Induction of HT-29 Colon Cancer Cells Apoptosis by Pyrogallol with Growth Inhibiting Efficacy Against Drug Resistant *Helicobacter pylori*

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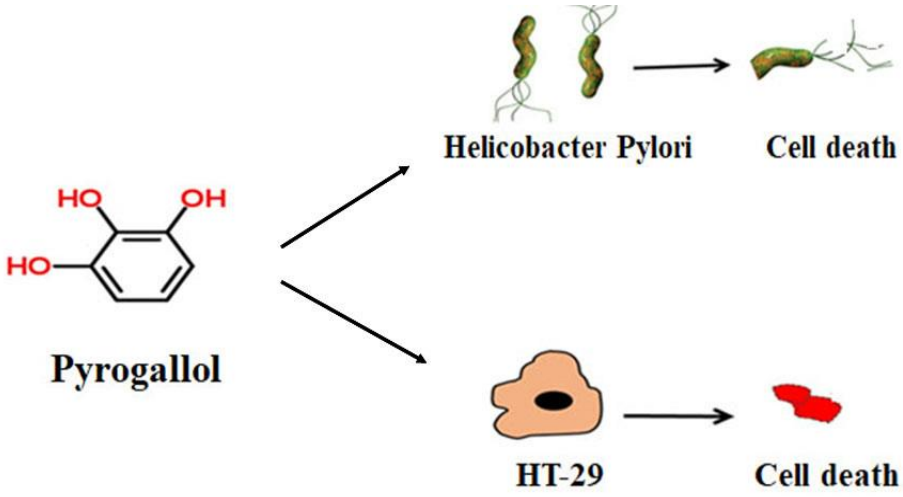
Graphical Abstract



Abstract

Background: Colon cancer is the most aggressive form of cancers, that causes 0.5 million deaths per year around the globe. Targeting colon cancer by conventional therapeutic options elicits toxicity. Traditional medicines take lead to alleviate the existing clinical challenges.

Objective: To investigate anti bacterial activity against *Helicobacter Pylori* and in vitro anti colon cancer activity by *Acacia nilotica* extract (ACE) and its active constituent pyrogallol.

Methods: Pyrogallol isolated from *A. nilotica* by column chromatography and HPLC and structure was elucidated by spectral analysis. Antibacterial activity was done by flow cytometry. Cyto-toxicity was measured by MTT assay. Apoptotic morphology and nuclear fragmentation was assessed with AO/ethidium bromide and DAPI staining. DNA fragmentation was done by electrophoresis. Western blot used to analyze the molecular mechanism of apoptosis. Cell cycle arrest was determined using flow cytometry of propidium iodide stained cells. Cell migration was determined by wound healing assay.

Results: ACE (20 µg/ml) and pyrogallol (10 µg/ml) treatment reduced the survival of *H.pylori* at 61% and 62% respectively. MTT results shows that HT-29 cells are more sensitive to pyrogallol with an IC_{50} value of 35µg/ml compare to ACE. Pyrogallol treated HT-29 cells reached dead state i.e. late apoptotic state with severe nuclear fragmentation. Pyrogallol induced apoptosis by simultaneous down regulation of Bcl-2 and up regulation of BAX and cytochrome c. Pyrogallol arrested HT-29 cells in S and G2/M phase of cell cycle. Further pyrogallol exhibited marked anti metastatic potential by inhibiting the migration of HT-29 cells dose dependently.

Conclusion: Both ACE and pyrogallol repressed the growth of *H.pylori* and as significant anti colon cancer agent.

Keywords: *Acacia nilotica*, pyrogallol, *Helicobacter pylori*, colon cancer, apoptosis

1. INTRODUCTION

Colon cancer is one of the leading causes of cancer related death worldwide with 0.5 million deaths every year and its incidence continues to rise in the eastern world [1]. As per the American Cancer Society, there will be an estimated 140,250 new cases of colon cancer and 27,390 men and 23,240 women will die from colon cancer in 2018 [2]. The evolution of colon cancer seems to follow a predictable pattern of histological changes and concurrent genetic and epigenetic changes, which ultimately provide a growth advantage resulting in the clonal expansion of transformed cells. At least three forms of genomic instability contribute to colon cancer, including microsatellite instability, chromosome instability, and chromosomal translocations **[3]**. Surgery can be an effective primary treatment in the early stage of colon cancer, but surgical resection is unsatisfactory in cases of metastasis and recurrence [4]. Chemotherapeutic agent 5-flurouracil (5-FU) widely used as a first line treatment for colon cancer patients over 50 years of age [5]. Due to extreme toxicity, drug resistant and inter-individual differences in response to treatment are major limitations [6]. Thus there is an urgent need on developing drug for colon cancer treatment with least toxicity and to target diverse array of colon cancer with different molecular signatures.

