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ANTICANCER ACTIVITY OF AN ETHANOL EXTRACT OF *COLEUS FORSKOHLII* ROOT AGAINST GASTRIC CANCER CELLS *IN-VITRO*

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Key words:

Coleus forskohlii; Gastric carcinoma cell lines; DNA damage; Apoptosis

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ABSTRACT: *Coleus forskohlii* is an Indian medicinal plant it treat various diseases of cardiovascular, respiratory, gastrointestinal, and central nervous systems. The purpose of this study was to investigate the anticancer activity of an ethanol extract of *Coleus forskohlii* against gastric cancer in AGS cancer cell lines. Gastric carcinoma cells were tested in vitro for cytotoxicity, Ao/EtBr staining for apoptotic cells, apoptotic DNA fragmentation in response to *Coleus forskohlii* extract. The protein expression of Bax, Bcl2 and Caspase-3 were analyzed through Western blotting. Treatment with *Coleus forskohlii* extract inhibited AGS cell proliferation dose and time dependently. Cells exposed to *Coleus forskohlii* extract showed typical hallmarks of apoptotic cell death. In conclusion, *Coleus forskohlii* ethanol extract has cytotoxic and apoptotic effects on Gastric carcinoma cells. Its mechanism of action might be associated with the apoptosis induction through up-regulation of the protein expressions of caspase-3 down-regulation of the protein expression of Bax and Bcl2.

INTRODUCTION: Gastric cancer is currently the most frequently occurring cancer and one of the leading causes of cancer-related death in the world. A total of 951,600 new Gastric cancer cases and 723,100 deaths are estimated to have occurred in 2012, accounting for 8% of the total cases and 10% of total deaths[1]. Although mechanism of gastric carcinogenesis is still not fully understood, it has been suggested that environmental factors combining with low-penetrance susceptibility genes may be important.

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For example, a high rate of Helicobacter pylori (HP) infection (70–90%) in developing countries (compared with 25–50% in developed countries) might be a potential risk factor for Gastric cancer [2, 3]. However, because only few Helicobacter pylori carriers eventually develop Gastric cancer, other factors must play a role in Gastric cancer risk. Life styles, such as tobacco smoking and diet, are also suggested as potential risk factors for Gastric cancer [4] but the relevant data are limited.

Plants have been a rich source of therapeutic agents and still an important source of new drugs for diseases that continue to lack a cure, such as cancer [5]. Certain plant components capable of killing cancer cells are also known to suppress the tumor promoting actions of immune and other tumor stromal cells [6]. *Coleus forskohlii* is a common indigenous medicinal plant belongs to Lamiaceae family [7]. It is native to India, where it has been used for centuries in Ayurvedic medicine to treat various diseases of the cardiovascular, respiratory, gastrointestinal, central nervous systems, digestive disorders, and dysentery treatment [8].

Coleus forskohlii which played a major role in stimulating cyclic adenosine monophosphate (cAMP) and other biological activities [9, 10] and further exhibits some anti-bacterial activity [11, 12, 13]. Thus, the aim of this study was to investigate the mechanisms of cell death induced by *Coleus forskohlii* extract in AGS cells.

MATERIALS AND METHODS:

Chemicals:

Coleus forskohlii root ethanol extract, Ethidium Bromide (EtBr), Rhodamine 123, Hoechst 33258, Fetal Bovine Serum (FBS), Antibiotics, 0.25% trypsin EDTA, Dulbecco's Modified Eagles Medium (DMEM) and Roswell Park Memorial Institute 1640 medium (RPMI-1640), MTT (3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyl-2 tetrazolium bromide) were purchased from Hi-media Lab Ltd, Mumbai, India.

Cell culture and Maintenance:

AGS Gastric carcinoma cell lines were obtained from the National Center for Cell Science Pune, India. Cells were grown in Dulbecco's Modified Eagles Medium (DMEM) and Roswell Park Memorial Institute 1640 medium (RPMI-1640) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The cells were maintained as monolayers in 25 cm^2 plastic tissue culture flasks at 37ºC in a humidified atmosphere of 5% $CO_2$and 95% air incubator under standard conditions. The cells were harvested using 0.25% trypsin EDTA then washed in the culture medium to inactivate the trypsin before reseeding or analysis. The cells were fed every 2-3 days and sub-cultured once they reached 70-80% confluence. Cells were plated at an appropriate density; exponentially growing cells were used in all the experiments.

Preparation of drug:

Coleus forskohlii root ethanol extract was suspended in 1% dimethyl sulfoxide (DMSO) just before treatment and the final concentration of DMSO in the culture medium was 0.01% W/V. 0.01% DMSO was used as the control.

