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Original Article

Anti proliferative Potential of Ergosterol : A Unique Plant Sterol on Hep2 Cell Line

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ABSTRACT

The anti proliferative effect of ergosterol (5,7, 22-ergostatrien-3b-ol) was studied, it is a type of plant sterol ,which are present in two main forms, free and esterifies. MTT assay showed that the ergosterol had marked cytotoxic activity in Hep2 cancer cells. We observed the higher concentrations (40, 80,160 and 320 µM/mL) of ergosterol treatment significantly showed increased cytotoxicity in Hep2 cells. The IC_{50} value was found to be 40 µM/mL of ergosterol could greatly inhibit the cell growth. So, we have chosen at 40 and 320 µM/mL concentration of ergosterol for further experiments. We observed increased levels of Lipid peroxidation and decreased levels of anti oxidants (SOD, CAT, GPx, and GSH) in Hep2 cell line, because of the pro oxidant mechanism of ergosterol. Present results showed that the treatment of Hep2 cells with 320 µM/mL of ergosterol significantly increased levels of TBARS and decreased enzymatic and non enzymatic levels when compared to other doses. These observations revealed the cytotoxic potential of ergosterol, which could be attributed to their pro-oxidant property on the Hep2 cells. It is evident from the observation made in the present study that the ergosterol has potential anticancer effect.

Key words: Anticancer, Antioxidant, cytotoxicity, Ergosterol, Hep2 cells .

1. INTRODUCTION

Laryngeal cancer is the most common malignancy of the head and neck. It is estimated that around 80-95% of them represents squamous cell carcinoma. Although there have been great advances in treatment of cancer, the struggle to combat cancer is one of the greatest challenges of mankind, due to the lack of widespread and comprehensive early detection methods and the poor prognosis of patient diagnosis in later stages of the diseases [1].Currently, conventional approaches to the treatment of cancer are includes radiotherapy, chemotherapy and hormonal therapy [2]. However, the available treatment regimens are not able to

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achieve a cure, nor improve survival substantially, except in rare cases. Thus new target for prevention and new agents for therapy need to be identified. Although many chemical anticancer agents are available, the emergence of chemotherapy among patients has made cancer research and discovery of new anti cancer agents. Further, synthetic anti cancer drugs evoke severe side effects in many cases; patients may recover cancer but may die due to side effects and severe immuno suppression. Natural dietary agents including fruits, vegetables and species have shown a great deal of attention from both the scientific community and general public owing to their demonstrated ability to suppress cancer [3]. Functional foods represent one of the most widely promoted and extensively investigated areas in the nutrition and food science [4]. The vast structural diversity of natural compounds found in mushroom provide unique opportunities for discovering new drugs that rationally target the abnormal molecular and biochemical signals leading to cancer. Edible mushrooms can be viewed as good candidates to be included in healthy diets. Besides their unique flavor and sensory properties, mushrooms provide excellent nutritional and healthy properties [5]. On dry weight basis, mushroom can be considered as a good source of proteins, carbohydrates and dietary fibre [6], in addition, to the significant amounts of bioactive metabolites involved in health promotion and disease prevention.

Besides their low fat content and high protein percentage [5], the greatest interest in mushroom is probably due to the occurrence of a wide range of phytochemical compounds, such as, sterols, ceramides or phenolic compounds [6]. These substances may promote human health since they seem to be involved in the prevention and treatment of several diseases including cancer. Edible mushrooms also contain sterols (ergosterol) with structural similarities to phytosterols and cholesterol.