Natural products derived from medicinal plants received attention due to their less toxicity. Natural products based cancer drugs such as vincristine, vinblastine, podophyllotoxin, taxol etc. are renowned examples. These discoveries inspired by traditional and folk medicine clearly gives an indication that natural products are the future source for lead structures, and these will be used as templates for the development of more promising novel compounds with improved biological properties. Therefore, it is essential to use the available traditional knowledge and investigate the active plant extracts for the isolation of new, less toxic and highly efficacious molecules. The genus *Acacia* belongs to the family *Leguminosae. Acacia* used as an antiseptic, demulcent, purgative [7] and an effective tonic in diabetes mellitus [8]. Several species of *Acacia* have been proven as an effective medicine in the treatment of a cough, toothache, diarrhea, dysentery, jaundice, and skin disorders [9-12]. *Acacia* containing more than 1350 species and various bioactive compounds such as (+) -catechin, (−)-epicatechin, (−)-epicatechin-3-*O*-gallate, epigallocatechin-3-*O*-gallate, quercetin, and (+)-cyanidanol

[13]. Various parts of *Acacia* possess significant antibacterial and antifungal properties [14, 15]. Compounds identified from *Acacia* species are known to modulate various signaling pathways in breast and prostate cancer [16, 17].

Among *Acacia* species, *Acacia nilotica* (also known as Gum Arabic tree, Babul, Egyptian thorn, or Prickly Acacia) is widely cultivated in the Indian subcontinent and also found on lateritic soil in the Himalayan foothills [13]. *A. nilotica* is also reported to be effective against multidrug-resistant strains of bacteria and fungus causing nosocomial and community-acquired infections [18]. *A. nilotica* reported to be an effective anti cancer agent in different tumor model. γ-Sistosterol isolated from *A. nilotica* induces apoptosis and cell cycle arrest in breast cancer (MCF-7) and lung cancer (A549) cells [19]. Phenolic compounds derived from Egyptian *A. nilotica* reported for arresting the proliferation of melanoma cells [20]. *A. nilotica* shown to be an effective anti cancer agent against Dalton's ascitic lymphoma [21]. Our previous study shows that *A. nilotica* extract can induce significant cell death in both MDA-MB-231 and HEp-2 cells [22]. However, the role of *A. nilotica* and its active constituent pyrogallol on colon cancer is not yet reported. As per our knowledge herein we report for the first time *in vitro* anticancer activity of *A. nilotica* extract and its active constituent pyrogallol on colon cancer cells and their role in inducing apoptosis and cell cycle arrest.

2. Materials and Methods

2.1. Plant Material

The fresh leaves of *A. nilotica* were collected from the Foundation for Revitalization of Local Health Traditions, Bangalore, India (Latitude 12.9715987; Longitude 77.5945627).

2.2. Preparation of *A. nilotica* Extract (ACE)

Extraction was done as described earlier [22]. Briefly, fresh leaves of *A. nilotica* was collected and washed in tap water. It was shade dried for 10 days and made into a fine powder of 40 mesh in size using the laboratory mill. 100 g of powder was filled in a clean flat-bottomed glass container and soaked in 70% acetone for 72 h. The container with its content was sealed and kept for a period of three days accompanying occasional shaking and stirring. Extract was filtered using whatman no. 1 filter paper and it was dried at room temperature.

2.3. Isolation and Characterization of Pyrogallol

ACE was fractionated by column chromatography using different proportion of ethyl acetate and hexane and fractions were subjected to HPLC and pyrogallol was isolated based on retention time and absorbance of reference standard pyrogallol (Sigma Aldrich, Louis MO, USA). Further structure of pyrogallol was elucidated by spectral analysis such as FTIR, ^{1}H NMR, ^{13}CNMR, GCMS, LCMS and XRD.

2.4. Effect of ACE and Pyrogallol on *Helicobacter pylori* Survival by Flow Cytometry Analysis

Bacteriostatic effect of ACE and pyrogallol on antibiotic resistant *H.pylori* 26695 was determined by flow cytometry using annexin V-FITC and propidium iodide. *H. pylori* ($1x10^5$ cells/ ml) was treated with ACE (20 µg/ml) and pyrogallol (10 µg/ml) separately and incubated for 3 h. Then, the treated cells was harvested three times at 1 hr intervals, washed and suspended in PBS. These cells were then centrifuged at 2000 g for 10 min in order to prepare cell pellet. To the cell pellet 1ml of annexin V-FITC staining solution (Strong Biotech Co., Taipei, Taiwan) was added and incubated for 10-15 min at

25˚C. Then cells were analyzed for cell cycle by flow cytometer (FACS verse, BD Bioscience, USA).

2.5. *In Vitro* Anti cancer Activity

2.5.1. Cell culture

The human colon cancer cell HT-29 and normal colon cell CRL-1831 cells were purchased from the National Center for Cell Sciences (NCCS), Pune, India. The cells were maintained in Dulbecco's modified eagles medium (DMEM) supplemented with 2mM l-glutamine and balanced salt solution (BSS) adjusted to contain 1.5 g/L Na_2CO_3, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM l-glutamine, 1.5 g/L glucose, 10 mM (4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid) (HEPES) and 10% fetal bovine serum (GIBCO, USA). 1% antibiotics (penicillin/streptomycin) mixture was added. The cells were maintained at 37˚C with 5% CO_2 in a humidified CO_2 incubator.

