Ergosterol Peroxide 3-Carbamate Derivatives and a fluorescent
568 mitochondria-targeting conjugate for enhanced anticancer activities. *ChemMedChem, 12*,
569 466-474. doi:10.1002/cmdc.201700021
570 Cardwell, G., Bornman, J. F., James, A. P., & Black, L. J. (2018). A review of mushrooms as a
571 potential source of dietary vitamin D. *Nutrients, 10*, 1498.
572 Chemat, F., Rombaut, N., Sicaire, A.-G., Meullemiestre, A., Fabiano-Tixier, A.-S., & Abert-Vian,
573 M. (2017). Ultrasound assisted extraction of food and natural products. Mechanisms,
574 techniques, combinations, protocols and applications. A review. *Ultrasonics*
575 *Sonochemistry, 34*, 540-560.
576 Chemat, F., Vian, M. A., & Cravotto, G. (2012). Green extraction of natural products: concept and
577 principles. *International journal of molecular sciences, 13*(7), 8615-8627.

578 Chen, S., Yong, T., Zhang, Y., Su, J., Jiao, C., & Xie, Y. (2017). Anti-tumor and anti-angiogenic
579 ergosterols from *Ganoderma lucidum. Frontiers in Chemistry, 5*(85).
580 Chen, W. J., Huang, Y. T., Wu, M. L., Huang, T. C., Ho, C. T., & Pan, M. H. (2008). Induction of
581 apoptosis by vitamin $D_2$, ergocalciferol, via reactive oxygen species generation,
582 glutathione depletion, and caspase activation in human leukemia Cells. *Journal of*
583 *Agricultural and Food Chemistry, 56*, 2996-3005.
584 Chou, W. T., Sheih, I. C., & Fang, T. J. (2013). The applications of polysaccharides from various
585 mushroom wastes as prebiotics in different systems. *Journal of Food Science, 78*, M1041-
586 M1048.
587 Correa, R. C. G., Barros, L., Fernandes, A., Sokovic, M., Bracht, A., Peralta, R. M., & Ferreira, I.
588 C. F. R. (2018). A natural food ingredient based on ergosterol: optimization of the
589 extraction from *Agaricus blazei*, evaluation of bioactive properties and incorporation in
590 yogurts. *Food & Function*, *3*, 1465-1474.
591 Dawson-Hughes, B., Mithal, A., Bonjour, J.-P., Boonen, S., Burckhardt, P., Fuleihan, G. E.-H.,
592 Josse, R. G., Lips, P., Morales-Torres, J., & Yoshimura, N. (2010). IOF position statement:
593 vitamin D recommendations for older adults. *Osteoporosis International, 21*, 1151-1154.
594 Dupont, S., Lemetais, G., Ferreira, T., Cayot, P., Gervais, P., & Beney, L. (2012). Ergosterol
595 biosynthesis: a fungal pathway for life on land? *Evolution, 66*, 2961-2968.
596 EFSA. (2006). Regulation 1924/2006 on nutrition and health claims made on foods. *Official*
597 *Journal of the European Union, L404*, 9-25.
598 EFSA. EU Register on nutrition and health claims. (2012).
599 https://ec.europa.eu/food/safety/labelling_nutrition/claims/nutrition_claims_en Accessed
600 22 December 2019
601 EFSA. (2016). Guidance on the preparation and presentation of an application for authorisation of
602 a novel food in the context of Regulation (EU) 2015/2283. *EFSA Journal, 14*, e04594.
603 EU Regulation. (2011). No 1169/2011 of the European Parliament and of the Council of 25
604 October 2011 on the provision of food information to consumers. *Official Journal of the*
605 *European Union*.
606 Food Manufacture. (2016). Tesco to sell mushrooms enriched with vitamin D.
607 Gallego, R., Bueno, M., & Herrero, M. (2019). Sub- and supercritical fluid extraction of bioactive
608 compounds from plants, food-by-products, seaweeds and microalgae – An update. *TrAC-*
609 *Trends in Analytical Chemistry, 116*, 198-213.
610 Gao, J.-M., Wang, M., Liu, L.-P., Wei, G.-H., Zhang, A.-L., Draghici, C., & Konishi, Y. (2007).
611 Ergosterol peroxides as phospholipase A2 inhibitors from the fungus Lactarius hatsudake.
612 *Phytomedicine, 14*(12), 821-824. doi:https://doi.org/10.1016/j.phymed.2006.12.006
613 Gąsecka, M., Siwulski, M., Magdziak, Z., Budzyńska, S., Stuper-Szablewska, K., Niedzielski, P.,
614 & Mleczek, M. (2019). The effect of drying temperature on bioactive compounds and
615 antioxidant activity of *Leccinum scabrum* (Bull.) Gray and *Hericium erinaceus* (Bull.)
616 Pers. *Journal of Food Science and Technology*. https://doi.org/10.1007/s13197-019-
617 04081-1
618 Gil-Ramirez, A., Aldars-Garcia, L., Palanisamy, M., Jiverdeanu, R. M., Ruiz-Rodriguez, A.,
619 Marin, F. R., Reglero, G., & Soler-Rivas, C. (2013). Sterol enriched fractions obtained
620 from Agaricus bisporus fruiting bodies and by-products by compressed fluid technologies
621 (PLE and SFE). *Innovative Food Science & Emerging Technologies, 18*, 101-107.

