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RESEARCH ARTICLE

ANTICANCER ACTIVITY OF ETHANOL EXTRACTS OF *ANISOCHILUS CARNOSUS* IN HUMAN LUNG CANCER A549 CELL LINES

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ABSTRACT

Cancer is a leading cause of deaths in developing as well as developed countries. Four most frequent serious cancers identified are lung, breast, colorectal and stomach. The currently available chemotherapy agents have drawbacks like sever side effect, poor solubility and non-specificity. In the present study, the anticancer activity of ethanol extracts of *Anisochilus carnosus* was checked against lung cancer A549 cell lines. The ethanol extract of *Anisochilus carnosus* showed dose dependent response against the human lung cancer A549 cells. Furthermore, the ethanol extract *Anisochilus carnosus* induced apoptotic cell death in the A549 lung cancer cells through generation of enhanced reactive oxygen species and altered mitochondrial membrane potential. Moreover, the isolated ethanol extract of *Anisochilus carnosus* steroid active compounds stigmasterol and β-sitosterol was docked with Bcl-2 protein. The steroid active compound stigmasterol and β-sitosterol displays high affinity to Bcl-2 protein. In conclusion, the ethanol extract of *Anisochilus carnosus* could be a novel anticancer agent.

INTRODUCTION

Cancer is one of the leading causes of human deaths in developing and developed countries. The International Agency for Research on Cancer reported worldwide burden rises to 14.1 million new cases and 8.2 million cancer deaths in 2012. The common causes of cancer death were cancers of the lung, liver, and stomach (Jemal *et al*., 2011). The currently available cancer chemotherapy agents are not distinguishing between cancer cells and normal cells. The progress of drug resistance, poor solubility, low therapeutic index and severe side effects also one of the major problems with the existing anticancer agents (Cho *et al*., 2008). Current studies recognized that herbs and herbal medicine are free from thoughtful side effects hence researchers started aiming towards the development of less toxic and more efficient anticancer agents from herbal sources (Muthuraman *et al*., 2012). Anisochilus Carnosus (L) Wall is an annual herb, found in the Western Ghats, belonging to the family of Lamiaceae (Setty *et al*., 2013). We recently reported that the identification and isolation of phytochemical compounds from different parts of the plant extraction of n-hexane. Furthermore, steroid compounds were also isolated from the n-hexane extract of the leaves from Anisochilus carnosus.

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Based on the spectral evidence, 1H-NMR and 13C-NMR, structures were determined to be stigmasterol and β-sitosterol (Kiruthiga and Sathish Sekar, 2014). Hence, in the present work we focused on ethanol extract of Anisochilus carnosus anticancer activity against in human non-small cell lung cancer A549 cell lines and also the extracted steroid active compounds stigmasterol and β-sitosterol was docked against Bcl-2 protein.

MATERIALS AND METHODS

Cell culture

Human non-small cell lung cancer (A549) cell line was obtained from the National Centre for Cell Science, Pune, India. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM: Hi Media Laboratories Mumbai, India), supplemented with 10% Fetal bovine serum and 1% penicillin/streptomycin (Hi Media Laboratories Mumbai, India) in a 5% CO2 humidified atmosphere at 37˚C.

Cytotoxicity assay

The human cancer A549 cells were harvested and diluted 1×10^4 cells/well were seeded into 96-well plates 100 µl of DMEM medium was added to well containing cells. The cells were allowed to adhere at an optimum condition (Overnight Incubation at 37°C in 5% Co_2 atmosphere). After overnight incubation, the culture medium was removed and cells were

rinsed with phosphate buffered saline (1xPBS) and incubated with different concentrations of extracts (50-500 µg) incomplete DMEM medium for 24 hrs. After 24 h of treatment, 20 µl MTT (5mg/ml in 1xPBS) was added to each well and incubated for an additional 4 h at 37°C to allow mitochondrial dehydrogenase to convert MTT into insoluble formazan crystals. The medium was then aspirated, and formazan was solubilized by adding 200 µl of DMSO. The absorption of solubilized formazan was measured at the wavelength of 590 nm with reference wavelength at 620nm in a micro titer plate reader (I Mark microplate reader Bio Rad Co.) (Sheeja *et al.,* 1997). The percent of inhibition of each concentration was calculated by following formula:

$$\text{Percentage of inhibition} = \frac{\text{Control optical density-Dose optical density}}{\text{Control optical density}} \times 100$$