MTT Assay:

The 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), proliferation assay was utilized in AGS cell lines to assess the dose-dependent effect of *Coleus forskohlii* root ethanol extract on cell proliferation. Cells were plated and grown in 200 µl of growth medium in 96-well microtiter plates. After an overnight attachment period, cells were treated with varying concentrations of *Coleus forskohlii* root extract (10, 20, 30, 40, 50, 60, 70, 80, 90, 100 µg/ml) for 24 h, 48 h and 72 h. All studies were performed in triplicates and repeated three times independently. Cell growth was quantified by the ability of living cells to reduce the yellow dye, MTT, to a purple formazan product. Cells were incubated with MTT at 37°C in a humidified 5% CO_2 atmosphere for 2 h. The MTT formazan product was then dissolved in DMSO, and absorbance was measured at 570 nm in a microplate reader. The percentage inhibition was calculated, from this data, using the formula:

$$\frac{\text{(Mean absorbance of control cells)} - \text{(Mean absorbance of treated cells)}}{\text{Mean absorbance of control cells}} \times 100$$

Apoptotic Morphology Analysis:

Unstained live Morphological Assay:

AGS cells were grown in glass cover slip (22 X 22 mm) placed in plates containing six well at a density of 5 X 10^5 cells/well and allowed to settle for 24 h before treatment with the 24 h IC_{50} values of Root ethanol extract. The medium was subsequently removed from each well of the treated and untreated AGS cells, and a cover slip inverted and placed over the slide. The gross morphological changes in the treated and untreated control cells were observed using a phase contrast light microscope (Axio Scope A1, Carl Zeiss, Germany) and photographed.

Ao/EtBr Staining

Acridine orange and Ethidium Bromide staining were performed as described by [14] 25 µl of cell suspension of each group (both attached, released to floating by trypsinization), containing 5 X 10^5 cells, were treated with Acridine Orange (AO) and

Ethidium Bromide (EtBr) solution (1 part of 100 µg/ml AO and 1 part of 100 µg/ml EtBr in PBS) and examined using a fluorescent microscope with an UV filter (450-490 nm) and photographed.

Hoechst 33258 Staining:
Based on the method of [15] the cell pathology was detected by staining of trypsinized cells (5X 10^5/ml) with 1 µl of Hoechst 33258 (1 mg/ml, aqueous) for 10 min at 37ºC. A drop of cell suspension was placed on a glass slide and a cover slip was laid over to reduce light diffraction and cell pathology were observed using a fluorescent microscope (Axio Scope A1, Carl Zeiss, Germany) fitted with a 377-355 nm filter and the cells reflecting pathological changes were observed and photographed.

Mitochondrial Membrane Potential:
Mitochondrial membrane potential of the treated and untreated cells were measured by the method of [16] using the fluorescent probe Rhodamine123. The cells (5 X 10^5) were grown in glass cover slip (22 X 22 mm), placed in six well plates and treated with the Root ethanol extract at the respective IC_{50} values.

The cells were stained with Rhodamine123 dye after 24 h and 48 h exposure. The mitochondrial depolarization patterns of the cells were observed using a fluorescent microscope fitted with 485-545 nm filters and photographed.

Measurement of intracellular ROS:
The measurement of intracellular ROS formation was based on the oxidation of 20, 70-dichlorodihydrofluorescein diacetate (DCHF-DA), which first gets hydrolyzed by the cellular esterases to DCFH, which on oxidation forms fluorophore dichlorofluorescein (DCF). The cells were treated with *Coleus forskohlii* root extract and incubation was continued for 12 and 24 h in the dark. After that the cells were harvested and washed twice with PBS, incubated with 10 mM DCFH-DA in PBS for 30 min, washed, and overlaid with RPMI 1640/10% FBS medium under strictly dark conditions to avoid any nonspecific artifacts. The fluorescence intensity was measured at 480 nm excitation and 530 nm emission in a Jasco F 6500 Spectrofluorimeter (Jasco, MD, USA).

Measurement of lipid peroxidation byproducts and antioxidants:
AGS cells were seeded in a T_{75} flask at a density of 1 X 106 cells/flask treated with 24 h IC_{50} values of Root ethanol extract for 24 h and 48 h. The cells were harvested by trypsinization and washed with PBS. The cells were suspended in 130 mM KCl, 50 mM PBS and 10 µM dithiothreitol and centrifuged at 20,000g for 15 min at 4 °C. The supernatant was collected and used for biochemical estimations. The concentrations of lipid peroxidation byproducts such as thiobarbituric acid reactive substances (TBARS) was measured by the method [17] and the activities of enzymic antioxidants such as superoxide dismutase (SOD) was assayed by [18] catalase (CAT) by the method of [19] and glutathione peroxidase (GPx) by the method of [20].