Ergosterol is a biological precursor of vitamin D_2, the chemical name of which is ergocalciferol. Exposure to ultraviolet light causes a photochemical reaction that converts ergosterol to ergocalciferol [7]. In addition, ergosterol has great importance because it undergoes photolysis when exposed to UV light (280–320 nm), yielding provitamin D2 as one of the main products. Under thermal rearrangement, provitamin D2 is spontaneously transformed into vitamin D2. On the other hand, ergosterol and derivatives have shown a wide range of health-promoting properties, such as the antioxidant [8], the anti-inflammatory [9] or the anti hyperlipidemic [10] activities. Integrity of the cell membrane requires that inserted sterols lack C-4 methyl groups. Several lines of evidence suggest that the primary target of azoles is the heme protein, which co-catalyzes cytochrome P-450-dependent 14α-demethylation of lanosterol [11]. Inhibition of 14α-demethylase leads to depletion of ergosterol and accumulation of sterol precursors, including 14α-methylated sterols (lanosterol, 4,14-dimethylzymosterol, and 24-methylenedihydrolanosterol), resulting in the formation of a plasma membrane with altered structure and function. The more recent triazole derivatives, such as fluconazole, itraconazole, and voriconazole (a triazole in development), owe their antifungal activity at least in part to inhibition of cytochrome P-450-dependent 14α-sterol demethylase [12].

Furthermore, ergosterol seems to be involved in the activated expression of a number of defense genes and, moreover, this sterol appears to increase the resistance of plants against pathogens [13]. Another important class of mycochemical comprises phenolic compounds, which have shown a protective effect in several human diseases, for instance, cancer, cardiovascular disease, or inflammatory processes [14]. The mechanism of action is commonly related to their excellent antioxidant properties [15]. The radicals formed by reactive oxygen species can be stabilized by the highly conjugated structure of phenolic derivatives, so that biological molecules such as lipids, proteins, and DNA [16] are prevented from radical attack and subsequent oxidation.

In the present study, we have made an attempt to confirm the antiproliferation effect of ergosterol on human laryngeal cancer cell line. (Hep2). To test this hypothesis, the following objectives were studied. To investigate the cytotoxicity effect of ergosterol in Hep2 cell line and to study the effect of ergosterol on lipid peroxidation status in Hep2 cancer cells by analyzing TBARS level, and also to determine the antioxidant potential of ergosterol by studying enzymatic and non- enzymatic antioxidant status in Hep2 cancer cells.

2. MATERIALS AND METHODS

Chemicals

Ergosterol, Thiobarbituric acid (TBA), Phenazine methosulphate (PMS), nitroblue tetrazolium (NBT),dimethyl sulfoxide (DMSO), 5, 5-dithiobis 2-nitrobenzoic acid (DTNB), 3-(4, 5-dimethyl-2-thiaozolyl)-2, 5-diphenyl-tetrazolium bromide (MTT), fetal calf serum (FBS), minimum essential medium (MEM), glutamine, penicillin-streptomycin, ethylene diamine tetra acetic acid (EDTA), trypsin and phosphate buffered saline (PBS) were purchased from Sigma Aldrich Mumbai, India. And all other chemicals used in this study are of analytical grade.

Preparation of drug

Ergosterol was dissolved in 0.2% dimethyl sulfoxide (DMSO). The stock solution was serially diluted with sterile Minimal Essential Media (MEM) to arrive at 0.007-1mg/mL of ergosterol and used for further studies.

Cell culture and maintenance

Human laryngeal cell line was purchased from National centre for cell science (NCCS), Pune, India. The cells were Minimum Essential Medium (MEM), supplemented with 10% heat-inactivated foetal bovie serum (FBS), 1% penicillin-steptomycin. The cells were maintained as monolayers in T_{25} tissue culture flasks at 37^{o} C in a humidified atmosphere of 5% co_2 and 95% air incubator under standard condition. The Hep2 cells were harvested by

trypsinization. The trypsinized cells were washed with phosphate buffered saline (PBS). The cells were suspended in 130 mM KCl plus 50 mM PBS containing 10 µM dithiothreitol and centrifuged at 20,000 × g for 15 min (4° C). The supernatant was collected and used for oxidant and anti - oxidant studies .

Measurement of cell proliferation (MTT assay)

The proliferation activity of cell populations under different treatment conditions were determined by the MTT assay based on the detection of mitochondrial de-hydrogenase activity in living cells. MTT (3-4,5-dimethyl thiazol-2yl)-2,5-di phenyl tetrazolium bromide) assay was first proposed by Mosmann et.,al [17].