2.5.2. Evaluation of Cytotoxicity

Cytotoxicity of ACE and pyrogallol was evaluated by MTT assay. HT-29 and CRL-1831 cells were cultured (1×10^4cells/well) in a 96-well plate for 48 h into 80% confluence. Then medium was replaced with fresh medium containing 20, 40, 60, 80 and 100 µg/ml pyrogallol or ACE, and the cells were further incubated for 24 h. Then culture medium was removed, and 100 µl of the MTT (3-4,5-dimethylthiozol-2-yl)-3,5-diphenyl tetrazolium bromide) (Hi-Media) solution was added to each well and incubated at 37˚C for 4 h. After removal of the supernatant, 50 µL of DMSO was added to each well and incubated for 10 min to solubilize the formazan crystals. The absorbance was measured at 620 nm in an ELISA multi well plate reader (Thermo Multiskan EX, USA). Absorbance

of control (without treatment) considered as 100% cell survival. Doxorubicin was used as positive drug control.

2.5.3. Fluorescence Microscopic Analysis of Apoptosis and Nuclear Fragmentation

CRL-1831 and HT-29, $1X10^5$ cells/ml each, were seeded separately in 96 well plates and allowed to adhere firmly. The cells were then treated with different concentration of ACE or pyrogallol (25, 50 and 100 µg/ml). After 24 h treatment, 20 µl of trypsin was added into each well. Trypsinized cell suspension washed with phosphate buffered saline (PBS) and 25 µl cell suspension was transferred to glass slides. Dual fluorescent staining solution (1 µl) containing 100 µg/ml acridine orange/ethidium bromide (AO/EB, Sigma, St. Louis, MO) was added to each suspension and then covered with a coverslip. The morphology of apoptotic cells was examined and visualized under a fluorescence microscope (Nikon Eclipse, Inc, Japan) at 400× magnification with an excitation filter at 480 nm. Likewise the cells were seeded on glass coverslip in a 24-well plate and treated with ACE or pyrogallol (25, 50 and 100 µg/ml) for 24 h. Then cells were fixed and permeabilised with 0.2% triton X-100 (50 µl) for 10 min at room temperature. Further cells were incubated for 3 min with 10 µl of DAPI by placing a coverslip over the cells to enable uniform spreading of the stain. The cells were then observed under fluorescent microscope (Nikon Eclipse, Inc, Japan).

2.5.4. *DNA Fragmentation by Agarose Gel Electrophoresis*

DNA ladder formation was done as described elsewhere. CRL-1831 and HT-29 were seeded in 6-well plates at a concentration of $1x10^6$ cells per ml of medium. Then cells were treated with ACE or pyrogallol (25, 50 and 100 µg/ml). Cells were harvested after 24 h of treatment and DNA fragmentation was assessed by gel electrophoresis.

2.5.5. Western Blot Analysis

The whole cell lysate was prepared from ACE or pyrogallol (25, 50 and 100 µg/ml) treated HT-29 cells after 24 hr as described earlier [23]. Then cell lysate were resolved in a 10% SDS polyacrylamide gel electrophoretically and electro transferred onto a nitrocellulose membrane. Then immunoblots were probed with Bcl-2, Bax and cytochrome c antibodies and visualized with the NBT/BCIP chromogenic substrate and documented.

2.5.6. Cell Cycle Analysis

HT-29 (2×10^{5}cells/10 cm dish) cells were treated with 25, 50 and 100 µg/ml of pyrogallol or ACE for 24h. Then cells were harvested by centrifugation, washed with ice-cold PBS, and then resuspended with ice-cold 70% ethanol overnight. Further, the cells were treated with 10 µg/ml of RNase at 37˚C, then spun down and stained with 40 µg/ml of propidium iodide (PI) for 30 min. The DNA content was then measured by flow cytometry (FACS, BD Bioscience).

2.5.7. Wound Healing Assay

HT-29 cells were seeded in 35 mm, collagen-coated dishes, at a density of $4X10^{5}$ cells/cm^{2}. Upon confluence, the monolayer cell cultures were treated with different concentration of ACE or pyrogallol (25 and 50 µg/ml). After 24 h of treatment, cell cultures were scratched with a sterile 200 µl tip to stimulate a wound *in vitro.* The cells were incubated in a 2% low serum medium and migration of cells across the scratch wound was measured from wounding up to complete coverage of the scratch. The cells were photographed under phase-contrast microscopy and scratch wound areas were measured using NIH Image J software. The mean scratch wound area was calculated from three independent experiments.

2.6. Statistical Analysis

The data presented mean ± STD. All the *in vitro* experiments were done in triplicate of three independent experiments. The statistical software SPSS version 17.0 was used for the analysis. *P* value <0.01 was considered significant.