DNA damage by comet assay:
DNA damage was studied by single cell gel electrophoresis (Comet assay) by the method of [21]. Frosted microscopic slides were covered with 200 µl of 1% normal melting agarose in PBS at 65 °c, cover slip removed and the second layer of 100 µl of 1% low melting agarose containing approximately 105 cells at 37ºC was added. Cover slip was placed immediately and the slides were placed at 4ºC. After solidification of the low melting agarose, the cover slip was removed and the slides were placed in the chilled lysis solution containing 2.5 M NaCl, 100 mM EDTA, 100 mM Tris–HCl, 1% Trisma base, 1% Triton X-100 and 10% DMSO for 16 h at 4ºC. The slides were removed from the lysing solution and placed on a horizontal electrophoresis tank filled with freshly prepared alkaline buffer (300 mM NaOH, 1 mM EDTA, pH >13.00). The slides were equilibrated in the same buffer for 20 min and electrophoresis was carried out at 25 V, 180 mA for 20 min.

After electrophoresis, the slides were washed gently with 2 M Tris–HCl buffer, pH 7.4 to remove the alkali. The slides were then stained with 50 µl of EB and visualized using a Nikon fluorescent microscope equipped with a 365 nm excitation filter and a 435 nm barrier filters. The quantification of DNA strand breaks of the stored images was done using the CASP software and % DNA in tail, tail length and olive tail moment were obtained directly.

Apoptosis related protein expressions:
The cells treated with 0-400 µg/mL SIE for 24 h were lysed in a protein extraction solution (Intron Biotechnology). The protein concentration was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA). For Western blotting, 30 micrograms of protein extraction was separated on 8-15% SDS- PAGE and electrotransferred to nitrocellulose membrane (Schleicher and Schuell, Germany). The membrane was blocked by incubation in 5% skim milk in TBST buffer (20mM Tris-HCl, pH 7.6, 140mM NaCl, 0.1% Tween 20) and then incubated with anti-caspase-3, Bax and $Bcl_2$for 13-15h. The blot was washed with TBST buffer and incubated with horse radish peroxidase-labeled secondary antibody (Santa Cruz Biotechnology) for 2h. The membranes were then washed again, and detection was performed using the enhanced chemiluminescence system (Amersham).

Statistical Analysis:
Data were analyzed by one-way analysis of variance (ANOVA) and a significant difference among treatment groups were evaluated by Duncan's Multiple Range Test (DMRT). The results were considered statistically significant at $p<0.05$. All statistical analyses were made using SPSS 17.0 software package (SPSS, Tokyo, Japan).

RESULTS AND DISCUSSION:
Natural products are known to be endowed with excellent properties as well as tolerability and reliability for the development of new drugs. Cytotoxicity tests generally possess a broad spectrum of sensitivity and are able to detect many novel anticancer drugs, which potentially inhibit the biochemical activity of a variety of cancer cells of animal and human origin [22]. The results clearly revealed that when the cancer cells were treated with Coleus forskohlii root ethanol extract for long times (72 h), the IC50 values were stand minimum dose. Supplementation with ethanol extract to the culture medium inhibits growth of AGS cells in a dose and time dependent manner, revealing the cytotoxic potential of extract. The AGS cell line required higher concentrations of *coleus forskohlii* root ethanol extract to induce 50% of cancer cell death at 24 h 73.04 µg, 48 h 62.69 µg and 72 h 48.78 µg (**Fig. 1**).

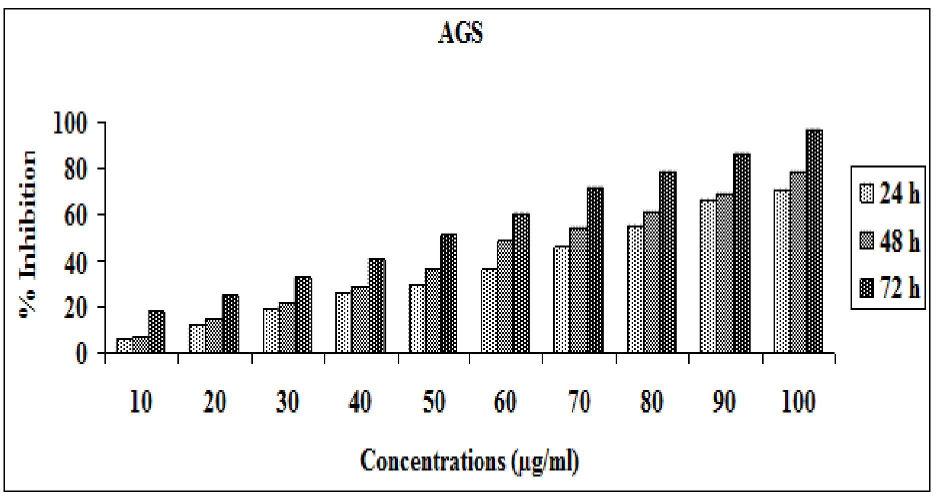


FIG.1: EFFECT OF *COLEUS FORSKOHLII* ETHANOL EXTRACT ON AGS CANCER CELL CYTOTOXICITY (MTT ASSAY)

