RESEARCH ARTICLE

Cytotoxic effects of *Kydia calycina* leaf fractions on different human cancer cell cultures

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Abstract

Objective: The objective of the study was to study the cytotoxic effects of effects of different fractions of *Kydia calycina* using various human cancer cell cultures, *in vitro* by MTT assay, reactive oxygen species (ROS) generation and caspase-3 activities. **Materials and Methods:** Human cervical carcinoma (HeLa) cells, human breast cancer (MCF-7) cells and human neuroblastoma (IMR-32) cells were maintained in a 5% CO_2 incubator at 37°C. Different concentrations of fractions of *K. calycina* such as toluene fraction (KT), ethyl acetate fraction (KE), butanone fraction (KB), and aqueous fraction (KAq) in serum-free culture medium were freshly prepared and used for cytotoxic activity by MTT assay, ROS generation and apoptotic effect by caspase-3 activity. **Results:** Among the four fractions, the KE and KB fractions have revealed that greater percentage inhibition in all types of cancer cells in a dose-dependent manner by MTT assay. The IC_{50} values of KE fraction were found to be 38.35, 40.47, and 36.83 µg/mL against HeLa, MCF-7, and IMR-32, respectively. The apoptotic activity was evaluated through ROS generation and caspase-3 activities of KE and KB. The results showed that both fractions have significantly increased the ROS production and caspase-3 levels in all the cell cultures in a dose-dependent manner. **Conclusions:** The present investigation has shown that the KE and KB fractions of *K. calycina* displayed significant cytotoxic activity against all three cancer cells by decreased cell viability, increased generation of ROS and caspase-3 activities.

Key words: Caspase-3, cell viability, *Kydia calycina,* reactive oxygen species and Reactive spcies

INTRODUCTION

Recently, medicinal plants occupy an important position for being the major sources of drug discovery, irrespective of its categorized groups (herb, shrub, or tree). Plants have been essential in treating different forms of diseases including cancer. According to the World Health Organization, 80% of the people living in the rural areas depend on medicinal plants as primary healthcare system. These practices are solely depends on the knowledge of the use of traditional medicinal plants.[1,2] Cancer chemoprevention is defined as the use of materials of natural source, biological agents, chemical or synthetic compounds to reverse, prevent, or suppress carcinogenic progression of invasive cancer.[3] Natural products are formulated to generate different types of effective drugs to augment anticancer activities. Proper understanding of the complex synergistic interaction of various constituents of anticancer herbs, would help in formulating the design to hit the cancerous cells without damaging the normal cells of the body.[1,2]

Cancer is a disease characterized by the uncontrolled proliferation of the cells. As a cell progresses from normal to cancerous, the biological imperative to survive and perpetuate forces fundamental alterations in cells behavior. The definite cause of the disease in different sections is still to be investigated clearly.[4] Cancer is thus, a class of diseases,

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classified by the type of cell that is primarily affected. To combat cancer United States National Cancer Institute has undergone 2070 anticancer clinical trials, in which 150 more drug combinations have been successfully recorded against cancer. The search for the cancer drug discovery from Natural sources started with the investigations done by Hartwell and his coworkers in the late 1960's with the application of a phytochemical, podophyllotoxin, and its derivatives from the plant *Podophyllum peltatum*. Further discoveries lead to isolate anticancer compounds from plants such as *Camptotheca acuminate, Catharanthus roseus,* and also *Taxus brevifolia.* Vinblastine, Vincristine, Camptothecin, and Taxol are the well-known potential anticancer agents derived from these plants which are found to be effective against various types of cancer.[5]

Advances in the clinical researchers for agents which treat various cancers have been increased over the years and as result numbers of agents have been initiated. The organic compounds present in various plants could exaggerate to reduce the toxicity caused due to chemotherapy. Task of modulating the adverse effect is possible only through requisite perspective regarding the specificity of these molecules with combination therapy.[6]

