Original Article

# Anticancer, Apoptotic, and Mitotic Effectiveness of *Lentinula edodes* Microwave-Assisted Extract against Certain 2D Monolayer Culture Cancer Cell Lines

**Ahmed Ghazi Sabbar Qaicy[1], Fatima A. R. Samarrai[1], Mohammed F. Haddad[2], Yassar Y. Al-Tamer[3]**

[1]Department of Medical Lab Techniques, Middle Technical University, Baghdad, Iraq, [2]Department of Medical Lab Techniques, Northern Technical University, Mosul, Iraq, [3]Department of Medical Lab Techniques, Al-Noor University, Mosul, Iraq

## Abstract

**Background:** *Lentinula edodes* (*L. edodes*), a wild edible mushroom, grows naturally when nitrogen is fixed in the soil by lightning strikes. It is found to possess different biological activities due to its active ingredients. **Objective:** The present study aimed to evaluate the anticancer, cytotoxic, and mitotic activity of *L. edodes* against certain types of cancer cell lines. **Materials and Methods:** Ethanolic extract of *L. edodes* was prepared using a microwave-assisted extraction method. The cancer cell lines were cultured in Dulbecco's modified eagle medium supplemented with 10% fetal bovine serum (FBS) and antibiotics, whereas the normal cell line was cultured in Roswell park memorial institute supplemented with 10% FBS. For one time of exposure at 72 h, the cytotoxic effect of the ethanolic extract of *L. edodes* was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole (MTT) assay, a non-radioactive, fast assay widely used to quantify cell viability and proliferation. Mitotic index was measured to detect the effect of the extract on lymphocytes, using colcemid and phytohemagglutinin to activate and inhibit mitosis as controls. **Results:** The results revealed that the initial significant concentration of *L. edodes* extract on the HepG2 (hepatocellular carcinoma) cell line started at 25 µg/mL in a dose-dependent manner after 72 h of exposure, with highly significant differences at $P < 0.05$. The results also revealed significant cytotoxicity of *L. edodes* extract on the HCT116 (human colorectal cancer cell line) after 72 h of exposure ($P < 0.05$). **Conclusion:** Besides the different biological activity of *L. edodes*, it may have important anticancer and cytotoxic activities, and it has no effect on blood lymphocyte mitosis. This encourages its use as a treatment for cancer in future.

**Keywords:** Cell line, HCT116, HepG2, *Lentinula edodes*, PANC-1

## Introduction

There are many distinct types of cancer, but they all have one thing in common: unregulated growth that advances to infinite expansion.[1] The primary cancer treatments include surgery, radiation, chemotherapy, stem cell transplant, biological therapies, angiogenesis inhibitors, and hormone therapy. The therapy chosen depends on a variety of criteria, including the patient's location, the extent of the cancer, and the stage of the disease.

The discovery of additional novel natural products and their semi-synthetic analogs as potential cancer chemotherapeutic drugs has replaced the use of treatments whose efficacy is frequently constrained by the toxicity to the body's normal tissues.[2] Among the huge number of medicinal herbs, many herbs have cytotoxic and anticancer activities, depending on their phytochemical constituents.

*Lentinula edodes* (*L. edodes*) is a wild edible mushroom, and the second most cultivated edible mushroom in the world, representing about 25% of worldwide mushroom production.[3] The bioactive compounds in *L. edodes* have

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**Address for correspondence:** Dr. Ahmed Ghazi Sabbar Qaicy, Department of Medical Lab Techniques, Middle Technical University, Baghdad, Iraq. E-mail: ahmed2421970@mtu.edu.iq

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been shown to have anti-tumor and antihypertensive effects.[4,5]

## Materials and Methods

### Plant samples

*L. edodes* were obtained from a local farm in Iraq, cut into small slices, and then extracted with 70% ethanol using microwave-assisted extraction with a time of 5 min, as mentioned by Gharekhani *et al.*[6] When the necessary temperature (75°C–85°C) was reached, the mixture was microwave-irradiated using pre-set protocols (10 s power on, 15 s power off for three times), followed by 3 s of heating and 10 s of cooling, until the pre-set extraction time was completed. The flask was then removed from the microwave, and the extract was filtered through Whatman no. 1 filter paper and then lyophilized, and the powder was kept in a freezer at −20°C. All steps are preceded by weighing the sample.