The cells were treated with different concentrations of *Coleus Forskohlii* ethanol extract (10-100 µg/mL), which affected cell viability in a time dependent manner 24 h, 48 h and 72 h respectively. Data are expressed as means ± SD of six independent experiments. Apoptosis is a physiological process leading to cell death far distinct from necrosis [23] deletion of excess cells from normal tissues and for specific pathologic events. Thus, apoptosis could be a therapeutic target for cancer cells, at the same time cell death or apoptosis induced by harmful stimuli should be prevented in normal cells. Biochemically and morphologically distinct from cellular necrosis, apoptosis involves chromatin condensation, cell shrinkage, DNA fragmentation, plasma membrane blebbing and the formation of membrane enclosed apoptotic bodies [24, 25].

In the study the AGS cancer cells treated with *Coleus forskohlii* root ethanol extract was stained with AO/EtBr and Hoechst 33285 staining, it exhibited many characteristics of apoptotic morphology, i.e., cell shrinkage, loss of cell membrane integrity, impaction of nuclei and more cells appeared in the granules in size and shape, No morphological change was observed in control cells (**Fig. 2**), chromatin condensation, membrane blebbing and apoptotic bodies (**Fig.3** and **Fig. 4).**

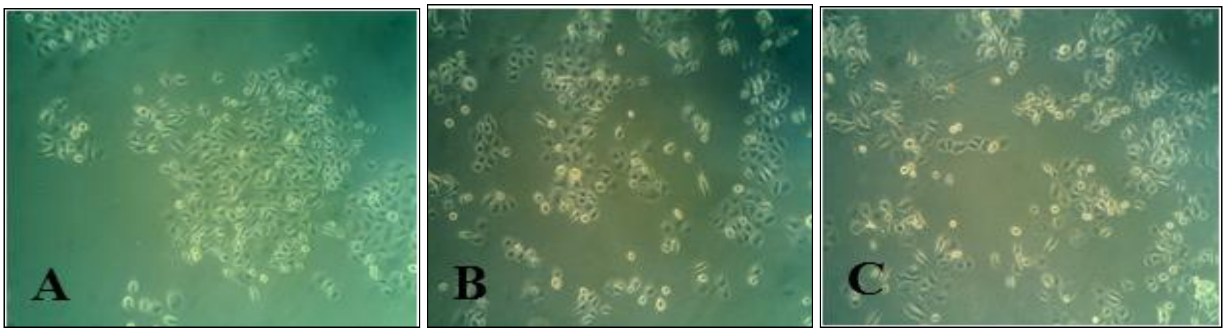


FIG.2: EFFECT OF *COLEUS FORSKOHLII* ETHANOL EXTRACT ON AGS CANCER CELL CYTOTOXICITY (MTT ASSAY) ON CELL MORPHOLOGY

A. Control cells show well defined cellular morphology. B. 24 h treated cells show characteristics of apoptosis i.e., cell shrinkage, loss of cell membrane integrity, impaction of nuclei many granules like appearance. C. 48 h treated cells show characteristics of apoptosis i.e., cell shrinkage, loss of cell membrane integrity, impaction of nuclei many granules like appearance

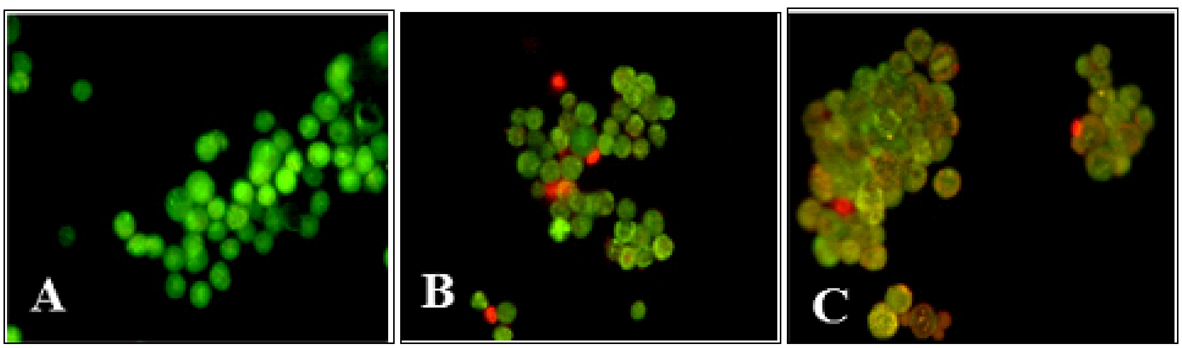


FIG. 3: EFFECT OF *COLEUS FORSKOHLII* ETHANOL EXTRACT ON CELL MORPHOLOGY WITH ACRIDINE ORANGE/ETHIDIUM BROMIDE (AO/EtBr) STAINING