Apoptosis study

The ethanol extract of *Anisochilus carnosus* apoptosis effect against lung cancer A549 cells were confirmed using an acridine orange (AO) and ethidium bromide (EB) staining method. For that, $5x10^5$ cells/well were cultured on cover slip in 6-cell plate and incubated overnight for attachment. After attachment, the cells were treated with ethanol extract of *Anisochilus carnosus*. After 24 h incubation, cover slip was removed and stained with A0/EB for 30 min and washed with PBS. Cover slip was mounted on objective glass and cells images were captured using an inverted fluorescence microscope.

Detection of intracellular reactive oxygen species levels

The 5×10^5 cells were seeded on a coverslip in 6-well plate and incubated overnight for attachment. Next day the cells were treated with fresh medium containing ethanol extract of *Anisochilus carnosus* and incubated for 24 h. At the end of incubation cover-slip was removed from the culture plate and stained with 40 µM of 2', 7'-dichlorofluorescein-diacetate (DCFHDA) dye for 30 min. The stained cover slip was washed with PBS solution. Cover slip was mounted on objective glass and cells images were captured using an inverted fluorescence microscope.

Assessment of mitochondrial membrane potential

The 5×10^5 cells/well were seeded in 6-well plates and incubated overnight for attachment. After overnight attachment the cells were treated with fresh medium containing ethanol extract of *Anisochilus carnosus* and incubated for 24 h. At the end of incubation cover-slip was removed from the culture plate and stained with 50 µl of Rhodamine-123 dye for 30 min, excess dye was removed by washing with PBS and cell images were captured using an inverted fluorescence microscope.

Molecular docking

Preparation of ligand for docking

3D structure of the ligand used in this study were taken in RCSB Protein Data Bank or it also be constructed using chemsketch and save the 3D structure of stigmasterol and β-sitosterol in a new.pdb file. Then converting the ligand file in the requested PDBQT format and sending the files to AutoDock engine for docking process.

Preparation of Proteins for Docking

The crystal structure of the target protein BCL2 protein was retrieved from the Protein Data Bank. Open the.pdb protein files in pyMol and remove all water molecules, manually and the polar and hydrogen atoms were added subsequently. Then converts the protein in the requested PDBQT format, and executes AutoDock Tools (ADT) in order to compute the corresponding grid maps. The pre-docking preparation of the ligand input files is automatically performed by GriDock without any manual intervention.

Autodock 4.0 method

The crystal structure of 2VM6 (BCL2) was downloaded from protein data bank. Molecular docking was performed with the program Autodock4.0 (Muthuraman *et al.,* 2012).

RESULTS AND DISCUSSION

Anticancer activity of ethanol extracts of *Anisochilus carnosus*

The ethanol extracts of *Anisochilus carnosus* toxicity was evaluated against lung cancer A549 cells with different concentration (50 to 500 µg/ml). Our result exhibited that, cancer cells respond to ethanol extract of *Anisochilus carnosus* with dose dependent concentration and the increasing in concentration of ethanol extract of *Anisochilus carnosus* revealed augmented cytotoxicity in cancer cells. The half maximal inhibitory concentration (IC50) of ethanol extracts of *Anisochilus carnosus* against A549 cells 250 µg/ml (Fig.1). The improved cytotoxicity may be due to the presence of bioactive molecules like alkaloids, flavanoids, steroids, glycosides in *Anisochilus carnosus* extracts (Aiyelaagbe and Osamudiamen, 2009; Edeoga *et al.,* 2005).

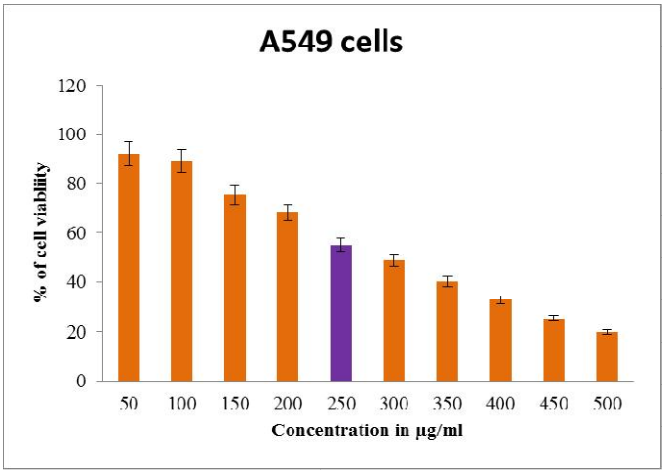

Fig.1. Ethanol extracts of *Anisochilus carnosus in vitro* cytotoxicity against A549 cells