622 Gil-Ramírez, A., Ruiz-Rodríguez, A., Marín, F. R., Reglero, G., & Soler-Rivas, C. (2014). Effect
623 of ergosterol-enriched extracts obtained from Agaricus bisporus on cholesterol absorption
624 using an *in vitro* digestion model. *Journal of functional foods, 11*, 589-597.
625 Han, X., & Zhou, Y. (2015). 4 - Application of lipidomics in nutrition research. In J. L. Sébédio
626 & L. Brennan (Eds.), *Metabolomics as a Tool in Nutrition Research* (pp. 63-84):
627 Woodhead Publishing.
628 He, L., Shi, W., Liu, X., Zhao, X., & Zhang, Z. (2018). Anticancer action and mechanism of
629 ergosterol peroxide from *Paecilomyces cicadae* fermentation broth. *International journal*
630 *of molecular sciences, 19*, 3935.
631 Heleno, S. A., Diz, P., Prieto, M. A., Barros, L., Rodrigues, A., Barreiro, M. F., & Ferreira, I. C.
632 F. R. (2016a). Optimization of ultrasound-assisted extraction to obtain mycosterols from
633 Agaricus bisporus L. by response surface methodology and comparison with conventional
634 Soxhlet extraction. *Food Chemistry, 197*, 1054-1063.
635 Heleno, S. A., Prieto, M. A., Barros, L., Rodrigues, A., Barreiro, M. F., & Ferreira, I. C. F. R.
636 (2016b). Optimization of microwave-assisted extraction of ergosterol from Agaricus
637 bisporus L. by-products using response surface methodology. *Food and Bioproducts*
638 *Processing, 100*, 25-35.
639 Ho-Pham, L. T., Vu, B. Q., Lai, T. Q., Nguyen, N. D., & Nguyen, T. V. (2012). Vegetarianism,
640 bone loss, fracture and vitamin D: a longitudinal study in Asian vegans and non-vegans.
641 *European Journal of Clinical Nutrition, 66*, 75-82.
642 Hsueh, Y.-W., Chen, M.-T., Patty, P. J., Code, C., Cheng, J., Frisken, B. J., Zuckermann, M., &
643 Thewalt, J. (2007). Ergosterol in POPC membranes: Physical properties and comparison
644 with structurally similar sterols. *Biophysical Journal, 92*, 1606-1615.
645 Huan, W., Tianzhu, Z., Yu, L., & Shumin, W. (2017). Effects of Ergosterol on COPD in Mice via
646 JAK3/STAT3/NF-kappaB Pathway. *Inflammation, 40*(3), 884-893.
647 Huang, S. J., Lin, C. P., & Tsai, S. Y. (2015). Vitamin $D_2$ content and antioxidant properties of
648 fruit body and mycelia of edible mushrooms by UV-B irradiation. *Journal of Food*
649 *Composition and Analysis, 42*, 38-45.
650 Jäpelt, R. B., & Jakobsen, J. (2013). Vitamin D in plants: a review of occurrence, analysis, and
651 biosynthesis. *Frontiers in plant science, 4*, 136-136.
652 Jasinghe, V. J., & Perera, C. O. (2005). Distribution of ergosterol in different tissues of mushrooms
653 and its effect on the conversion of ergosterol to vitamin $D_2$ by UV irradiation. *Food*
654 *Chemistry, 92*, 541-546.
655 Jasinghe, V. J., & Perera, C. O. (2006). Ultraviolet irradiation: The generator of Vitamin $D_2$ in
656 edible mushrooms. *Food Chemistry, 95*(4), 638-643.
657 Jasinghe, V. J., Perera, C. O., & Sablani, S. S. (2007). Kinetics of the conversion of ergosterol in
658 edible mushrooms. *Journal of Food Engineering, 79*(3), 864-869.
659 Jin, H., McCaffery, J. M., & Grote, E. (2008). Ergosterol promotes pheromone signaling and
660 plasma membrane fusion in mating yeast. *The Journal of cell biology*, *180*, 813-826.
661 Ina, K., Kataoka, T., & Ando, T. (2013). The use of lentinan for treating gastric cancer. *Anti-cancer*
662 *agents in medicinal chemistry*, *13*, 681-688.
663 Kageyama-Yahara, N., Wang, P., Wang, X., Yamamoto, T., & Kadowaki, M. (2010). The
664 inhibitory effect of ergosterol, a bioactive constituent of a traditional Japanese herbal
665 medicine saireito on the activity of mucosal-type mast cells. *Biological and*
666 *Pharmaceutical Bulletin, 33*, 142-145.