The cytotoxic and antitumor drugs are generally non-selective and kill normal proliferating cells. Identification of active cancer specific agents remains a needful area in drug screening and drug discovery mechanisms. Much more emphasis has been put on discovering new agents that target tumor cells more efficiently and selectively with negligible toxic effects on normal cells.[7,8] Although the molecular mechanisms of its antiproliferative and apoptotic effects have not been cleared, increasing evidence has supported that the increase of reactive oxygen species (ROS) generation contributes to the treatment of cancer cells.[9,10]

Kydia calycina is distributed in Himalayas from the Indus eastwards to Myanmar and in peninsular India from Maharashtra and Madhya Pradesh, principally in mixed, moist, deciduous forests. The leaves were alternate, 7.5–15 cm long and wide, usually 3–7 lobed, apex angled or rounded, base cordate, palmate 7-nerved, hoary-tomentose beneath, and petioles 2.5–5 cm. The leaves are applied to relieve body pains, lumbago and arthritis, a poultice of the leaves is used to treat skin diseases.[11] *K. calycina* leaf and stem bark paste applied for ulcers and skin diseases.[12] The *K. calycina* seed oil was reported to contain cyclopropenoid fatty acid apart from normal fatty acids,[13] and Hibiscone C, Hibiscoquinone B, 8-formyl-2,7-dihydroxy-5-isopropyl-1-methoxy-3-methyl naphthalene were isolated from stem heartwood of *K. calycina*.[14] The *K. calycina* methanolic leaf extract was reported to having analgesic, anti-inflammatory and hepatoprotective activity.[15,16] Hence, the present study evaluated the cytotoxic effects of different leaf fractions of *K. calycina* using various human cancer cell cultures, *in vitro*.

MATERIALS AND METHODS

Plant Material

The leaves of *K. calycina* were collected from Tirupati hills, Andhra Pradesh, India. It was authenticated by Prof. V. Raju, Department of Botany, Kakatiya University, Warangal, India.

Preparation of Extracts

Leaves of *K. calycina* were made free from the adherent foreign material and air-dried. Then they were coarsely powdered and macerated with methanol in a round bottom flask for 7 days separately. The content of the flask was stirred intermittently to ensure the efficiency of the extraction. After a week, they were filtered and concentrated under reduced pressure to yield corresponding extracts, and the extracts were kept in a desiccator to remove moisture and stored properly until used.

The methanolic extracts of *K. calycina* were dispersed in sufficient amount of distilled water separately and fractionated with toluene, ethyl acetate and butan-2-one in succession. The obtained fractions and the aqueous residues were concentrated under reduced pressure to yield corresponding extracts.

Cell Culture and Treatment

Human cervical carcinoma (HeLa) cells, human breast cancer (MCF-7) cells and human neuroblastoma (IMR-32) cells were procured from NCCS, Pune. The cells were used between passages 10 and 15. All these cells were grown in suitable culture media supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin-streptomycin antibiotic solution. Cells were seeded at 250,000 cells/flask in a total volume of 10 mL. When confluent, all the cells were trypsinized as described above and seeded in 96 well plates at the rate of 1.0×10^4 cells/0.1 mL. All the cell cultures were maintained in a 5% CO_2 incubator at 37°C. Different concentrations of fractions of *K. calycina* such as toluene fraction (KT), ethyl acetate fraction (KE), butanone fraction (KB), and aqueous residue (KAq) in serum-free culture medium were freshly prepared and used for the cytotoxic activity.

MTT Assay Method

Measurement of cell viability and proliferation forms the basis for numerous *in vitro* assays of a cell population's response to external factors. The reduction of tetrazolium salts is widely believed as a reliable way to study cell proliferation. The effect of test fractions on the cellular proliferation and viability was determined using MTT assay method.[17] The yellow tetrazolium salt was reduced by dehydrogenase enzymes present in metabolically active cells, to produce

reducing equivalents such as NADH and NADPH. The formazan product has low aqueous solubility and was present as purple crystals. The resulting formazan was dissolved by using dimethyl sulfoxide (DMSO) permitted the convenient quantification of product development. The intensity of the product color was measured at 562 nm and was directly proportional to the number of living cells in the culture.[18]