### Cytotoxic effect of ethanolic extract on certain cell lines

Three types of cancer cell lines, such as "human colorectal cancer cell line (HCT116)," "hepatocellular carcinoma cell line (HepG2)," and "pancreatic ductal adenocarcinoma (PANC-1)," along with the rat embryo fibroblast (REF) cell line (a normal cell line), were kindly purchased from the Iranian Biological Resource Center and used in the present study. The cell lines were cultured according to the method described by Capes-Davis and Freshney.[7]

The sub-culturing for those four types of cell lines was done for the purpose of monolayer.

HCT116 were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) + 100 units/mL penicillin + 100 µg/mL streptomycin. Hepatocellular HepG2 cells were cultured in DMEM + 10% FBS + 1% pen-strep. Pancreatic PANC-1 cells were cultured in DMEM + 2 Mm glutamine + 10% FBS; furthermore, the REF, a normal cell line, was maintained in Roswell park memorial institute-1640 containing 10% FBS. All cell lines were incubated at 37°C in 5% $CO_2$. To pursue subculture, the *in vitro* cell culture was treated with a trypsin/versine mixture when the cells formed a monolayer.

The freeze-dried extract was thawed and dissolved in dimethylsulfoxide (DMSO), and a serial dilution was made to obtain a set of gradual concentrations (12.5, 25, 50, 100, 200, and 400 µg/mL), all used in triplicate. The DMSO concentration was kept below 0.3% throughout the cell culture and treatment period and did not exert any detectable effect on cell growth or cell death.[8] Figures 1–4 scanned by an inverted microscope were representative of different scopes of the cultured cell lines.

The cytotoxic effect of the ethanolic extract of *L. edodes was* evaluated by MTT assay, a non-radioactive, fast assay widely used to quantify cell viability and proliferation. The exposure time for the assay was 72 h. For all types of cell lines, the inhibition rate was measured according to Qaicy.[8] Cytotoxicity was described by Capes-Davis and Freshney[7] and expressed as inhibitory concentration 50