667 Kalač, P. (2016). Chapter 4 - Health-stimulating compounds and effects. In P. Kalač (Ed.), *Edible*
668 *Mushrooms Chemical* (pp. 137-153): Academic Press.
669 Kalaras, M. D., Richie, J. P., Calcagnotto, A., & Beelman, R. B. (2017). Mushrooms: A rich source
670 of the antioxidants ergothioneine and glutathione. *Food Chemistry, 233,* 429-433.
671 Kang, J.-H., Jang, J.-E., Mishra, S. K., Lee, H.-J., Nho, C. W., Shin, D., Jin, M., Kim, M. K., Choi,
672 C., & Oh, S. H. (2015). Ergosterol peroxide from Chaga mushroom (*Inonotus obliquus*)
673 exhibits anti-cancer activity by down-regulation of the β-catenin pathway in colorectal
674 cancer. *Journal of Ethnopharmacology, 173*, 303-312.
675 Kaur, N., & Singh, A. K. (2016). Ohmic Heating: Concept and Applications-A Review. *Critical*
676 *Reviews in Food Science and Nutrition, 56*, 2338-2351.
677 Kim, D. S., Baek, N.-I., Oh, S. R., Jung, K. Y., Lee, I. S., Kim, J. H., & Lee, H.-K. (1997).
678 Anticomplementary activity of ergosterol peroxide fromNaematoloma fasciculare and
679 reassignment of NMR data. *Archives of Pharmacal Research, 20*, 201-205.
680 Ko, J. A., Lee, B. H., Lee, J. S., & Park, H. J. (2008). Effect of UV-B exposure on the concentration
681 of vitamin $D_2$ in sliced shiitake mushroom (*Lentinus edodes*) and white button mushroom
682 (*Agaricus bisporus*). *Journal of Agricultural and Food Chemistry, 56*, 3671-3674.
683 Kobori, M., Yoshida, M., Ohnishi-Kameyama, M., & Shinmoto, H. (2007). Ergosterol peroxide
684 from an edible mushroom suppresses inflammatory responses in RAW264.7 macrophages
685 and growth of HT29 colon adenocarcinoma cells. *British journal of pharmacology, 150*,
686 209-219. doi:10.1038/sj.bjp.0706972
687 Koyyalamudi, S. R., Jeong, S.-C., Song, C.-H., Cho, K. Y., & Pang, G. (2009). Vitamin D2
688 Formation and Bioavailability from Agaricus bisporus Button Mushrooms Treated with
689 Ultraviolet Irradiation. *Journal of Agricultural and Food Chemistry, 57*, 3351-3355.
690 Kumar, C., S., Sivacumar, M., & Ruckmani, K. (2016). Microwave-assisted extraction of
691 polysaccharides from Cyphomandra betacea and its biological activities. *International*
692 *Journal of Biological Macromolecules, 92*, 682-693.
693 Kuo, C.-F., Hsieh, C.-H., & Lin, W.-Y. (2011). Proteomic response of LAP-activated RAW 264.7
694 macrophages to the anti-inflammatory property of fungal ergosterol. *Food Chemistry, 126*,
695 207-212.
696 Kuo, L. M., Chen, K. Y., Hwang, S. Y., Chen, J. L., Liu, Y. Y., Liaw, C. C., Ye, P. H., Chou, C.
697 J., & Shen CC, Kuo, Y. H. (2005). DNA topoisomerase I inhibitor, ergosterol peroxide
698 from *Penicillium oxalicum. Planta Medica, 71*, 77-79.
699 Kuo, Y. C., Weng, S. C., Chou, C. J., Chang, T. T., & Tsai, W. J. (2003). Activation and
700 proliferation signals in primary human T lymphocytes inhibited by ergosterol peroxide
701 isolated from Cordyceps cicadae. *British Journal of Pharmacology, 140*(5), 895-906.
702 Lee, N. K., & Aan, B. Y. (2016). Optimization of ergosterol to vitamin $D_2$ synthesis in *Agaricus*
703 *bisporus* powder using ultraviolet-B radiation. *Food science and biotechnology, 25,* 1627-
704 1631.
705 Leiva, F., Saenz-Díez, J., Martínez, E., Jiménez, E., & Blanco, J. (2015). Environmental impact of
706 *Agaricus bisporus* cultivation process. *European Journal of Agronomy, 71*, 141-148.
707 Lemieszek, M., & Rzeski, W. (2012). Anticancer properties of polysaccharides isolated from fungi
708 of the Basidiomycetes class. *Contemporary oncology*, *16*, 285-289.
709 Li, X., Wu, Q., Xie, Y., Ding, Y., Du, W. W., Sdiri, M., & Yang, B. B. (2015). Ergosterol purified
710 from medicinal mushroom Amauroderma rude inhibits cancer growth *in vitro* and *in vivo*
711 by up-regulating multiple tumor suppressors. *Oncotarget, 6*, 17832-17846.