3. RESULTS

3.1. Isolation and Characterization of Pyrogallol

Acetone extract of *A.nilotica* (ACE) was fractionated by column chromatography using different proportion of ethyl acetate and hexane. Totally 15 fractions were collected and fractions were checked for anti bacterial against H*elicobacter pylori*. By adopting bioassay guided approach for active principle isolation, active anti bacterial fraction was subjected to HPLC for pyrogallol isolation. FT-IR spectrum showed the broad peak at 3321.42cm^{-1}. This is due to the presence of phenolic OH group present on the pyrogallol then the aromatic stretching C-H bond appeared at 3093.82cm^{-1}. The aromatic ring C=C stretching bond shown in 1620, 1517 and 1481cm^{-1}. In plane of O-H bond exhibiting at bending mode at 1359 cm^{-1} and C-O stretching appeared at 1242 cm^{-1} which again confirmed the presence of C-O. The out of plane of C-H bending shown at 838.767cm^{-1} and the out of plane O-H bond about 697 cm^{-1} (Fig. 1A). The molecular weight of pyrogallol (126.11) identified by GCMS analysis (Fig. 1B). ^{1}H NMR, a broad singlet appeared at δ 6. 25 ppm, which represents the presence of hydroxyl moiety next the aromatic proton, appeared at δ 7.26-6.46 ppm which represents the presents of aromatic protons. ^{13}C NMR the peaks appeared at δ 102. 6, δ 114.9, δ 126. 5 and 139.7 ppm these again confirmed the presence of the aromatic carbon shown in the pyrogallol unit (Fig. 1C and D). HPLC profile purified compound displayed a sharp intense peak at retention time 4.028 and it is compared with reference pyrogallol (Fig. 1E). LCMS data revealed the molecular mass of isolated pyrogallol and it is also in agreement with GCMS (Fig. 1F). XRD pattern of pyrogallol powder revealed that it is according to the line width of the plane and refraction peak of m.p. 131° - 135°C. F (Fig. 1G). Further XRD patterns of the pyrogallol in diffraction angle range 2θ = 3 - 80° using monochromatic Cu ka radiation reveals presence of hydroxyl group of pyrogallol and can adopt different confirmations. Our result is in agreement with Thakuria et al. [24].

3.2. Flow Cytometry Analysis of *Helicobacter pylori* Inhibition

Drug resistant *H. pylori* was treated with ACE (20 µg/ml) or pyrogallol (10 µg/ml) and observed for percent of cells attained the bacteriostatic state. Flow cytometry data shows that the viability of *H. pylori* is decreased in time dependent manner viz. 37%

(1h), 42% (2h) and 61% (3h) upon the treatment of ACE whereas pyrogallol (10 µg/ml) also reduced viability of *H. pylori* viz. 47% (1h), 57% (2h), and 62% (3h). This data clearly indicates that pyrogallol (10 µg/ml) and ACE (20 µg/ml) have similar potency against *H. pylori* (Fig. 2).

3.3. Cytotoxicity of ACE or Pyrogallol on HT-29 and CRL-1831 cells

HT-29 and CRL-1831 cells were treated with different concentration (20, 40, 60, 80 and 100 µg/ml) of ACE or pyrogallol for 24 h. Viability of both HT-29 and CRL-1831 are reduced in a dose dependent manner (Fig. 3). IC_{50} value of ACE on HT-29 and CRL-1831 cells is >100 µg/ml. Whereas IC_{50} value of pyrogallol on HT-29 and CRL-1831 cells are 35 µg/ml and > 100 µg/ml respectively. HT-29 cells are more sensitive to pyrogallol than ACE and this data clearly evidences the anti-proliferative potential of pyrogallol.

3.4. Role of ACE or Pyrogallol on Apoptosis and Nuclear Fragmentation

The morphological changes of the CRL-1831 and HT29 cells-treated with ACE or pyrogallol for 24 h at concentration of 25, 50 and 100 µg/ml were analyzed by AO/EB fluorescence staining. ACE treatment exhibited pronounced morphological changes in HT-29 cells compared to CRL-1831 cells. HT-29 cells reached late apoptotic state where the irreversible damage occurs. Further DAPI staining infers that 24 h of ACE/pyrogallol treatment elicits significant apoptotic morphology such as cell shrinkage, nuclear condensation and fragmentation and formation of apoptotic bodies in HT-29 cells compare to CRL-1831 cells (Fig. 4A and B).

3.5. DNA Fragmentation Ability of ACE or Pyrogallol on CRL-1831 and HT29 cells

To ensure the DNA targeting ability of ACE or Pyrogallol, CRL-1831 and HT-29 cells were treated with different concentration (25, 50 and 100 µg/ml) of ACE or Pyrogallol for 24 h and observed for DNA damage. ACE or Pyrogallol induced profound dose dependent DNA ladder formation in HT-29 cells compare to CRL-1831 (Fig. 5).

3.6. Molecular Mechanism of Inducing Apoptosis by ACE or Pyrogallol in HT-29 cells

To investigate the role of ACE or pyrogallol on pro apoptotic and anti apoptotic signaling, we analyzed the expression level of Bcl-2, BAX and cytochrome c by immunoblot. ACE or pyrogallol treatment down regulates the expression of Bcl-2 and up-regulate the expression of BAX and cytochrome c (Fig. 6A and B). This data reveals that apoptosis induction in HT-29 cells by ACE or pyrogallol mediated by simultaneous up regulation of BAX and cytochrome c and down regulation of Bcl-2.