A. Control shows uniformly green fluorescing, viable cells. B. 24 h treated cells shows chromatin condensation, membrane blebbing and apoptotic bodies, which fluoresce uniformly in bright red and orange. C. 48 h treated cells show chromatin condensation, membrane blebbing and apoptotic bodies, which fluoresce uniformly in bright red and orange.

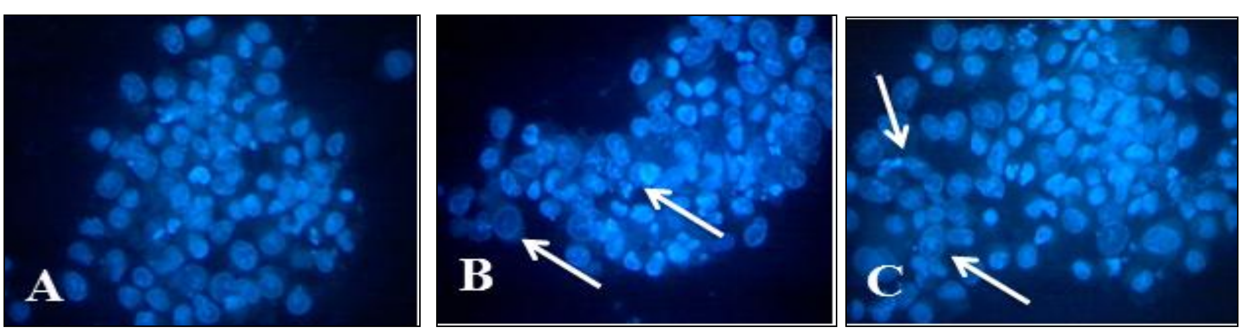


FIG. 4: EFFECT OF *COLEUS FORSKOHLII* ETHANOL EXTRACT ON NUCLEAR MORPHOLOGY WITH HOECHST 33258 STAINING

A. Control cells show normal nuclei. B. 24 h treated cells show loss of membrane integrity, nuclear swelling and dot-like chromatin fragmentation. C. 48 h treated cells show loss of membrane integrity, nuclear swelling and dot-like chromatin fragmentation

Mitochondria as main targets for anticancer agents because they have a central role in the induction and regulation of both necrotic and apoptotic cell deaths [26]. In our study loss of mitochondrial membrane potential (Δψm) was observed on *Coleus forskohlii* root ethanol extract treated AGS cell line staínined with Rhodamine 123. In the non-apoptotic cancer cells the dye (Rhodamine 123) accumulated and aggregated within the mitochondria, resulting in green fluorescence while the apoptotic cells revealed weak staining underlining the anticancer effects of root ethanol extract (**Fig.5**).

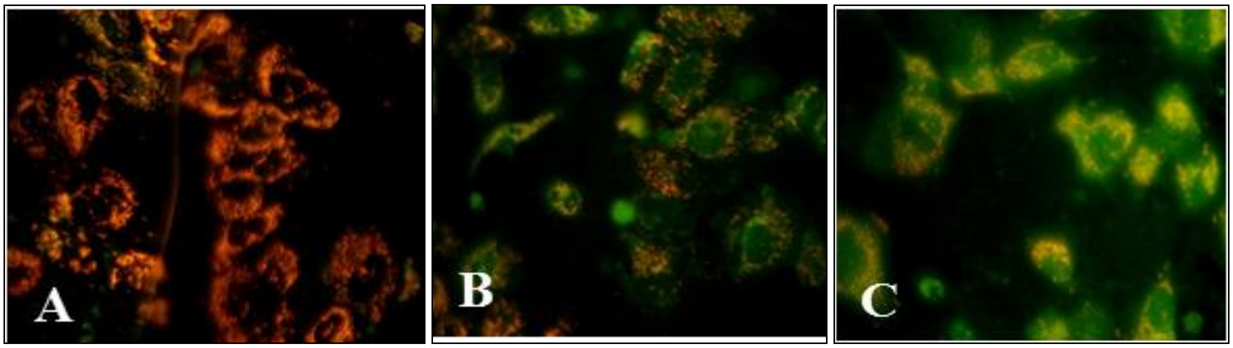


FIG.5: EFFECT OF *COLEUS FORSKOHLII* ETHANOL EXTRACT ON MITOCHONDRIAL MEMBRANE POTENTIAL (Δψm) (JC-1)