The ethano extract of *Anisochilus carnosus* induced apoptotic in the A549 lung cancer cells. As compared to control the

ethanol extract of *Anisochilus carnosus* treated cells was apoptotic with orange fragmented nuclei when compared with control, agreement with low cell viability (Fig.2).

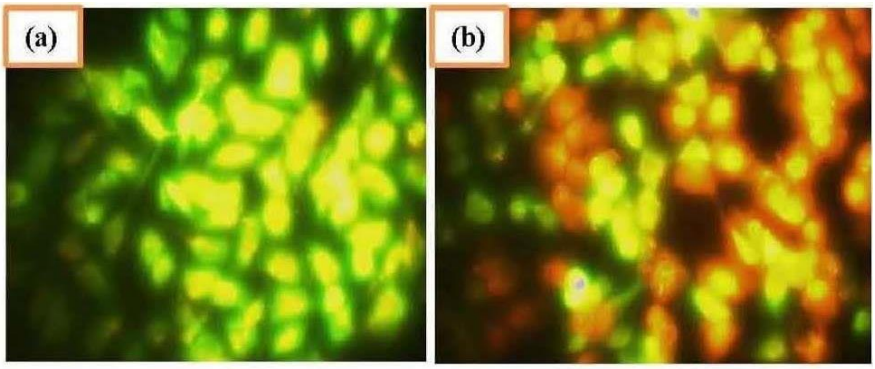

Fig.2. Apoptotic activity in A549 cells after treatment of (a) control; (b) ethanol extract of *Anisochilus carnosus*

The results showed that ethanol extract of *Anisochilus carnosus* could induce cell death through apoptosis. To approve the apoptosis cell death associated with reactive oxygen species (ROS) formation, the intracellular ROS generation level was evaluated using fluorescent probe DCFH-DA (Maurya *et al.,* 2011). The fluorescence microscopy analysis results exhibited ethanol extract of *Anisochilus carnosus* treated cells produced increased fluorescence intensity, indicating the generation of ROS, whereas the control cells had not been produced (Fig.3). The enhanced ROS level in A549 cancer cell alters the mitochondrial functions and play as a key role in apoptosis induction (Gibellini *et al.,* 2010).

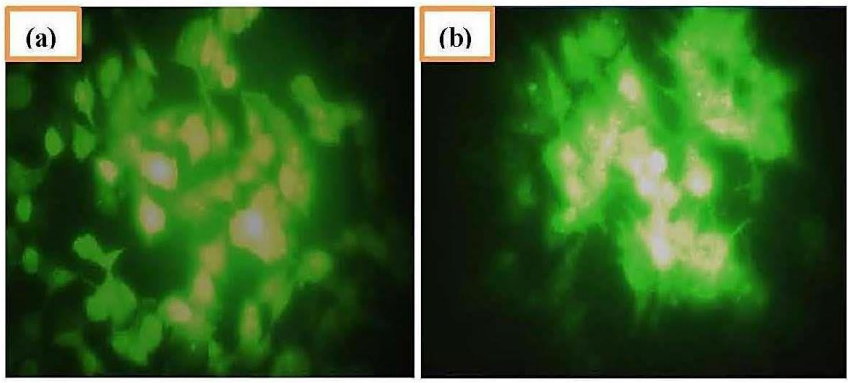

Fig.3. Reactive oxygen species generation in A549 cells after treatment of (a) control; (b) ethanol extract of *Anisochilus carnosus*

The lipophilic cationic Rhodamine-123 is well-organized probe of vital mitochondria, it precisely accumulates in the inner mitochondrial membrane. The high fluorescence indicates healthy mitochondria. Our results exhibited, significantly less amount of Rhodamine-123 dye was taken up in the ethanol extract of *Anisochilus carnosus* treated cells, leads to reduced fluorescence intensity, when compared with control cells (Fig.4). The decreased fluorescence intensity accompany with the loss of mitochondrial membrane, the promise for apoptosis cascade. Our results pay for decisive evidence for the anticancer activity of ethanol extract of *Anisochilus carnosus* in the lung cancer A549 cells.