3.7. Efficacy of ACE or Pyrogallol on HT-29 Cell Cycle

HT-29 cells were treated with different concentrations (25, 50, and 100 μg/ml) of ACE or pyrogallol for 24 h and analyzed for cell cycle arrest by flow cytometry. ACE or pyrogallol treatment resulted in dose dependent reduction of number of cells in S and G2/M phase (Fig. 6A and B). Data indicates that ACE or pyrogallol halt DNA synthesis and subsequent mitosis in HT-29 cells.

3.8. Effect of ACE or Pyrogallol on HT-29 Migration by Wound Healing Assay

Cancer cell migration is an hallmark of metastasis. HT-29 cells were treated with different concentration (25 and 50 μg/ml) of ACE or pyrogallol for 24 h and observed for migration. Both ACE and pyrogallol elicited significant dose dependent migration inhibitory activity compared to untreated control (Fig. 7A and B).

4. Discussion

Focus on complementary and alternative medicine has been emerged rapidly for the past two decades. Active ingredients present in natural resources possess significant biological activity to combat deadliest form of diseases such as cancer. Natural products derived from medicinal plants attracted lots of attention since the fact that major breakthroughs in cancer drug discovery have been owed either to the natural products or the natural product scaffolds; vincristine, vinblastine, podophyllotoxin, taxol are few to be quoted. These natural products based scaffolds can be used as templates for the source for lead structures, and for the development of more promising novel compounds with improved biological properties. Therefore in this study we delineate the role of *A. nilotica* extract (ACE) and its active constituent pyrogallol for anti bacterial activity against *helicobacter pylori* and as an anti colon cancer agent *in vitro*.

It is well known that *H. pylori* disrupts gastric function and induce carcinogenesis in colon. As per International Agency for Research on Cancer, *H. pylori* is recognized as a class I human carcinogen [25]. A possible pathogenetic mechanism involves the persistent *H. pylori* colonization and inflammation of the gastric mucosa, particularly when the *H. pylori* strains express the cytoxin-associated gene (CagA) which often results in the development of chronic atrophic gastritis and subsequently hypergastrinemia and it leads to be a possible risk factor for the development of colon cancer [26–28]. In this study we tested the efficacy of ACE and pyrogallol on survival of *H. pylori*. Earlier, ACE reported for inhibition of *H. pylori* and its minimum inhibitory concentration (MIC) was determined by agar well diffusion method [29]. In our study we used flow cytometry analysis to assess the antibacterial activity of ACE and pyrogallol since minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC) assays has limitations such as non-quantitative, do not discriminate between bacteriostatic and bactericidal which requires quantitative assays. By using flow cytometry technique disruption of the cytoplasmic membrane of *H. pylori* can be monitored by membrane permeable fluorescent (Annexin FITC-A) intercalating DNA dyes and it is directly reflecting viability of a microbial culture. Noticeably flow cytometry employed by others to assess the growth inhibition of *H. pylori* by natural products [30]. After 3 hr of treatment with ACE (20 µg/ml) or pyrogallol (10 µg/ml)

more than 60% of *H. pylori* cells reached bacteriostatic state (Fig. 2A and 2B). It is noteworthy that less dose of pyrogallol can induce prominent *H. pylori* cell death compare to ACE. However mechanism of inducing cell death in *H. pylori* by pyrogallol is unclear. In agreement with our study active ingredients extracted from traditional medicinal plants exhibited potent antibacterial activity against *H.pyroli* [29].

After 24 h of treatment with ACE or pyrogallol HT-29 and CRL-1831 cells showed dose dependent decline in cell survival (Fig. 3A and 3B). Interestingly HT-29 cells are more sensitive to pyrogallol with an IC_{50} value of 35 μg/ml. High chemo sensitivity of pyrogallol towards HT-29 cells is unclear. However the observed cytotoxicity of pyrogallol can be inferred based on structure activity relationship. It is well reported that dihydroxylated phenolic compounds shown less cytotoxicity than orthotrihydroxylated phenolic compounds in breast cancer (MCF-7 cells) [31]. Hydroxyl groups can intercalate with DNA and execute irreversible DNA damage and it leads to nuclear fragmentation. Phenolic compounds are well reported as an inducer of apoptosis in HEK293T and K562 cells [32]. In our study, observed cytotoxicity of pyrogallol on HT-29 cells due to its phenolic nature with three hydroxyl group at ortho position in ring. Based on the structure of pyrogallol we hypothesize that molecular mechanism of inducing cell death by pyrogallol on HT-29 cells due to nuclear fragmentation and apoptosis. To address this issue in this study we monitored HT-29 and CRL-1831 cells for apoptotic morphology and nuclear fragmentation by acridine orange/ethidium bromide and DAPI staining after 24 h of ACE or pyrogallol treatment. HT-29 and CRL-1831 cells reached necrotic and apoptotic state with treatment of ACE or pyrogallol (Fig. 4A and B). In particular more number of pyrogallol treated HT-29 cells reached dead state compared to CRL-1831 cells. It indicates that specific chemo sensitive property of pyrogallol on HT-29 cells. Further DAPI staining reveals that pyrogallol treatment elicits significant change in nucleus morphology in the form of crescents around the periphery of the nucleus or the entire chromatin was present as one or a group of featureless, bright spherical beads compared to ACE (Fig. 4A and B). This nuclear morphological features clearly indicates the induction of apoptosis. Biochemical signatures accompanied with apoptosis include chromosomal DNA cleavage into inter-nucleosomal fragments, phosphatidylserine externalization and a number of intracellular substrate cleavages by

specific proteolysis (Cohen et al., 1994) [33]. To ensure the extent of DNA damage, in this study, CRL-1831 and HT-29 cells were treated with ACE or pyrogallaol for 24 h and DNA ladder formation was examined by electrophoresis. Fig. 5 shows that ACE or pyrogallol induced dose dependent DNA damage in HT-29 cells compare to CRL-1831 cells [33].