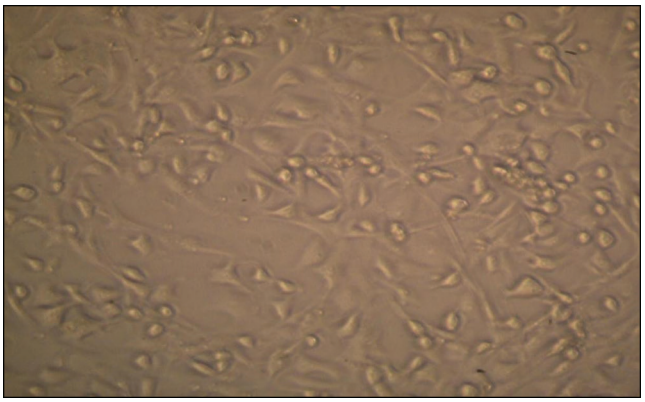

**Figure 1:** HepG2 cell line image using inverted cell culture microscope Leica DM IL LED

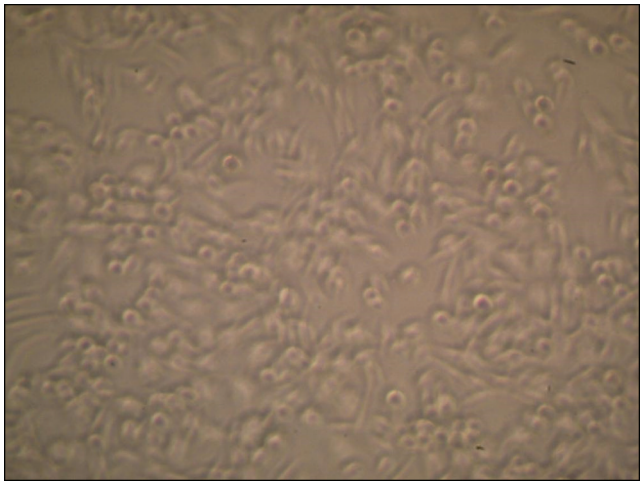

**Figure 2:** HCT116 cell line image using inverted cell culture microscope Leica DM IL LED

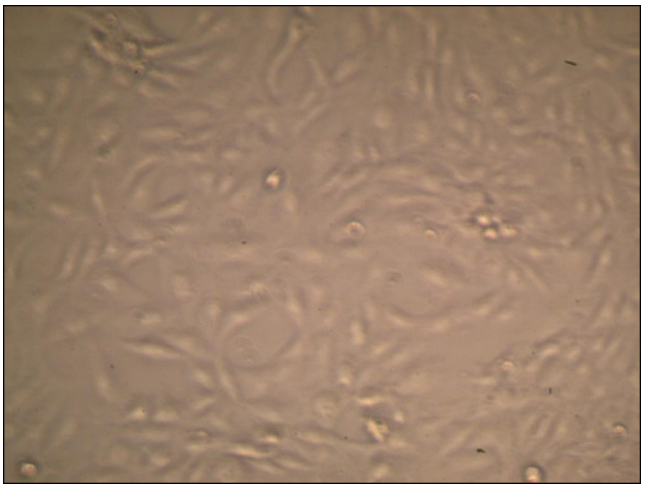

**Figure 3:** PANC1 cell line image using inverted cell culture microscope Leica DM IL LED

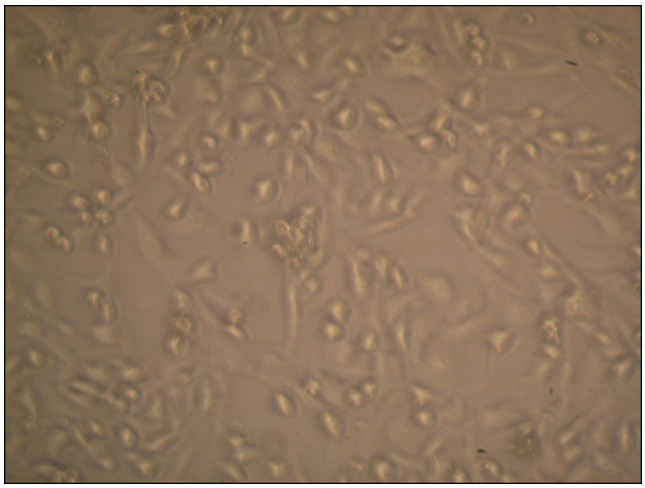

**Figure 4:** Ref cell line image using inverted cell culture microscope Leica DM IL LED

($IC_{50}$), which is the concentration that reduces the viability of treated cells by 50% compared to the control (untreated cells).

### Effect of *Lentinula edodes* on normal human lymphocytes (calculation of mitotic index)

A total of 5 mL of blood was collected from two normal adult persons in a sterile syringe containing heparin. Management's procedure of mitotic index was carried out as mentioned by Capes-Davis and Freshney[7] and Qaicy,[8] and the calculation formula for estimation of the mitotic index was described by Ikeda *et al.*[9]

### Statistical analysis

GraphPad Prism 5 software (GraphPad, Inc., La Jolla, CA, USA) was used for statistical analysis and calculation of the half maximal $IC_{50}$, and one-way analysis of variance was used to indicate a statistically significant difference.

## Results

The present study was carried out to assess the cytotoxic effect of ethanolic extract of *L. edodes* against three cancer cell lines and one normal cell line.