Cultured Hep-2 cells ($1x10^6$ cells/mL) were taken into a 96 well plate. Ten microliters of MTT solution (5 mg/mL in PBS) was added to each culture well after 48 h of incubation with ergosterol treatment. The colour was allowed to develop for additional 4 h incubation. An equal volume of DMSO was added to stop the reaction and to solubilize the blue crystals. The absorbance was taken using UV–visible spectrophotometer (Elico SL159, India) at a wavelength of 570 nm. Absorbance was measured in a microplate reader at 540 nm. Images were captured under microscope. Percentage cytotoxicity was calculated as follows:

$$\% \text{ Cytotoxicity} = \frac{\text{Mean absorbance Test cells-Mean absorbance Control cells}}{\text{Mean absorbance Control cells}} \times 100$$

Biochemical estimation

The levels of thiobarbituric acid reactive substances (TBARS) in plasma were estimated by the method of Niehaus and samuelson et al.,[18]. SOD status was estimated by the method of Kakkar et al. [19]. CAT activities were examined by the method of Sinha [20]. GPx activities were determined by the method of Rotruck et al., [21]. GSH levels were determined by the method of Ellman [22].

Statistical analysis

All quantitative measurements were expressed as means ± SD for untreated and ergosterol treated cells. The data were analyzed using one way analysis of variance (ANOVA) on SPSS 11.5/PC (statistical package for social sciences, personal computer) and the group means were compared by Duncan's Multiple Range Test (DMRT). The results were considered statistically significant if the *p* value were less than 0.05.

3. RESULTS

Cytotoxic effect of ergosterol on human laryngeal cancer (Hep2) cells

Figure 1 represented the cytotxicity effect of ergosterol on human laryngeal cancer Hep2 cell line was determined by MTT assay. Various concentrations of ergosterol (10, 20, 40, 80, 160 and 320 µM/mL) were treated with Hep2 cancer cells for 48 h and observed for cytotoxicity by MTT assay as shown in Table 1 and cytotoxicity microscopically Figure 2. The results from MTT assay reaveled that Hep-2 at lower concentration did not inhibit the cell growth (10 and 20µM/mL). However, the higher concentrations (40, 80, 160 and 320 µM/mL) of ergosterol treatment significantly showed increased cytotoxic effect on Hep-2 cells. Moreover, the IC_{50} value of ergosterol was found to be 40 µM/mL could greatly inhibit the cell growth. So, we have chosen the 80, 160 and 320 µM/ml concentration of ergosterol for further experiments.

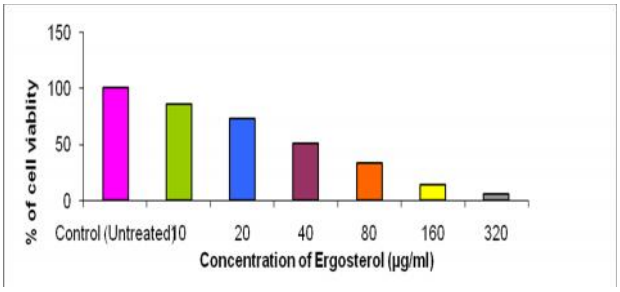

Fig 1: Cytotoxicity of ergosterol in Hep2 cells after 48 hrs of treatment.

Table 1: Measurement of cell viability.