Chemotherapeutic agent triggers apoptosis with irreversible death of cancer cells is a promising drug candidate [34]. Apoptosis is induced by two alternative pathways, an extrinsic pathway mediated by the death receptor and the intrinsic pathway mediated by mitochondria [35, 36]. In the intrinsic pathway, Bcl-2 family includes key regulators of apoptosis and the molecule is over-expressed in many types of cancer cells [37]. Mitochondria activated by proapoptotic Bcl-2 family members (Bax, Bak, Bid, etc.) with release of cytochrome *c* [38]. Reduced Bcl-2 expression accompanied with high expression of Bax and cytochrome c may promote apoptotic response to anticancer drugs, increased expression of Bcl-2 leads to resistance to chemotherapeutic drugs [39]. To reveal the molecular mechanism of apoptosis induced by ACE or pyrogallol in HT-29 cells, in this study expression of Bcl-2, BAX and cytochrome c were analysed by immunoblot. Both ACE and pyrogallol decline Bcl-2 expression in HT-29 cells dose dependently whereas BAX and cytochrome c expression were up regulated (Fig. 6A and B). These findings suggest that the ACE and pyrogallol induced apoptosis in HT-29 cells via down regulating the apoptosis activator Bcl-2 with high expression of cytochrome c which leads to formation of apoptosome in the cytosol and initiation of caspase signaling cascade. This might be due to free radical scavenging potential of ACE or pyrogallol. Similarly, luteolin an antioxidant induces apoptosis in HT-29 cells by upregulating Bax, down regulating Bcl-2, and inducing the release of cytochrome c from mitochondria to the cytosol [40].

Cell cycle checkpoints are important control mechanisms that ensure the proper execution of cell cycle events which leads to cell proliferation. S and G2/M checkpoint blocks the entry into mitosis when DNA is damaged [41]. Protein p53 can regulate the G2/M transition either through the induction of p21 and 14-3-3σ, a protein that normally sequesters cyclin B1-Cdc2 complexes in the cytoplasm [42, 43] or through the induction of apoptosis [44, 45]. In our study both ACE and pyrogallol induced significant

cytotoxicity and apoptosis in HT-29 cells; however the inhibitory role of ACE and pyrogallol on HT-29 cell cycle remains elusive. To reveal this HT-29 cells were analyzed for cell cycle after 24 h of treatment with ACE or pyrogallol. We found that both ACE and pyrogallol is able to arrest S and G2/M phase of cell cycle in a dose dependent manner (Fig. 7A and 6B). Data suggests that both ACE and pyrogallol halt the S and G2/M stage of cell division where DNA synthesis and cell division were initiated. Molecular mechanism of cell cycle arrest induced by ACE or pyrogallol is not clear. Previously it was demonstrated that pyrogallol induces G2/M arrest in human lung cancer cells [46]. Phenolic compound treatment arrest S phase of cell cycle in prostate carcinoma DU145 cells and G2/M phase arrest in HeLa cells accompanied by mitotic catastrophe, and formation of cells with multiple nuclei, followed by impaired centrosomal clustering [47, 48]. Further Snchez-Carranza et al. reported that phenolic compounds isolated from *Caesalpinia coriaria* induce S and G2/M phase cell cycle arrest by inhibiting microtubule [49]. These reports are in agreement with our results both ACE and pyrogallol are phenolic compounds arrest the cell cycle at S and G2/M phase in HT-29 cells possibly by inhibiting microtubule function however, further research in needed to test this hypothesis. Cancer cell migration is an imperative step before the formation of micro or macro metastasis. Chemotherapeutic agent halt the migration of cancer cells can be an ideal drug candidate to arrest metastasis. In this study we performed *in vitro* wound healing assay to assess anti migration property of ACE or pyrogallol on HT-29 cells. We found that 24 h treatment of ACE or pyrogallol restricts the migration of HT-29 cells significantly (Fig. 8A and 7B). However the molecular mechanism of migration inhibition by ACE or pyrogallol is unclear. Basically loss of adhesion molecule E-cadherin facilitates early detachment from primary tumor and migration of tumor cells [50]. Molecules can upregulate the expression of E-cadherin could be an ideal anti metastatic agent. Phenolic compound such as curcumin inhibited the colon cancer cells (SW620) migration by upregulating E-cadherin expression [51]. Our previous study reveals that polyphenol crocin inhibited melanoma metastasis by up regulating the E-cadherin expression [52]. Bases on these previous reports we speculate that both ACE and pyrogallol might play pivotal role in modulation of E-cadherin expression in HT-29 cells however, this hypothesis should be addressed experimentally with conclusive data.