Molecular docking Stigmasterol and β-sitosterol with Bcl-2 protein

Structure-based drug design begins with the identification of a molecular target such as a protein such as Bcl-2 in this study. This structure is then used as a blueprint for the drug design of a lead compound. The compounds are modeled for their fit in the active site of the target, considering both steric aspects and functional group interactions, such as hydrogen bonding and hydrophobic interactions (doi:10.4172/scientificreports.458). The compounds obtained from ethanol extract of *Anisochilus carnosus* active compounds stigmasterol and β-sitosterol were docked with Bcl-2 Protein (doi:10.4172/scientificreports.458). The qualities of the homology modeled proteins were evaluated using the procheck tool. It estimates the stereo-chemical quality of the modeled structures. On analysis of the Ramachandran plot, it was observed that in the BCl2 protein around 96.7% of the residues were present in the favored regions. The Q-Site Finder analysis produced the ten top most ranked binding sites. The higher cavity site was taken as the most favorable site to dock the ligands. The stigmasterol showed affinity with the BCl2 protein 3MAX (-5.43 kcal/mol) (Fig. 5) and β-sitosterol showed affinity with the BCl2 protein 3MAX (-6.13 kcal/mol) (Fig. 6).

Our investigations shows that stigmasterol and β-sitosterol has good inhibitory activity on Bcl-2 and this can be helpful for further investigations. The docking results data supports the inhibitory activity of ethanol extract of *Anisochilus carnosus* active compounds.

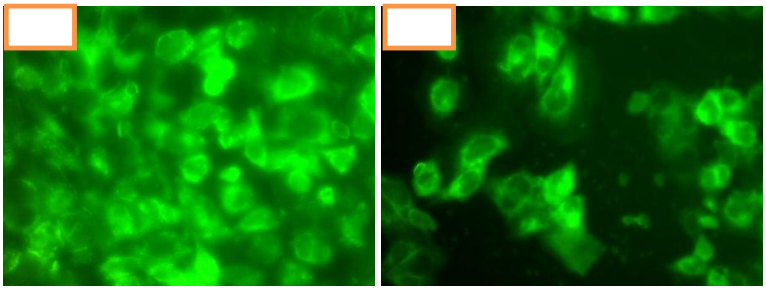

Fig.4. Mitochondrial membrane potential alterations in A549 cells after treatment of (a) control; (b) ethanol extract of *Anisochilus carnosus*