S.No	Concentration (µM/mL)	Average Absorbance	Cell viability
1	Control (Untreated)	1.576	100
2	10	1.476	86.04
3	20	1.346	73.15
4	40	1.05	50.50
5	80	0.78	33.37
6	160	0.42	14.59
7	320	0.22	6.34

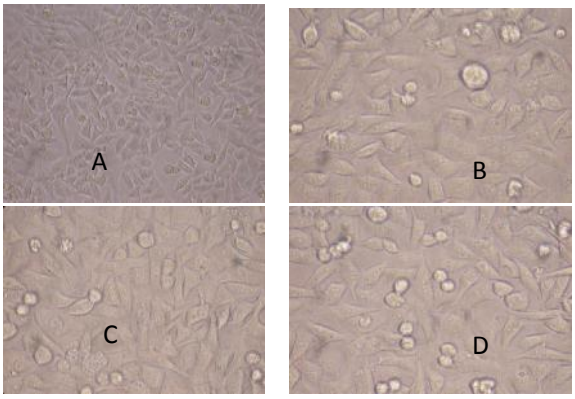

Fig 2: Microscopic observation of Hep-2 cells after 48 hrs of treatment a) Control (Untreated), b) Ergosterol (80 µM/mL), c) Ergosterol (160 µM/mL), d) Ergosterol (320 µM/mL)

Effect of ergosterol on oxidant and antioxidant status

Lipid peroxidation

To study the effect of Ergosterol on oxidative damage, the lipid peroxidation (TBARS) indices in Hep-2 cell line was measured (Figure 3). Ergosterol treatment showed increased levels of lipid peroxidation in Hep-2 cells. Among the three doses (80, 160 and 320 µM/mL) tested, 320 µM/mL of ergosterol showed maximum levels of lipid peroxidation.

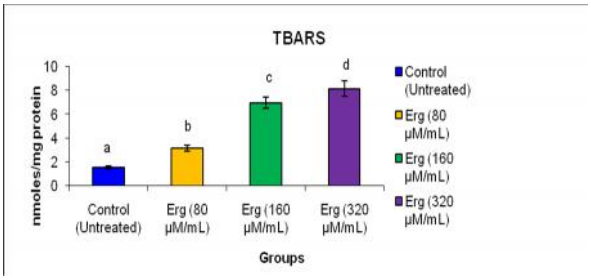

Fig 3: The levels of lipid peroxidation marker (TBARS) in Hep-2 cells of control and experimental groups.

Antioxidants

In this study, the effect of on SOD, CAT GPx and GSH activities in ergosterol treated Hep2 cells were examined. Activities of enzymatic antioxidants such as SOD, CAT, GPx and GSH were depicted in (figure 4,5,6 & 7). Ergosterol treatment significantly decreased the activities of SOD, CAT, GPx and GSH in Hep2 cancer cells. Among the three doses (80, 160 and 320 µM/mL) tested, 320 µM/mL of ergosterol showed maximum levels of depletion activities in enzymatic and non-enzymatic antioxidants when compare to other doses.

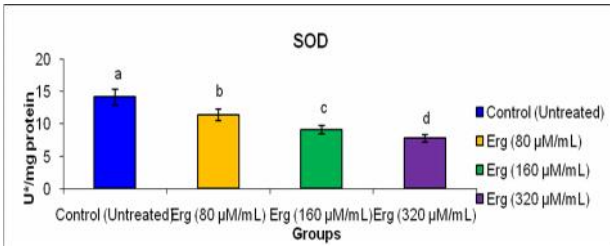

Fig 4: Effect of ergosterol on the activity of SOD in Hep-2 cells of control and experimental groups.

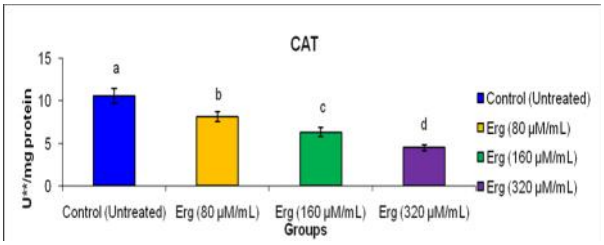

Fig 5: Effect of ergosterol on the activity of CAT in Hep-2 cells of control and experimental groups

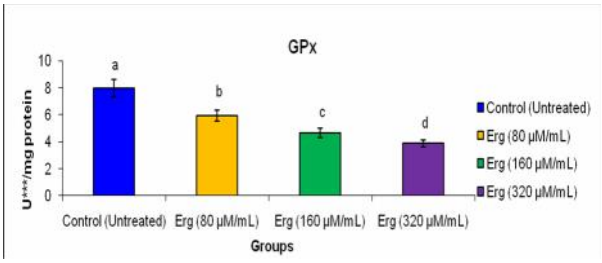

Fig 6: Effect of ergosterol on the activity of GPx in Hep-2 cells of control and experimental groups.