712 Lingwood, D., & Simons, K. (2010). Lipid rafts as a membrane-organizing principle. *Science, 327*,
713 46-50.
714 Ma, L., Chen, H., Dong, P., & Lu, X. (2013). Anti-inflammatory and anticancer activities of
715 extracts and compounds from the mushroom Inonotus obliquus. *Food Chemistry, 139*, 503-
716 508.
717 Maeng, J. H., Muhammad Shahbaz, H., Ameer, K., Jo, Y., & Kwon, J. H. (2017). Optimization of
718 Microwave-Assisted Extraction of Bioactive Compounds from *Coriolus versicolor*
719 Mushroom Using Response Surface Methodology. *Journal of Food Process Engineering,*
720 *40*. doi:10.1111/jfpe.12421
721 Mahfuz, S., Song, H., Miao, Y., & Liu, Z. (2019). Dietary inclusion of mushroom (*Flammulina*
722 *velutipes*) stem waste on growth performance and immune responses in growing layer hens.
723 *Journal of the Science of Food and Agriculture, 99*, 703-710.
724 Martínez-Montemayor, M. M., Ling, T., Suárez-Arroyo, I. J., Ortiz-Soto, G., Santiago-Negrón, C.
725 L., Lacourt-Ventura, M. Y., Valentín-Acevedo, A., Lang, W. H.,& Rivas, F. (2019).
726 Identification of biologically active *Ganoderma lucidum* compounds and synthesis of
727 improved derivatives that confer anti-cancer activities *in vitro*. *Frontiers in Pharmacology,*
728 *10*. doi:10.3389/fphar.2019.00115
729 Morales, D., Gil-Ramirez, A., Smiderle, F. R., Piris, A. J., Ruiz-Rodriguez, A., & Soler-Rivas, C.
730 (2017). Vitamin D-enriched extracts obtained from shiitake mushrooms (*Lentinula edodes*)
731 by supercritical fluid extraction and UV-irradiation. *Innovative Food Science & Emerging*
732 *Technologies, 41*, 330-336.
733 Nallathamby, N., Guan-Serm, L., Vidyadaran, S., Abd Malek, S. N., Raman, J., & Sabaratnam, V.
734 (2015). Ergosterol of Cordyceps militaris Attenuates LPS Induced Inflammation in BV2
735 Microglia Cells. *Natural Product Communications, 10*, 885-886.
736 Nam, K. S., Jo, Y. S., Kim, Y. H., Hyun, J. W., & Kim, H. W. (2001). Cytotoxic activities of
737 acetoxyscirpenediol and ergosterol peroxide from Paecilomyces tenuipes. *Life Scinces, 69*,
738 229-237.
739 Nölle, N., Argyropoulos, D., Müller, J., & Biesalski, H. K. (2018). Temperature stability of vitamin
740 D2 and color changes during drying of UVB-treated mushrooms. *Drying Technology, 36*,
741 307-315.
742 Patil, J., Ghodke, S., Jain, R., & Dandekar, P. (2018). Extraction of Vitamin D from Button
743 Mushroom (Agaricus bisporus) Using Deep Eutectic Solvent and Ultrasonication. *ACS*
744 *Sustainable Chemistry and Engineering, 6*(8), 10578-10586.
745 doi:10.1021/acssuschemeng.8b01915
746 Peng, Y., Tao, Y., Wang, Q., Shen, L., Yang, T., Liu, Z., & Liu, C. (2014). Ergosterol Is the Active
747 Compound of Cultured Mycelium Cordyceps sinensis on Antiliver Fibrosis. *Evidence-*
748 *Based Complementary and Alternative Medicine, 2014*, 12. doi:10.1155/2014/537234
749 Picotto, G., Liaudat, A. C., Bohl, L., & Talamoni, N. T. d. (2012). Molecular Aspects of Vitamin
750 D Anticancer Activity. *Cancer Investigation, 30*(8), 604-614.
751 Prabu, S. L., Suriya Prakash, T. N. K., & Thirumurugan, R. (2015). Cleaning Validation and Its
752 Regulatory Aspects in the Pharmaceutical Industry. In R. Kohli & K. L. Mittal (Eds.),
753 *Developments in Surface Contamination and Cleaning* (pp. 129-186). Oxford: William
754 Andrew Publishing.
755 Ramos, M., Burgos, N., Barnard, A., Evans, G., Preece, J., Graz, M., Ruthes, A. C., Jimenez-
756 Quero, A., Martinez-Abad, A., Vilaplana, F., Ngoc, L. P., Brouwer, A., van der Burg, B.,