The adherent cells were trypsinized according to protocol and were re-suspended in fresh medium after centrifugation. Cell suspension was mixed thoroughly by pipetting several times to get a uniform single cell suspension. 10–15 passages were conducted before performing the experiment to evaluate the cytotoxicity. Different dilutions of fractions were made in media with final phosphate buffer solution (PBS)+1% PEG (solvent control) concentration in the well to be <1%. 100 µL (0.1 mL) of cell suspension was transferred aseptically to each well of a 96 well plate and it 100 µL of solvent/fraction (in triplicate) in media was added. The plate was then incubated at 37°C for 48 h in 5% CO_2 incubator. After 48 h of incubation, 20 µL of MTT was added to each well, and the microtiter plate was again incubated for 2 h. 80 µL of lysis buffer was added to each well; the plate was wrapped in aluminum foil to prevent the oxidation of the dye. The plate was placed on a rotary shaker for 2 h to solubilize the purple formazan crystals. The absorbances were recorded on the ELISA reader at 562 nm wavelength. The absorbance of the test was compared with that of solvent control to get the percent cytotoxicity.[19,20]

Measurement of ROS Generation

Generation of ROS was assessed using a cell-permeable fluorescent signal H2DCF-DA as an indicator for ROS.[21,22] As described previously, H2DCF-DA is oxidized to a highly green fluorescent 2070-dichlorofluorescein (DCF) by the generation of ROS. Cancer cell lines were pretreated with various concentrations of KE and KB fractions for 24 h. After 24 h incubation period, the cells were washed with cold PBS and incubated with 100 mmol/L H2DCF-DA for another 30 min at 37°C. DCF fluorescence intensity was measured using the fluorescence plate reader (Varioskan Flash Multimode Reader, Waltham, MA) at excitation/emission of 488/525 nm. The determinations were carried out thrice in triplicate, ensuring each time that the number of cells per treatment group was the same to ensure reproducibility. The values were expressed as % relative fluorescence compared to the control.

Caspase-3 Assay

Caspases are members of the aspartate-specific cysteinyl protease family. Caspase-3 exists in cells as an inactive 32 kDa proenzyme, called procaspase-3. Procaspase-3 is cleaved into active 17 and 12 kDa subunits by upstream proteases such as caspase-6, caspase-8, and granzyme B during apoptosis. The overexpression of caspase-3 can result in the initiation of apoptosis. Likewise, the inhibition of caspase-3 can prevent cells from entering into the apoptotic pathway. The activation of caspase-3 is used as a biomarker in the evaluation of apoptosis and in understanding mechanisms of apoptosis induction.[23]

The assay is based on a colorimetric finding of the chromophore *p*-nitroaniline (*p*NA) after cleavage from the DEVD-*p*NA, a labeled substrate. The *p*NA light emission can be quantified using a microtiter plate reader at 405 nm. Assessment of the absorbances of *p*NA from an apoptotic sample with an uninduced control allows determination of the fold raise in caspase-3 activity.[24]

Statistical Analysis

Data were expressed as mean±SEM. Significance was calculated using one-way ANOVA followed by Dunnett's multiple comparison tests compared to control.

RESULTS AND DISCUSSION

Natural products or their derivatives have been exhibited to have significant anticancer potentials due to their ability to inhibit tumor growth, angiogenesis and metastasis without many side effects.[25] Recent publications showed the effect of many herbal plants in the treatment of wide range of illnesses. Furthermore, several naturally produced herbal formulations are currently available for cancer patients. Most of the chemotherapeutic agents were cytotoxic to normal cells and build up drug resistance. Therefore, scientific consideration and test of traditionally used herbs for the treatment of various malignancies could be also considered as a very important source for new chemotherapeutic drugs.[26]