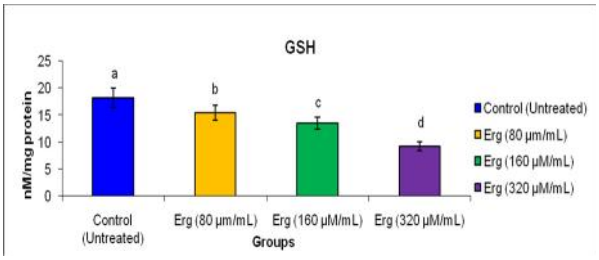

Fig 7: Effect of ergosterol on the activity of GSH in Hep-2 cells of control and experimental groups.

4. DISCUSSION

Mushrooms have been a perennial component of the human diet, consumed since antiquity not only as part of the normal diet but also as a delicacy, because of their texture and highly desirable taste and aroma. Research conducted during the last decades has indicated that mushrooms exert a number of nutritional properties and they are source of beneficial bioactive compounds [23]. Mushrooms are quite rich in protein, providing all the essential amino acids, they have a low fat content, and they contain relatively high amounts of carbohydrates and fiber [24]. They are considered as low-energy functional foods, which could notably contribute to the design of healthy dietary patterns. Besides macronutrients, mushrooms contain significant amounts of bioactive substances such as vitamins and vitamin precursors, minerals and trace elements [24], specific b-glucans, and exert antioxidant properties which are mainly attributed to their phenolic content [25]. A large body of evidence supports the implication of oxidative stress in the pathogenesis of several chronic and degenerative diseases such as atherosclerosis, diabetes mellitus, chronic inflammation, cancer and aging [26].Therefore, the enhancement of the antioxidant systems for the prevention of cellular oxidative damage via the consumption of antioxidant rich foods is of great interest.

Among the bioactive constituents in mushroom sterols are the, predominance mainly ergosterol, the precursor of vitamin D. In mushrooms, ergosterol is converted to vitamin D2 (ergocalciferol) when exposed to UV radiation. Vitamin D2 from fungi and mushrooms serves as the only available dietary source of vitamin D for those who eat no animal products. The crucial role of vitamin D for bone health is well established, while during the last decade its role in immune system modulation and cancer prevention [27] has been recognized. In this present study, we evaluated the anticancer effect of ergosterol in Hep2 cell line.

We discussed antiproliferation activity of ergosterol on human laryngeal cancer cell lines (Hep2) in vitro. The initial screening of bioactive compounds for their anticancer properties use cell-based assays and established cell lines, in which the cytotoxic effects of isolated compounds could be measured. We used MTT (3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay to detect the anti-proliferative activity of ergosterol in Hep2 cells. Decreased

mitochondrial function in cells exposed to ergosterol in a dose-dependent manner as seen in the MTT assay. We observed ergosterol treatment (48-h incubation) significantly decreased percentage of cell viability in Hep2 cells. This result was in line with the previous reports demonstrating that ergosterol peroxide and (22E)-ergosta-7,22-dien-5_-hydroxy-3,6-dione attenuate the growth of prostate cells, at least in part, triggering an apoptotic process, permit to confirm the use of mushrooms as origin of compounds to be used as novel therapeutic agents for cancer treatment.

In recent years, researchers have focused on the pathological role of free radicals in a variety of diseases including cancer [30]. High levels of oxidative stress result in the peroxidation of cell membrane lipids by generating lipid peroxides that can decompose into multiple mutagenic carbonyl products. They are considered to be mutagenic and carcinogenic [28]. They can also modulate the expression of genes that are related to tumour promotion [29]. The levels of TBARS and LOOH reflect the extent of lipid peroxidation. Many of the agents that induce apoptosis are oxidants or stimulators of cellular oxidative metabolism, while many inhibitors of apoptosis show antioxidant activity [30].