In conclusion *A. nilotica* extract and its active constituent pyrogallol inhibited the growth of carcinogenic *H.pylori*. Both ACE and pyrogallol elicited significant cytotoxicity on HT-29 cells by sparing normal colon epithelial cells (CRL-1831) with minimal toxicity. Pyrogallol induced apoptosis, nuclear fragmentation and arrest S and G2/M phase of cell cycle in HT-29 cells. Further pyrogallol exhibited *in vitro* anti metastatic potential by inhibiting the migration of HT-29 cells. However, the molecular mechanism of anti cancer potential of pyrogallol should be studied in detail.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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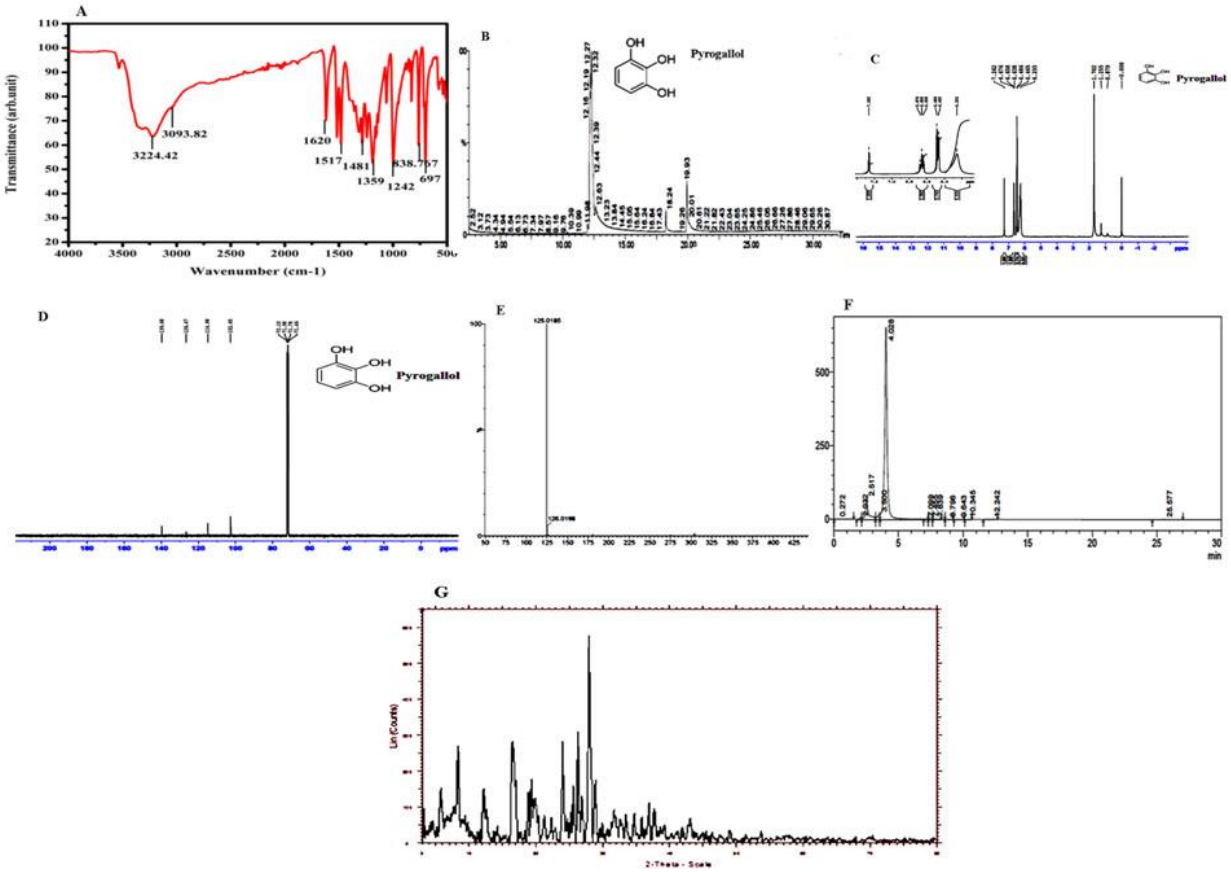
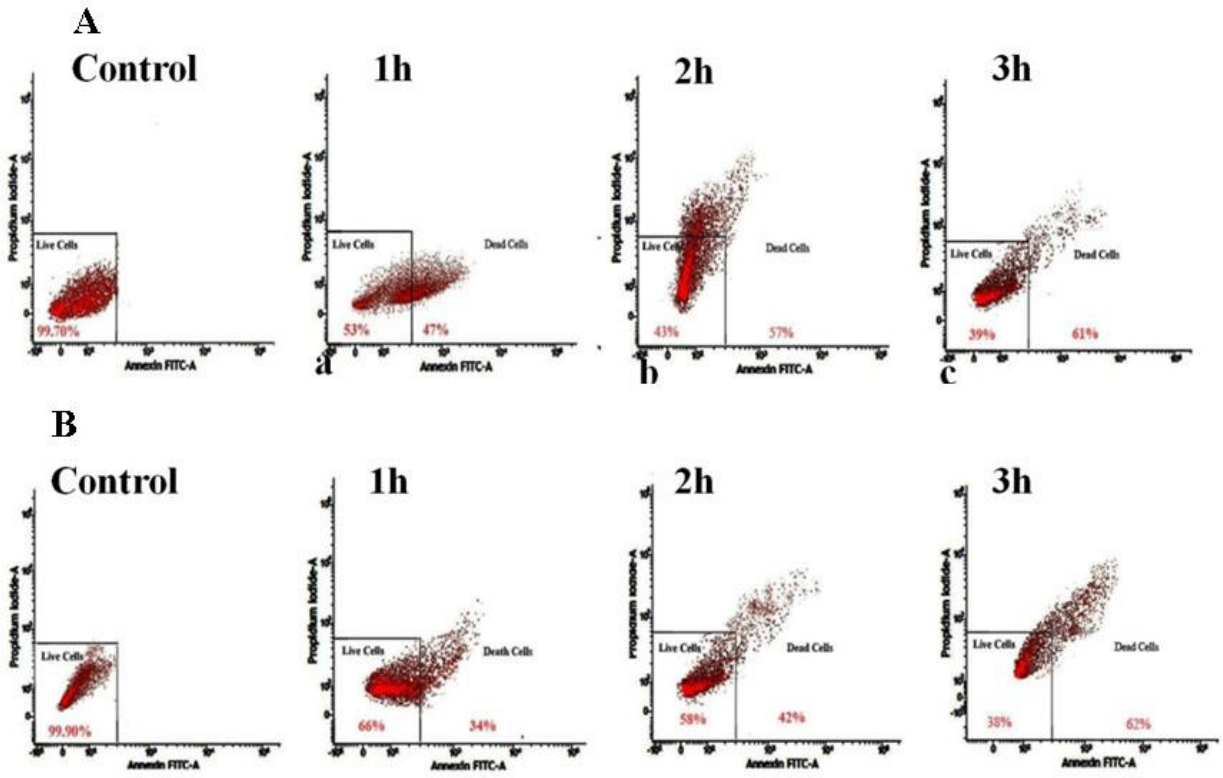
Figure 1: Characterization of Purified pyrogallol from *Acacia nilotica*