The *in vitro* cytotoxicity of four different fractions of *K. calycina* such as toluene fraction (KT), ethyl acetate fraction (KE), butanone fraction (KB), and aqueous residue (KAq) were evaluated against three different cancer cell cultures, such as human cervical carcinoma (HeLa), human breast cancer (MCF-7), and human neuroblastoma (IMR-32) cells using MTT assay, which is based on the reduction of MTT at different concentrations (10, 30, 100, 300, and 500 µg/ml). After 48 h of treatment, KE and KB fractions exhibited higher inhibitory effect against all tumor cells, with varying efficiencies and selectivities while others caused marginal cell inhibition [Figure 1]. The IC_{50} (concentration of the fraction causing 50% cell death) values of test fractions were given in Table 1. Among the four fractions, the KE and KB fractions have revealed that greater percentage inhibition in all types of cancer cells in a dose-dependent manner. Moreover, the KE fraction has shown superior cytotoxicity than KB fraction. The IC_{50} values of KE fraction were found

to be 38.35, 40.47, and 36.83 µg/mL against HeLa, MCF-7, and IMR-32, respectively.

Since the cytotoxicity of KE and KB was greater, we further evaluated to reveal the mechanism for its cytotoxicity. For this purpose, we evaluated the apoptotic activity through ROS generation and caspase-3 activities of KE and KB against all three cancer cell lines. The results showed that both fractions have significantly increased the ROS production in all the cell cultures [Figure 2]. ROS production was significantly elevated with KE fraction ($P < 0.01$) in all cancer cells. Whereas, KB fraction showed increased ROS generation ($P < 0.05$) in all cancer cells. Excess accumulation of ROS leads to cellular damage and inflammation of the tissues.[27] ROS plays a major role in cellular senescence paving way to cell death; therefore, there is an urgent need for potential therapeutics that may prevent oxidative stress-induced neurodegeneration. It is highly possible that the pro-oxidant effect is responsible for the apoptotic activity of these extracts and ROS are key signaling molecules to modulate cell death.[28] Accumulating evidence indicates that cancer cells produce high levels of ROS that lead to a state of increased basal oxidative stress. The increased production of ROS in cancer cells was observed in *in vitro* studies.[29] Ahamad *et al*. demonstrated that naringenin leads to cell death in cancer cells through inducing ROS generation.[30] We, therefore, investigated the effectiveness of KE and KB fractions in generation of ROS. We found that exposure of all three cancer cells with these fractions dramatically enhanced generation of intracellular ROS at different levels in a dose-dependent manner in all cell lines.

Table 1: The IC_{50} values of different *Kydia calcycina* fractionss using various cell lines

Extract	IC_{50} values (µg/mL)		
	HeLa	MCF-7	IMR-32
KT	108.47±2.72*	114.14±3.16*	76.44±2.16**
KE	38.35±1.34***	40.47±2.13***	36.83±1.78***
KB	69.23±2.89***	80.82±2.45***	93.4±2.67**
KAq	98.92±3.14*	97.82±3.45*	109.32±3.78*

KT: *Kydia calcycina* toluene fraction, KE: *Kydia calcycina* ethyl acetate fraction, KB: *Kydia calcycina* butanone fraction, KAq: *Kydia calcycina* aqueous residue, data were mean±SEM, *P<0.05, **P<0.01, ***P<0.001 compared to control

Intracellular caspase-3 activation is a key stage in the apoptotic pathway. Hence, we tested the effect of treatment with our plant fractions on intracellular caspase-3 enzymatic activity. Different human cancer cells were treated with the plant fractions and caspase-3 enzymatic activity within the cells was measured [Figure 3]. Since caspase-3 activity rises while cells die and cell numbers drop, it was essential to normalize caspase-3 activity to the number of cells, to obtain more accurate results. The results here are expressed as the percent increase in caspase-3 activity in treated cells compared to cells added with DMSO. Caspase-3 levels were significantly increased with KE fraction in HeLa ($P < 0.05$), MCF-7 ($P < 0.05$), and IMR-32 ($P < 0.01$) cancer cells. Whereas, KB fraction showed increased caspase-3 levels in HeLa ($P < 0.05$) and