757 Del Carmen Garrigos, M., & Jimenez, A. (2019). *Agaricus bisporus* and its by-products as a source of valuable extracts and bioactive compounds. *Food Chemistry*, *292*, 176-187.
759 Roberts, J. S., Teichert, A., & McHugh, T. H. (2008). Vitamin $D_2$ Formation from post-harvest UV-B treatment of mushrooms (*Agaricus bisporus*) and retention during storage. *Journal of Agricultural and Food Chemistry, 56*, 4541-4544.
762 Rodrigues, M. L. (2018). The multifunctional fungal ergosterol. *mBio, 9*, e01755-01718.
763 Roncero-Ramos, I., & Delgado-Andrade, C. (2017). The beneficial role of edible mushrooms in human health. *Current Opinion in Food Science, 14*, 122-128.
765 Roselló-Soto, E., Parniakov, O., Deng, Q., Patras, A., Koubaa, M., Grimi, N., Boussetta, N., Tiwari, B. K., Vorobiev, E., Lebovkova, N., & Barba, F. J. (2016). Application of Non-conventional Extraction Methods: Toward a Sustainable and Green Production of Valuable Compounds from Mushrooms. *Food Engineering Reviews, 8*, 214-234.
769 Royse, D. J. (2014). *A global perspective on the high five: Agaricus, Pleurotus, Lentinula, Auricularia & Flammulina.* Paper presented at the Proceedings of the 8th International Conference on Mushroom Biology and Mushroom Products (ICMBMP8).
772 Russo, A., Cardile, V., Piovano, M., Caggia, S., Espinoza, C. L., & Garbarino, J. A. (2010). Pro-apoptotic activity of ergosterol peroxide and (22E)-ergosta-7,22-dien-5alpha-hydroxy-3,6-dione in human prostate cancer cells. *Chemico-Biological Interactions, 184*, 352-358.
775 Sinanoglou, V. J., Zoumpoulakis, P., Heropoulos, G., Proestos, C., Ciric, A., Petrovic, J., Glamoclija, J., & Sokovic, M. (2015). Lipid and fatty acid profile of the edible fungus *Laetiporus sulphurous*. Antifungal and antibacterial properties. *Journal of Food Science and Technology, 52*, 3264-3272.
779 Sineva, G. S., & Pospelov, V. A. (2014). β-Catenin in Pluripotency: Adhering to Self-Renewal or Wnting to Differentiate? In K. W. Jeon (Ed.), *International Review of Cell and Molecular Biology* (pp. 53-78): Academic Press.
782 Slawinska, A., Fornal, E., Radzki, W., Skrzypczak, K., Zalewska-Korona, M., Michalak-Majewska, M., Parfieniuk, E., & Stachniuk, A. (2016). Study on vitamin D(2) stability in dried mushrooms during drying and storage. *Food Chemistry, 199*, 203-209.
785 Takei, T., Yoshida, M., Ohnishi-Kameyama, M., & Kobori, M. (2005). Ergosterol peroxide, an apoptosis-inducing component isolated from *Sarcodon aspratus* (Berk.) S. Ito.
787 *Bioscience, Biotechnology, and Biochemistry, 69*, 212-215.
788 Tan, W., Pan, M., Liu, H., Tian, H., Ye, Q., & Liu, H. (2017). Ergosterol peroxide inhibits ovarian cancer cell growth through multiple pathways. *OncoTargets and Therapy, 10*, 3467-3474.
790 Taofiq, O., Correa, R. C. G., Barros, L., Prieto, M. A., Bracht, A., Peralta, R. M., Gonzalez-Paramas, A. M., Barreiro, M. F., & Ferreira, I. C. F. R. (2019). A comparative study between conventional and non-conventional extraction techniques for the recovery of ergosterol from *Agaricus blazei* Murrill. *Food Research International, 125*, https://doi.org/10.1016/j.foodres.2019.108541.
795 Taofiq, O., Fernandes, Â., Barros, L., Barreiro, M. F., & Ferreira, I. C. F. R. (2017). UV-irradiated mushrooms as a source of vitamin $D_2$: A review. *Trends in Food Science & Technology, 70*, 82-94.
798 Teichmann, A., Dutta, P. C., Staffas, A., & Jägerstad, M. (2007). Sterol and vitamin $D_2$ concentrations in cultivated and wild grown mushrooms: Effects of UV irradiation. *LWT - Food Science and Technology, 40*, 815-822.
801 Tewtrakul, S., Tansakul, P., Daengrot, C., Ponglimanont, C., & Karalai, C. (2010). Anti-inflammatory principles from Heritiera littoralis bark. *Phytomedicine, 17*, 851-855.