A. Control cells show intense red fluorescence indicating no changes in mitochondrial membrane potential. B. 24 h treated cells show weak red fluorescence and which showed green fluorescence at 24 h and 48 h due to mitochondrial membrane depolarization. C. 48 h treated cells show weak red fluorescence and which showed green fluorescence at 24 h and 48 h due to mitochondrial membrane depolarization. ROS is involved in triggering apoptotic signalling by inducing depolarization of the mitochondrial membrane (Δψm) which eventually leads to an increase in the levels of pro-apoptotic molecules intracellularly [27]. Treatment with *Coleus forskohlii* root ethanol extract to AGS cell lines showed significant ($p<0.05$) increase in the intracellular ROS production in a time dependent manner (24 h and 48 h) as compared to the control cells. However more amount of ROS production was observed at 48 h time point as compared to the 24 h time point. (**Fig. 6).**

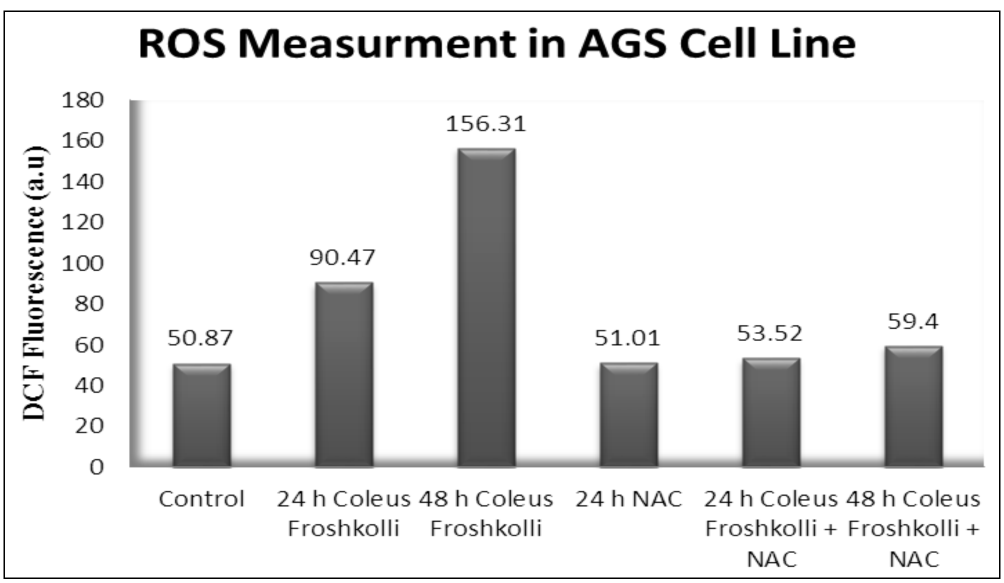


FIG.6: EFFECT OF *COLEUS FORSKOHLII* ETHANOL EXTRACT ON INTRACELLULAR ROS

Data are presented as the means ± SD of six independent experiments in each group. Values not sharing a common superscript letter ($^{a-e}$) differ significantly at $p<0.05$ (DMRT).

Furthermore, enhanced lipid peroxidation by products (TBARS) and decreased activities of antioxidant enzymes (SOD, CAT and GPx) observed in the present study correlates with *Coleus forskohlii* root ethanol extract induced ROS production (**Fig.7**) and **(Table 2**). Thus over all root ethanol extract exerts a beneficial action in cancer cells in the presence of low antioxidant defense. These effects by *Coleus forskohlii* root ethanol extract could be associated with inhibition of cell proliferation, induction of tumor cell death and alterations in the levels of oxidative stress markers. Induction of apoptosis is an ideal cancer therapy strategy.

In our study root ethanol extract exploits this process by selectively inducing cell death through the ROS dependent apoptotic pathway in AGS cells. Thus our findings suggest that root ethanol extract selectively possesses potent anticancer properties.

TABLE 2: EFFECT OF *COLEUS FORSKOHLII* ON ANTIOXIDANT ENZYMES (SOD, CAT AND GPX)

Cell lines	AGS		
Groups	Control	24 h	48 h
SOD #	4.02 ± 0.20^{a}	2.96 ± 0.30^{b}	2.37 ± 0.36^{c}
CAT @	8.36 ± 0.76^{a}	5.36 ± 0.41^{b}	3.63 ± 0.28^{c}
GPx $	7.70 ± 0.40^{a}	6.07 ± 0.27^{b}	4.07 ± 0.26^{c}

#50% NBT chromogen reduction/min/mg protein.
@µmoles of H_2O_2 consumed /min/mg protein.
$µmoles of GSH consumed /min/mg protein.
Data are presented as the means ± SD of six independent experiments in each group. Values not sharing a common superscript letter are (a-c) differ significantly at p <0.05 (DMRT).