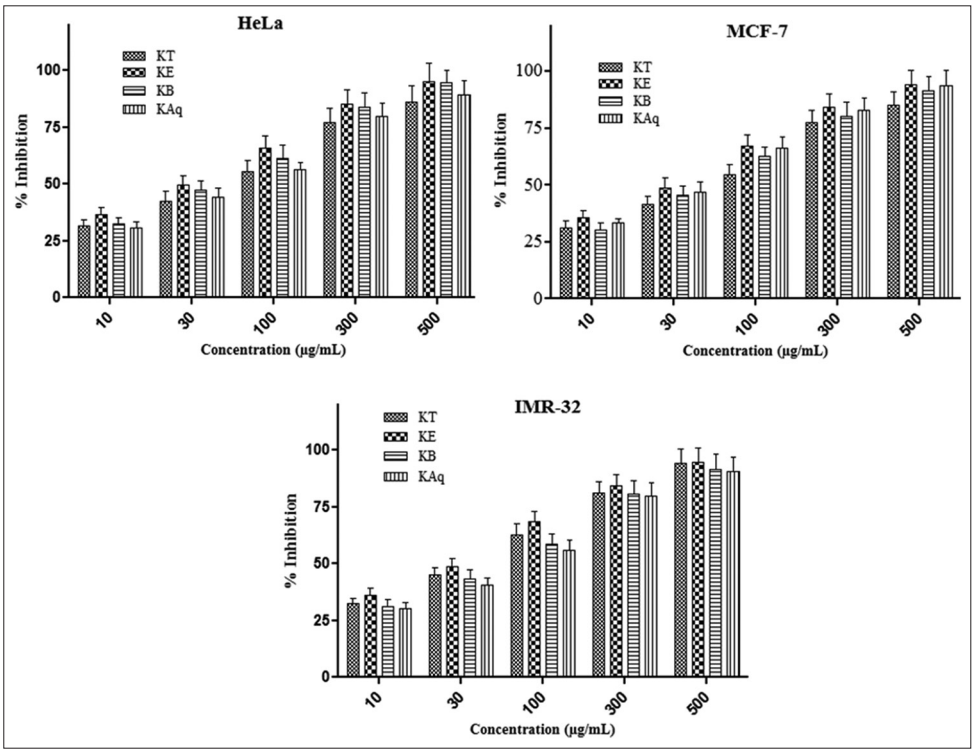

Figure 1: Effect of different fractions on cytotoxicity of HeLa, MCF-7, and IMR-32 cancer cell lines; data were mean±SEM

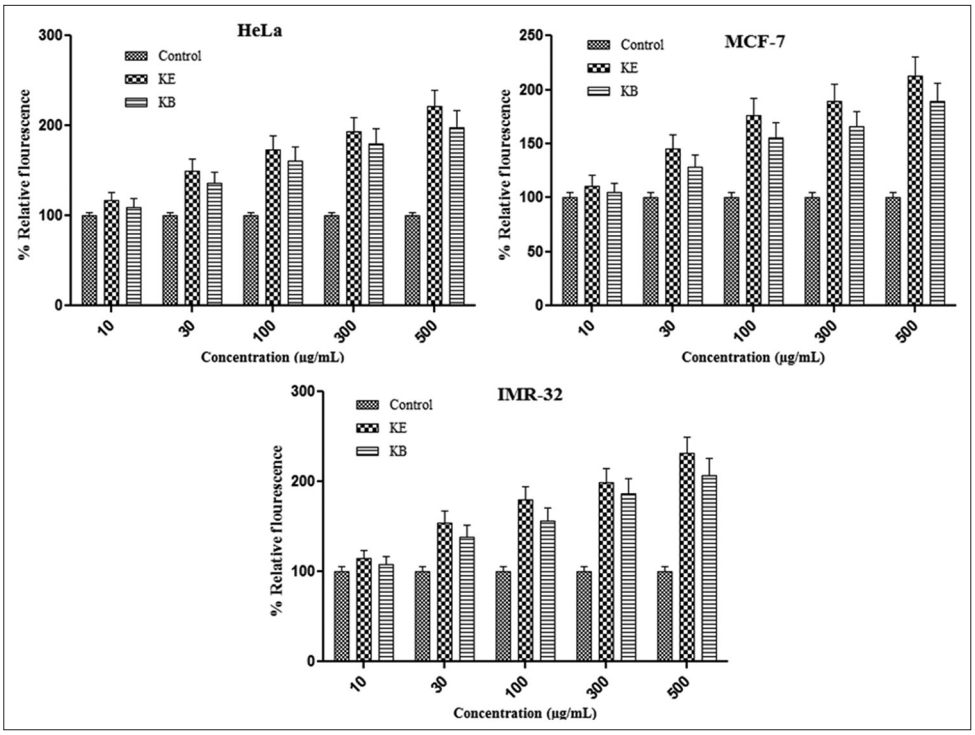

Figure 2: Reactive oxygen species generation effect of different fractions on HeLa, MCF-7, and IMR-32 cancer cell lines, data were mean±SEM