When the cancer cell lines were treated with the ethanolic extract of *L. edodes*, the results showed significant growth inhibition % in a dose-dependent manner. The cytotoxic effect of the extract varied among the cancer cell lines used in the present study. The $IC_{50}$ was estimated, and the result varied among different cell lines.

The result in Table 1 revealed that the initial significant concentration of *L. edodes* extract on the HepG2 cell line started at 25 µg/mL in a dose-dependent manner after 72 h of exposure, with highly significant differences at $P < 0.05$.

**Table 1: Initial significant concentration and inhibition rate of *Lentinula edodes* extract on HepG2 cancer cell line after 72 h of exposure**

| Concentration (µg/mL) | Inhibition rate ± SE | Initial significant concentration (µg/mL) | CS |
|---|---|---|---|
| 12.5 | 0 | 25 | One-way ANOVA |
| 25 | 5.46 ± 0.1855 | | |
| 50 | 15.033 ± 0.145 | | $^{*}P < 0.0001$ |
| 100 | 40.83 ± 0.4409 | | |
| 200 | 60.333 ± 0.333 | | |
| 400 | 85.1666 ± 0.218 | | |

$^{*}P < 0.05$: highly significant difference

On the other hand, Figure 5 represents the $IC_{50}$ of *L. edodes* ethanolic extract on the HepG2 cancer cell line, which is 155.5.

After 72 h of exposure, the inhibitory rate and significant cytotoxicity of *L. edodes* extract regarding HCT116 are shown in Table 2. It is clear that the initial significant concentration starts at 50 µg/mL, and there were highly significant differences among inhibition rates with gradually increasing concentrations ($P < 0.05$). Moreover, in the same way, the $IC_{50}$ of *L. edodes* on HCT116 was 141.7, as shown in Figure 6.

The results in Table 3 represent the effect of our interested extract on PANC-1 cell line, where it shows us a highly significant difference ($P < 0.05$), among inhibition rates by increasing the concentration of *L. edodes* extract, knowing that the initial significant concentration starts with 50 µg/mL in the case of PANC-1 cancer cell line after 72 h of exposure.

Figure 7 indicates that the $IC_{50}$ of *L. edodes* extract against the PANC-1 cancer cell line was 260.1.

The normal fibroblastic cells REF were cultured and used for evaluating the cytotoxicity of *L. edodes* extract. Results showed slight cytotoxicity at each concentration in a dose-dependent manner but with a non-significant difference ($P > 0.1$) on that particular primary cell, as shown in Table 4.

The $IC_{50}$ regarding the reference cell line is outside the concentration range, so a higher concentration 400 is used, as shown in Figure 8.

In the study of mitotic index, the ethanolic extract of *L. edodes* exhibited no ability to stimulate or inhibit the human peripheral blood lymphocytes to proliferate at different concentrations; therefore, no dividing cells were detected in metaphase. On the other hand, the ethanolic extract neither exerted colchicine-like nor phytohemagglutinin (PHA) activity, and no blood lymphocyte was arrested at metaphase. Furthermore, the lymphocytic cells exposed to different concentrations showed no deformation.

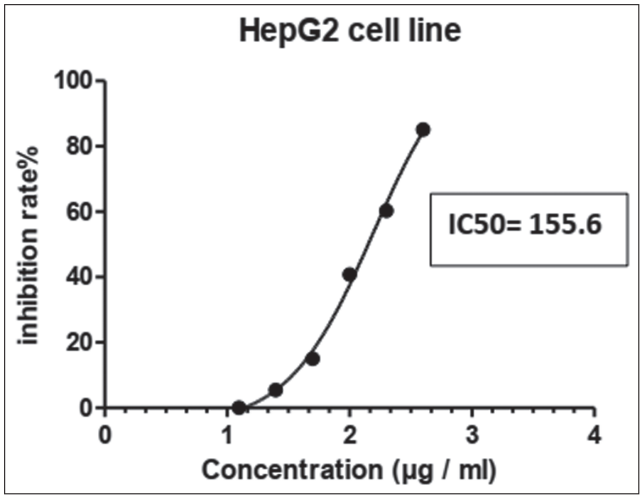


**Figure 5:** $IC_{50}$ for HepG2 cancer cell line

**Table 2: Inhibitory rate and initial significant concentration of *Lentinula edodes* extract on HCT116 cancer cell line after 72 h of exposure**