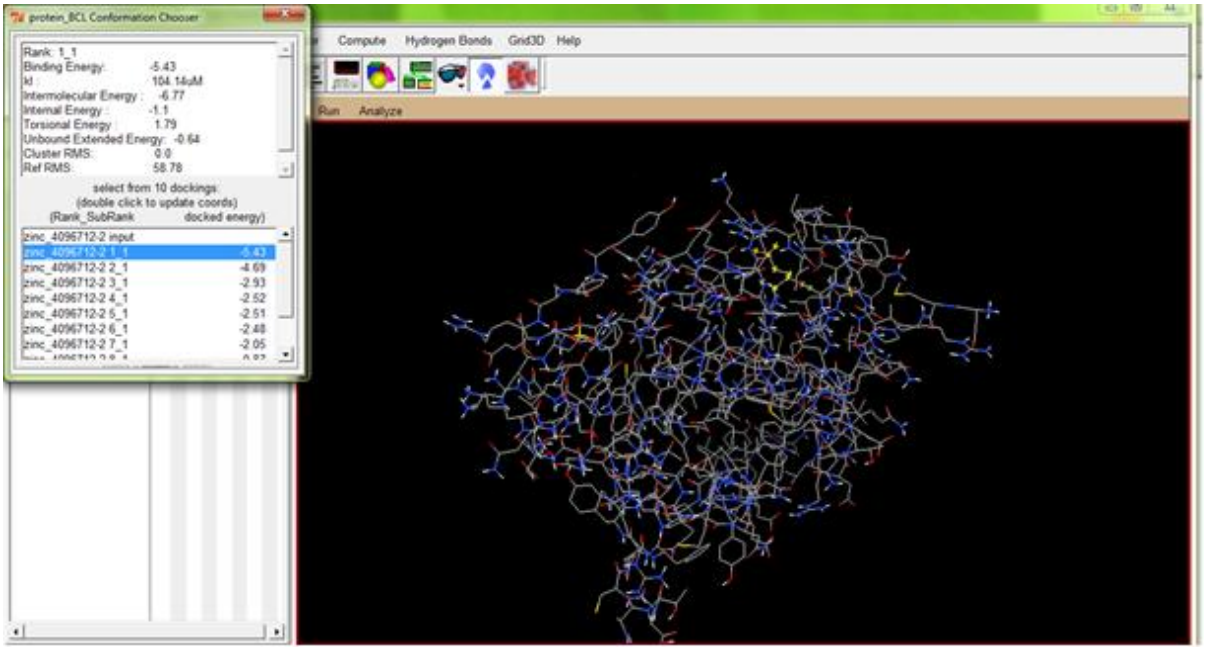

Fig.5. Stigmasterol with Bcl-2 protein (Binding energy: -5.43)

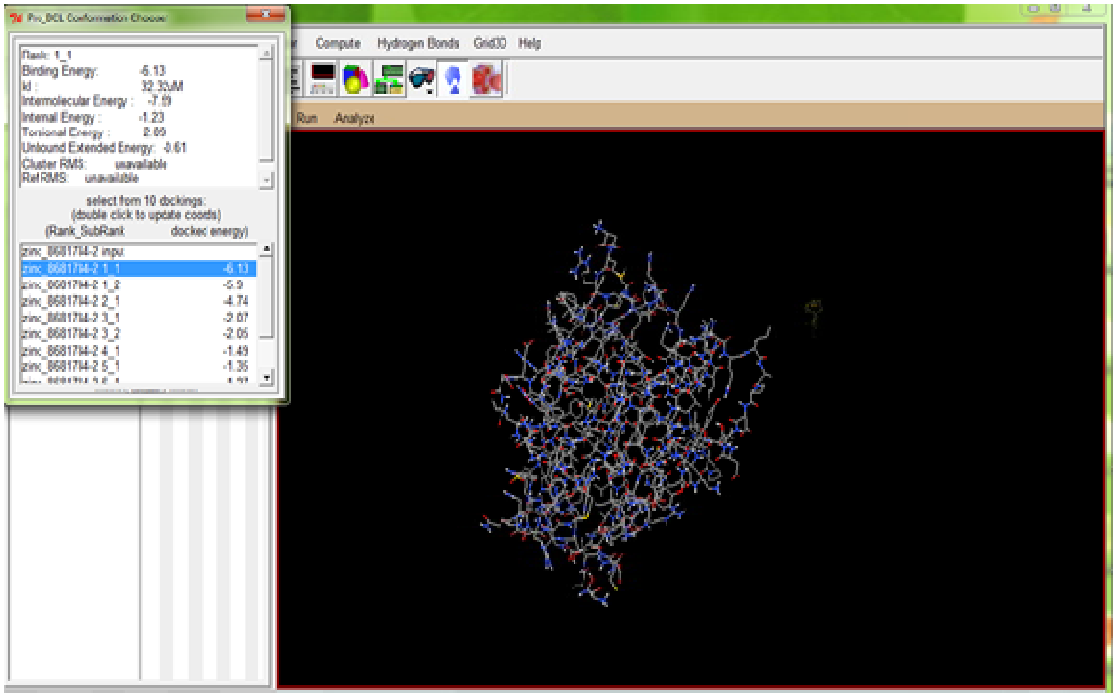

Fig.6. β-sitosterol with Bcl-2 protein (Binding energy: -6.13)

In conclusion, the ethanol extract of *Anisochilus carnosus* showed dose dependent cytotoxic activity against human lung cancer A549 cells. Furthermore, the ethanol extract of *Anisochilus carnosus* induced apoptotic cell death through generation of reactive oxygen species and altered mitochondrial membrane potential. Moreover, an isolated steroid active compounds stigmasterol and β-sitosterol displayed high affinity with the anti-apoptotic Bcl-2 protein. Based on the results obtained from human cancer cell lines and molecular docking studies the ethanol extract of *Anisochilus carnosus* could be a novel Anti cancer agent in the near future.

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