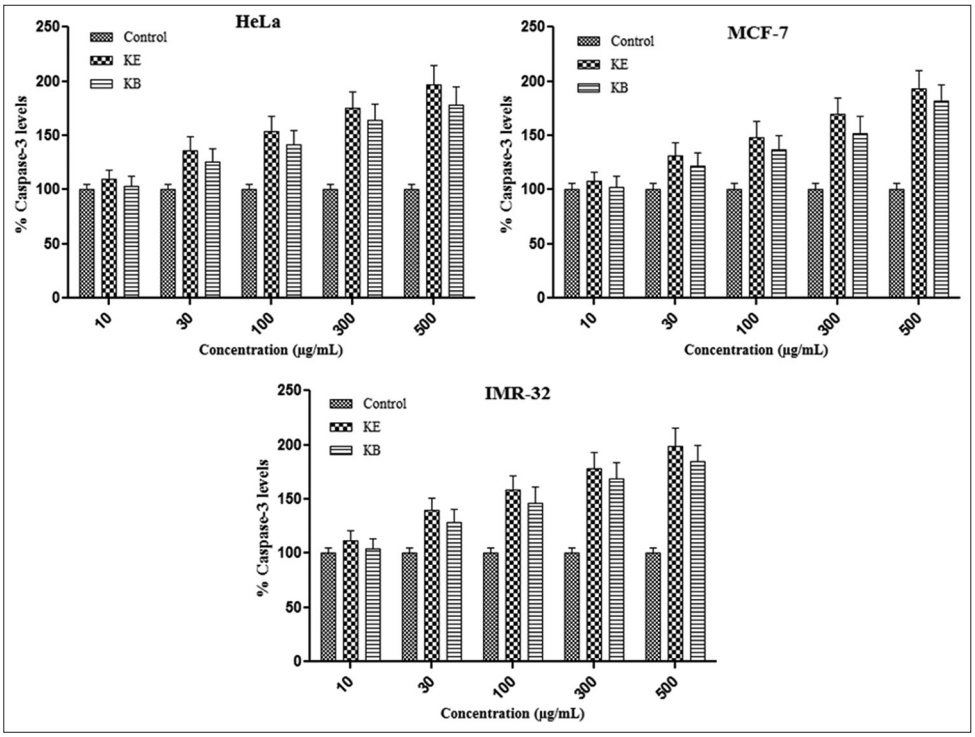

Figure 3: Caspase-3 levels of different fractions on HeLa, MCF-7, and IMR-32 cancer cell lines, data were mean±SEM

IMR-32 ($P < 0.05$) cancer cells. Sherine *et al*. evaluated the mechanism of cell death induced by *Petunia punctata* by caspase-3 colorimetric assay, before and after treatment with the extract. The results showed that the treatment of Panc-1 cells with *P. punctata* strongly induces increased caspase-3 activity. Furthermore, caspase-3 activity in *P. punctata*-treated cells was reduced in the presence of a caspase-3 specific apoptosis inhibitor. This suggests the involvement of caspase-3 in triggering apoptosis in *P. punctata*-treated Panc-1 cells.[31]

CONCLUSIONS

In summary, among the four *K. calycina* fractions, the KE and KB fractions were showed increased percentage inhibition of HeLa, MCF-7, and IMR-32 cells by MTT assay. Moreover, the apoptotic activity revealed that both KE and KB fractions have displayed significant increased generation of ROS and caspase-3 activities in all cancer cell lines in a dose-dependent fashion. Further studies are warranted to elucidate the molecular mechanisms of isolated compounds of *K. calycina.*

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