| Concentration (µg/mL) | Inhibition rate ± SE | Initial significant concentration (µg/mL) | CS |
|---|---|---|---|
| 12.5 | 0 | 50 | One-way ANOVA |
| 25 | 0 | | |
| 50 | 18.1 ± 0.4618 | | $^{*}P < 0.0001$ |
| 100 | 32.5 ± 0.8717 | | |
| 200 | 76.4 ± 0.7094 | | |
| 400 | 95.8 ± 0.5507 | | |

$^{*}P < 0.05$: highly significant difference

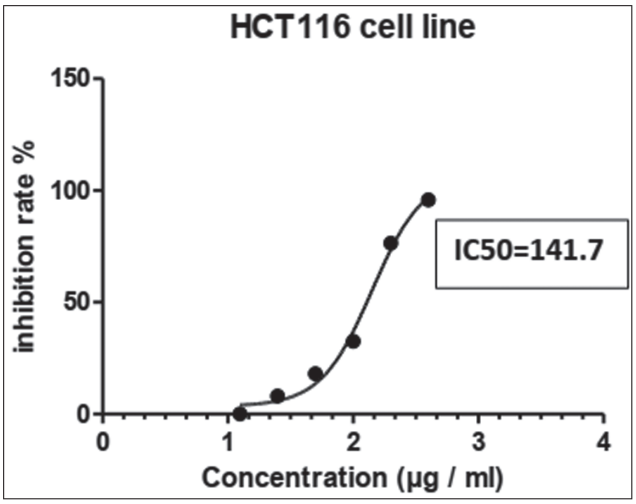


**Figure 6:** $IC_{50}$ for HCT116 cancer cell line

## Discussion

Statistically, it can be documented that the ethanolic extract of *L. edodes* possesses a cytotoxic effect on the cancer cells used in the present study (HepG2, HCT116, and PANC-1), with low toxicity against the reference normal cell lines. The severity of cytotoxicity increases in a dose-dependent manner, whereas no significant cytotoxic effect towered REF normal cell line.

The ethanolic extract of *L. edodes* exhibits different activity on different cell lines. This selectivity could be due to the sensitivity of the cell lines to the active compounds in the extract or to tissue-specific response.

Six concentrations of the extract (12.5, 25, 50, 100, 200, and 400 µg/mL) were used, respectively, in one time of

**Table 3: Inhibitory rate and initial significant concentration of *Lentinula edodes* extract on PANC-1 cancer cell line after 72 h of exposure**

| Concentration (µg/mL) | Inhibition rate ± SE | Initial significant concentration (µg/mL) | CS |
|---|---|---|---|
| 12.5 | 0 | 50 | One-way ANOVA |
| 25 | 0 | | |
| 50 | 20.475 ± 0.6076 | | $^{*}P < 0.0001$ |
| 100 | 34.7 ± 0.2739 | | |
| 200 | 66.325 ± 0.4404 | | |
| 400 | 97.95 ± 0.6062 | | |