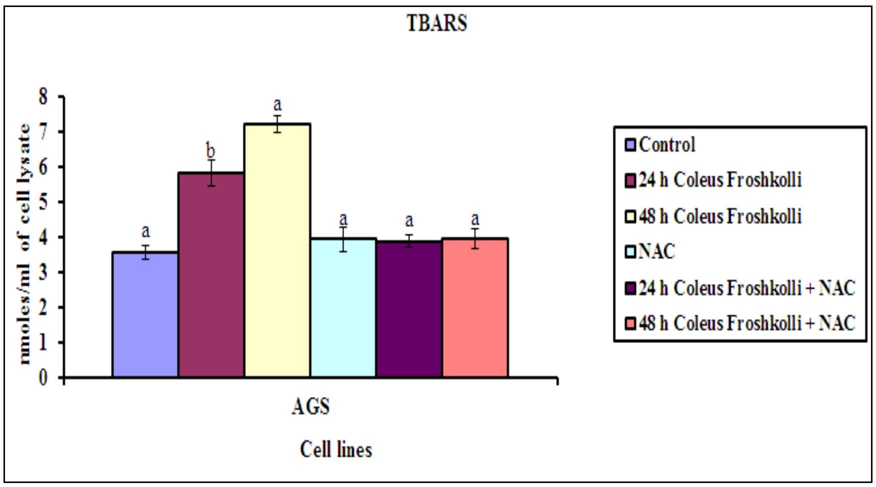
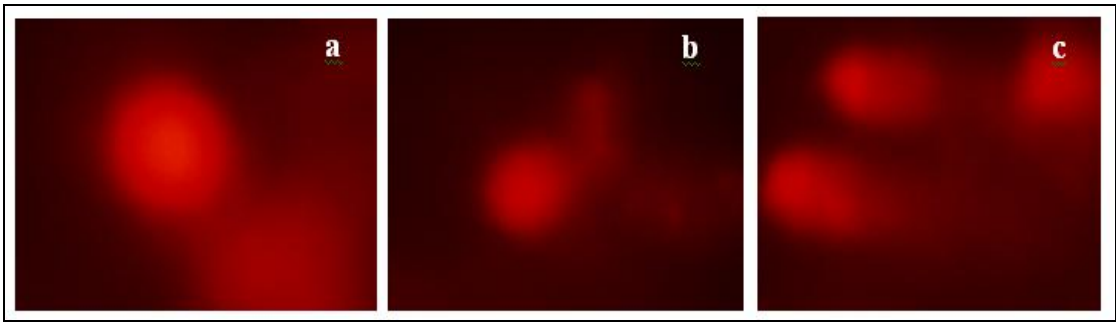
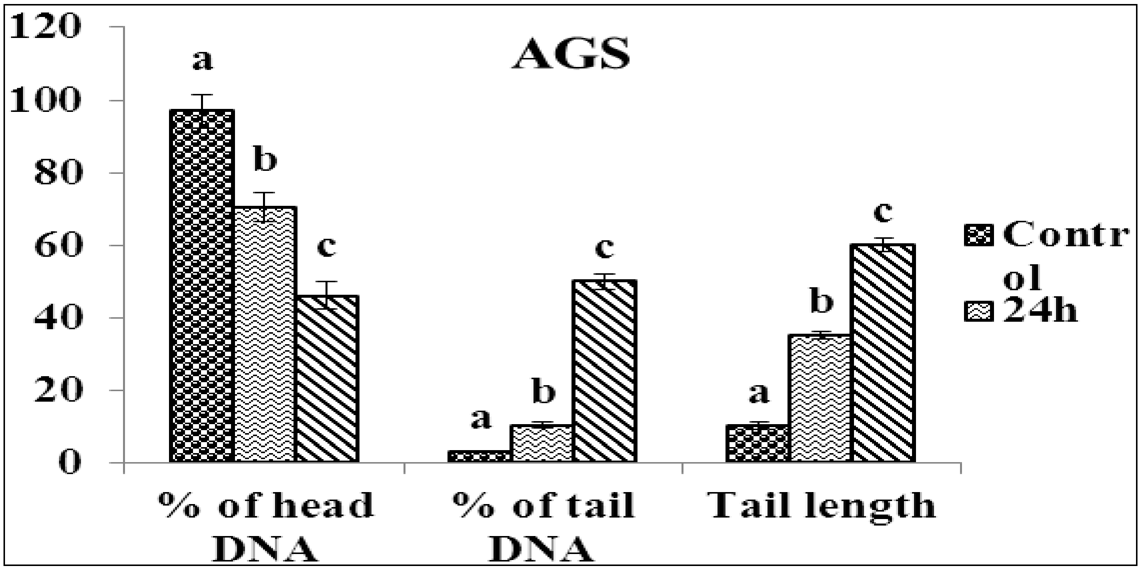