803 Tian, Y., Zhao, Y., Huang, J., Zeng, H., & Zheng, B. (2016). Effects of different drying methods
804 on the product quality and volatile compounds of whole shiitake mushrooms. *Food*
805 *Chemistry, 197*, 714-722.
806 Valadez-Carmona, L., Ortiz-Moreno, A., Ceballos-Reyes, G., Mendiola, J. A., & Ibáñez, E.
807 (2018). Valorization of cacao pod husk through supercritical fluid extraction of phenolic
808 compounds. *The Journal of Supercritical Fluids, 131*, 99-105.
809 Vasylenko, L., Loewe, K., & Wegener, J. (2008). Applicability of agricultural by-products from
810 the mushroom industry for fueling. *Landtechnik, 63*, 34-35.
811 Villares, A., Garcia-Lafuente, A., Guillamon, E., & Ramos, A. (2012). Identification and
812 quantification of ergosterol and phenolic compounds occurring in *Tuber* spp. truffles.
813 *Journal of Food Composition and Analysis, 26*, 177-182.
814 Villares, A., Mateo-Vivaracho, L., Garcia-Lafuente, A., & Guillamon, E. (2014). Storage
815 temperature and UV-irradiation influence on the ergosterol content in edible mushrooms.
816 *Food Chemistry, 147*, 252-256.
817 Wen, X., Nigel, B., James, G. L., Sabene, H., Solange, C., & Konstantinos, P. (2020). Changes in
818 volatile and non-volatile compounds of shiitake mushrooms treated with pulsed ultraviolet
819 light after 24h storage at different conditions. *Food Bioscience*. Accepted manuscript
820 Wittig, M., Krings, U., & Berger, R. G. (2013). Single-run analysis of vitamin D photoproducts in
821 oyster mushroom (*Pleurotus ostreatus*) after UV-B treatment. *Journal of Food*
822 *Composition and Analysis, 31*, 266-274.
823 Won, D. J., Kim, S. Y., Jang, C. H., Lee, J. S., Ko, J. A., & Park, H. J. (2018). Optimization of UV
824 irradiation conditions for the vitamin D2-fortified shiitake mushroom (*Lentinula edodes*)
825 using response surface methodology. *Food science and biotechnology, 27*, 417-424.
826 Wu, T., Zivanovic, S., Draughon, F. A., & Sams, C. E. (2004). Chitin and chitosan value-added
827 products from mushroom waste. *Journal of Agricultural and Food Chemistry, 52*, 7905-
828 7910.
829 Xu, D. P., Zheng, J., Zhou, Y., Li, Y., Li, S., & Li, H. B. (2016). Extraction of natural antioxidants
830 from the Thelephora ganbajun mushroom by an ultrasound-assisted extraction technique
831 and evaluation of antiproliferative activity of the extract against human cancer cells.
832 *International journal of molecular sciences, 17*, 1664.
833 Young, J. C. (1995). Microwave-assisted extraction of the fungal metabolite ergosterol and total
834 fatty acids. *Journal of Agricultural and Food Chemistry, 43,* 2904-2910.
835 Yuan, J. P., Kuang, H. C., Wang, J. H., & Liu, X. (2008). Evaluation of ergosterol and its esters in
836 the pileus, gill, and stipe tissues of agaric fungi and their relative changes in the
837 comminuted fungal tissues. *Applied Microbiology and Biotechnology, 80*, 459-465.
838 Yuan, J. P., Wang, J. H., Liu, X., Kuang, H. C., & Zhao, S. Y. (2007). Simultaneous determination
839 of free ergosterol and ergosteryl esters in Cordyceps sinensis by HPLC. *Food Chemistry,*
840 *105*, 1755-1759.
841 Zhang, S. Y., Xu, L. T., Li, A. X., & Wang, S. M. (2015). Effects of ergosterol, isolated from
842 *Scleroderma Polyrhizum* Pers., on lipopolysaccharide-induced inflammatory responses in
843 acute lung injury. *Inflammation, 38*, 1979-1985.
844 Zhu, R., Zheng, R., Deng, Y., Chen, Y., & Zhang, S. (2014). Ergosterol peroxide from *Cordyceps*
845 *cicadae* ameliorates TGF-beta1-induced activation of kidney fibroblasts. *Phytomedicine,*
846 *21*, 372-378.