Indeed, factors that induce oxidative stress, such as ROS production, lipid per oxidation, down regulation of the antioxidant defences characterized by reduced glutathione levels, and reduced transcription of superoxide dismutase, catalase, and thioredoxin, have been observed in some apoptotic processes [31]. Interestingly, we observed significantly increased in lipid peroxidation indices in ergosterol treated Hep2 cells. This could be attributed to the increased formation or inadequate clearance of free radicals by the cellular antioxidants. Thus, based on the present findings it can be conclude that ergosterol possesses cytotoxic effect on Hep2 cells [32].Oxidative damage of Ergosterol in Hep-2 cells was observed in this study. An increased concentration of end products of lipid peroxidation is the evidence which is most frequently quoted for the involvement of free radicals in human diseases. Lipid peroxidation plays a major role in diseases processes and oxidative stress. [33].

Antioxidants have been shown to inhibit both the initiation and promotion in carcinogenesis as well as counteract cell immortalization and transformation. Cellular enzymatic antioxidants are also known as free radical scavengers that protect a cell against toxic free radicals. Reduced GSH is the chief constituent of the thiol pool and a vital intracellular scavenger of free radicals [34, 40]. It has been also observed decreased activities of antioxidant enzymes, i.e., SOD, CAT, and GPx in ergosterol treated cancer cells. This may be due to pro-oxidant property of ergosterol on Hep2 cell line.

Apart from enzymatic antioxidants and non-enzymatic antioxidants play an excellent role in protecting the cells from oxidative damage. Non enzymatic antioxidant system such as GSH is considered as the second line of defense against free radicals. GSH a major non-protein thiol, presumed to be an important endogenous defense against per oxidative destruction of cellular membranes. Glutathione reacts directly with ROS and electrophilic metabolites, protects essential thiol groups from oxidation and serves as a substrate for several enzymes including GPx [35].

In normal healthy cells, enzymatic and non-enzymatic antioxidant serve to balance the intracellular production of ROS, thereby delaying or inhibiting the destructive oxidation of molecular components. Many studies suggested that antioxidant systems are critical in protecting against tumor promoting agents. Interestingly, cell malignancy or transformation is often accompanied by a decrease in the activities of antioxidant enzymes (SOD, CAT and GPx), which may increase the cell sensitivity to Pro-oxidant compounds.

In the present study, we noticed a prominent decrease of GSH levels in cancer cells treated with ergosterol. Previous study showed that phytochemicals depletes intracellular antioxidants there by induced cancer cell death [37, 38]. Diosgenin a plant derived steroid and cumarin possess an effective pro-oxidant activity in cancer cell line by decreasing antioxidant and enhancing lipid peroxidation [36, 39, 41, 42] .Our results also correlates with the above finding. Thus ergosterol is a potent pro-oxidant and antiproliferative effect on Hep2 cell line and there by inhibits laryngeal carcinoma promotion.

5. CONCLUSION

Our work summarize the effect of ergosterol initiate the cancer cell death by inducing cytotoxicity, which modulates oxidant and antioxidant status in Hep2 cell line. Enhanced lipid peroxidation during ergosterol may be the reason for decreased activities of antioxidant enzymes. Ergosterol treatment depletes GSH level via increasing the oxidation of GSH and inhibiting GSH recycling mechanisms. Present results shows that the treatment of cells with 320 µM/mL of ergosterol significantly shows decreased enzymatic and non enzymatic levels. These observations revealed the cytotoxic potential of ergosterol, which could be attributed to their pro-oxidant property on the cancer cells. It is evident from the observation made in the present study that the ergosterol has potential anticancer effect. Thus the present study strengthens our hypothesis and hope that this novel formulation can open a new avenue for laryngeal cancer therapy.

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