$^{*}P < 0.05$: highly significant difference

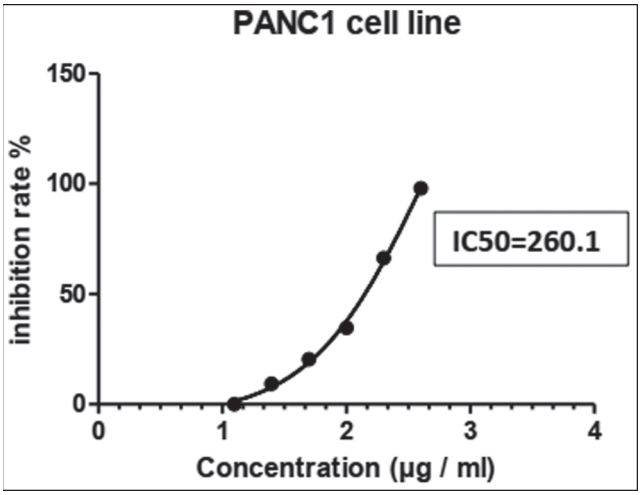


**Figure 7:** $IC_{50}$ for PANC-1 cancer cell line

**Table 4: Inhibitory rate and initial significant concentration of *Lentinula edodes* extract on rat embryo fibroblast (normal fibroblastic cell line after 72 h of exposure)**

| Concentration (µg/mL) | Inhibition rate ± SE | Initial significant concentration (µg/mL) | CS |
|---|---|---|---|
| 12.5 | 0 | 100 | $^{*}P > 0.1$ |
| 25 | 0 | | |
| 50 | 0 | | |
| 100 | 4.163 ± 0.1789 | | |
| 200 | 12.233 ± 0.2027 | | |
| 400 | 21.666 ± 0.2848 | | |

$^{*}P > 0.05$ is non-significant

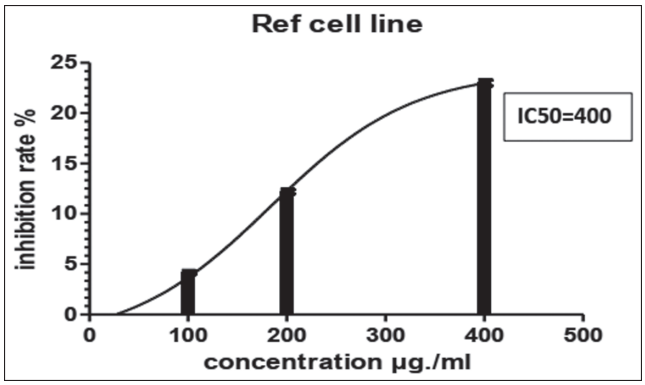


**Figure 8:** $IC_{50}$ for rat embryo fibroblast cell line. *There is no $IC_{50}$, so the highest concentration was used

exposure 72 h. These concentrations are the best range to give the least significant inhibitory effect of *L. edodes* extract *in vitro*.[8]

The final outcome from the MTT assay was a variable cytotoxic effect with significant differences on three types of cancer cell lines (HepG2, HCT116, and PANC-1) in a dose-dependent manner. From the data in Tables 1–3 and Figures 5–7, it was concluded that the *L. edodes* extract possesses a highly cytotoxic effect on HepG2, HCT116, and PANC-1 cancer cell lines, and this may be attributed to the presence of many specific compounds in this extract. Choi *et al.*[10] revealed that the ethanolic extract of *L. edodes* consists of different phytocomponents varied in percentages, and they contain polyphenols, flavonoids, β-carotene, and lycopene. Furthermore, Das *et al.*[11] investigated the presence of about 36 different types of fatty acid, including polyunsaturated fatty acids in *L. edodes* extract.

Kim *et al.*[12] mentioned that lycopene promotes apoptosis in gastric carcinoma cells by inhibiting the nuclear translocation of *β*-catenin and expression of most cancer cell survival genes. Lycopene increased deoxyribonucleic acid degradation and the Bcl-2-associated X protein (BAX/BCL-2) ratio, decreasing the viability of the human gastric cancer cells. Lycopene-mediated reactive oxygen species (ROS) reduction also lowered activation of the extracellular-signal-regulated kinase and p38 pathway.[13] Furthermore, lycopene reduces the viability of the PANC-1 cell line by a similar mechanism of action,[14] activating apoptosis of PANC-1 cells by lowering ROS level.[15]

Several studies showed that lycopene reinforces the expression of the pro-apoptotic protein BAX, whereas it inhibits the anti-apoptotic protein BCL-2 in ovarian,[16] oral,[17] and breast cancer.[18] The studies showed that cell treatment with lycopene inhibited insulin-like growth factor I-stimulated cell cycle progression from G1 to S phase and decreased phosphorylation of retinoblastoma protein.