FIG.7: EFFECT OF *COLEUS FORSKOHLII* ETHANOL EXTRACT ON LIPID PEROXIDATION BYPRODUCTS
Data are presented as the means ± SD of six independent experiments in each group. Values not sharing a common superscript letter are (a-c) differ significantly at p <0.05 (DMRT)

Molecular studies and preclinical trials are warranted to know the exact mechanism of apoptosis by *Coleus forskohlii* root ethanol extract. As it is known that chemotherapeutic agents induce apoptosis and that DNA strand breaks may be an indication of on-going programmed cell death [28]. We searched for morphological signs of apoptosis and for its molecular hallmark, the DNA damage by comet assay. The cells were scored using comet analysis software (CASP). Control groups showed no significant DNA damage while treated with *Coleus forskohlii* root ethanol extract cells showed significant DNA damage. The nuclear head, comet tail and other comet parameters were recorded and the comparative data were generated and depicted in (**Fig. 8 a**). It was observed that the treatment with extract caused damage to DNA at 24 h and the percentage of damaged cells increased on treatment with extract treated cells at 48 h, which is evident by the appearance of prominent comet with tails (**Fig. 8**). The extent of DNA damage was increased with increasing duration of exposure.



(a) Round intact nucleus of AGS control cells. (b) The image of *Coleus forskohlii* ethanol extract treatment at 24 h. (c) The image of *Coleus forskohlii* ethanol extract treatment at 48 h showing comet like DNA tail.



(a) Round intact nucleus of AGS control cells. (b) The image of *Coleus forskohlii* ethanol extract treatment at 24 h. (c) The image of *Coleus forskohlii* ethanol extract treatment at 48 h showing comet like DNA tail.

FIG.8a: DNA DAMAGE WAS ANALYSED BY IMAGE ANALYSIS SOFTWARE (CASP).

To explore the potential signaling pathways underlying the *Coleus forskohlii* root ethanol extract induced apoptosis of cancer cells and apoptosis related protein expressions, cleaved caspase-3, bax, bcl 2 were evaluated with western blotting. The expression levels of cleaved caspase-3, were increased in dose-dependent manner, and the expression levels of Bax and Bcl2 were decreased in dose-dependent manner (**Fig. 9**)

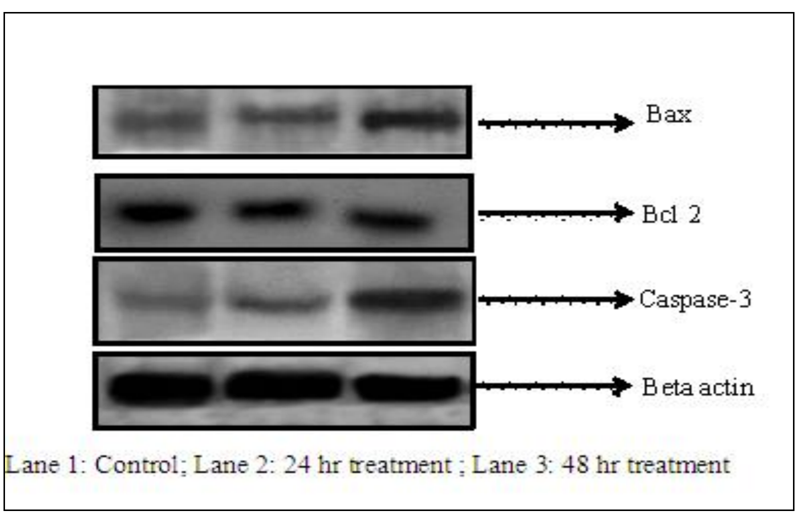


FIG. 9: APOPTOSIS RELATED PROTEIN EXPRESSIONS USING WESTERN BLOT

CONCLUSION: Our data clearly demonstrates that *coleus forskohlii* root ethanol extract could inhibit the proliferation of AGS cells by inducing apoptosis via mitochondrial pathway involving oxidant/antioxidant imbalance. Furthermore, *coleus forskohlii* root ethanol extract could be a potential candidate for development of an anticancer drug for the treatment of human gastric cancer.

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