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848 **Figures**

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

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

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

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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) |

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|---|---|---|---|---|---|
| *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) |

878 *A. Bisporus*: *Agaricus bisporus*
879 DES: Deep eutectic solvents
880 Dw: Dry weight
881 HAE: Heat-assisted extraction
882 *L. edodes*: *Lentinula edodes*
883 LLE: Liquid-liquid extraction
884 MAE: Microwave-assisted extraction
885 PLE: Pressurized liquid extraction
886 SE: Soxhlet extraction
887 SFE: Supercritical fluid extraction
888 *T. melanosporum*: *Tuber melanosporum*
889 *T. aestivum*: *Tuber aestivum*
890 *T. indicum*: *Tuber indicum*
891 UAE: Ultrasound-assisted extraction
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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) |

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|---|---|---|---|---|
| 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) |

913 RP: Reverse phase
914 UHPLC: Ultra-high-performance liquid chromatography
915 N.M.: Not mentioned
916 UV: Ultraviolet
917 DAD: Diode-array-detector
918 PAD: Photodiode array detector
919 MS: Mass spectrometer
920 d.w.: dry weight
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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*<br>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*<br>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) |

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| Ergosterol peroxide | Chemical standard | *In vitro*<br>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*<br>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*<br>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*<br>HCT116, HT-29, SW620 and DLD-1 human colorectal cancer cells<br><br>*In vivo*<br>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 sub$G_1$ stage. Suppressed the nuclear levels of β-catenin leading to reduced transcription of c-Myc, cyclin D1, and CDK-8. | (Kang et al., 2015) |

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| Ergosterol | *Amauroderma rude* | *In vitro*<br>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*<br>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*<br>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*<br>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*<br>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) |

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| | | | | 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 |
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| 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) |

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| | | | | 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-κB, 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-κB 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-κB 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) |

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| 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) |

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| | *In vivo*<br>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 $\alpha$-SMA in vivo. Reduced- liver/BW or spleen/BW, hepatic inflammation and collagen deposition in vivo. |