These actions were in response to a decrease in cyclin D1 and cyclin-dependent kinase inhibitor 1 levels, with no variation in the levels of cyclin E and cyclin-dependent kinase 2 (CDK2)/CDK4 in Michigan cancer foundation-7 (MCF-7) and enterochromaffin cell-1 cell lines.[19] In 2020, Huang *et al.*[20] in the first evidence suggested that *β*-carotene regulates the cancer microenvironment by IL-6/signal transducers and activators of transcription3-mediated inhibition of M2 macrophage polarization and fibroblast activation. *β*-carotene also has a self-renewal of colon cancer stem cells via epigenetic regulation.[12]

In the mechanism of carcinogenesis, flavonoids interfere with different signal transduction pathways and thus inhibit proliferation, angiogenesis, and metastasis or increase apoptosis.[19] Polyphenols, known for their potential antioxidant effectiveness and free radical scavenging capability, were also found to activate cellular cell mortality in human esophageal carcinoma109 cells by increasing ROS and decreasing glutathione levels, confirming intrinsic mitochondrial cell damage.[21]

Similar pro-apoptotic effects were connected to HT-29, MCF-7, and MD Anderson-metastasis breast cancer (MDA-MB-231) cells.[22,23] By same way, a type of flavonoid called naringenin was used to study its activity against MCF-7 cell lines, resulting in the activation of caspase-3.[24] Generally, flavopiridol, a type of flavonoids, initiates cell cycle stop and apoptosis in a p53-independent mechanism that includes the downregulation of anti-apoptotic MCL-1 and X-IAP and the induction of ER stress.[15,25-27]

Several studies documented the biological effects of the polyphenol constituent quercetin on autophagy in various types of cancer. Quercetin is an important member of the flavonoids subclass of flavanol, and it is the most common flavanol in the diet of the Western population.[28] Klappan *et al.*[29] confirmed that quercetin (90 µM) activates autophagy-mediated cell death by inhibiting the proteasome activity and the mTOR signaling pathway in epithelial cancer cells (MCF-7, HeLa).

Many studies have estimated the role of flavones on autophagy. Brunelli *et al.*[30] evaluated the effects of increasing concentrations of 8-prenylapigenin and its 3'-methoxylated analog, isocannflavin B (IsoB), on the proliferation of estrogens-sensitive $ER^+$ T47-D and insensitive $ER^-$ MDA-MB-231 cells. Brunelli *et al.*[30] noted that IsoB (25 µM) activated autophagic cell damage in $ER^+$ breast cancer cells. Moreover, many studies have documented that luteolin, a type of flavonoid, induces cell damage by the activation of autophagy. For example, Park *et al.*[31] reported that luteolin-activated endoplasmic reticulum stress-mediated apoptosis and Beclin 1-independent autophagy in NCI-H460 lung carcinoma cells.

The present work revealed that there are no effects of *L. edodes* ethanolic extract on normal peripheral lymphocytes, as it did not stimulate or inhibit the human

peripheral blood lymphocytes at different concentrations; therefore, no dividing cells were detected in metaphase. In other words, the ethanolic extract neither exhibited colchicine-like nor PHA activity, and no blood lymphocytes were arrested at metaphase. Furthermore, the lymphocytic cells exposed to different concentrations showed no deformation. Interpretation for these results might be that the phytocomponents of *L. edodes* were not stimulating lymphocyte proliferation, and this is good news encouraging to use of *L. edodes* in the treatment of cancers.

## Conclusion

L. *edodes*, as a wild and cultivated edible mushroom, has significant activity against certain cancer cell lines with no cytotoxic effect on normal cell lines and has no harmful effect on human lymphocyte mitosis.

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Nil.

### Conflicts of interest

There are no conflicts of interest.

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