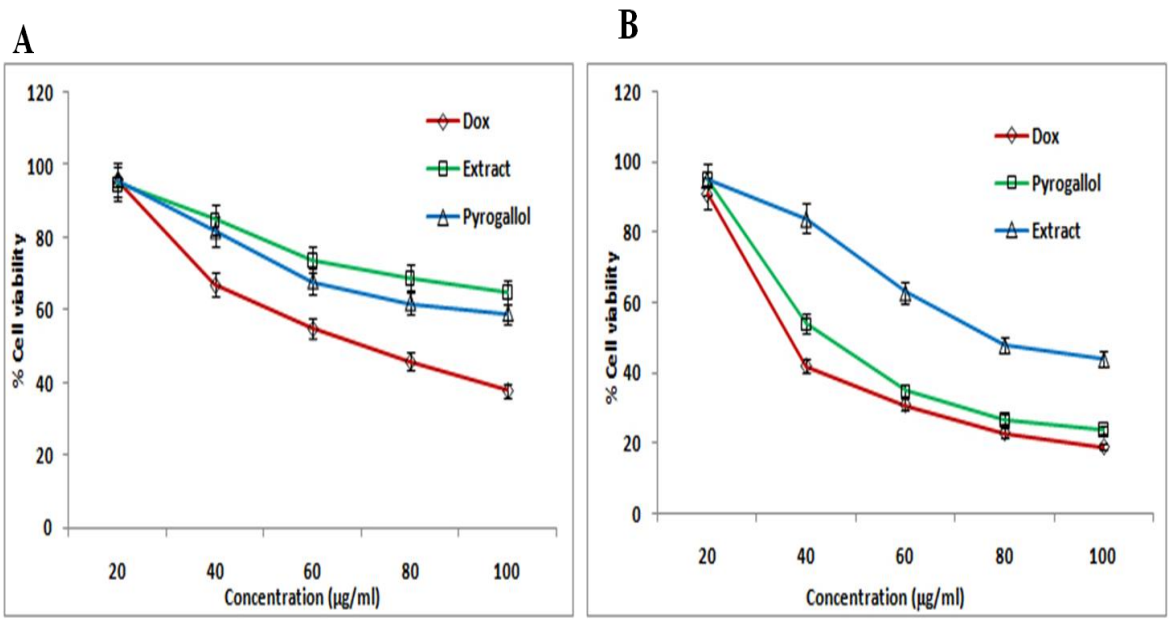
Isolated pyrogallol characterized by spectral analysis. a) FTIR b) GC MS, c) ^{1}H NMR, d) ^{13}C NMR, e) LC-MS, f) HPLC, g) P.XRD

Figure 2: Flow cytometry dot plots of antibiotics Resistant *H. pylori* 26695 treated with ACE extract and pyrogallol



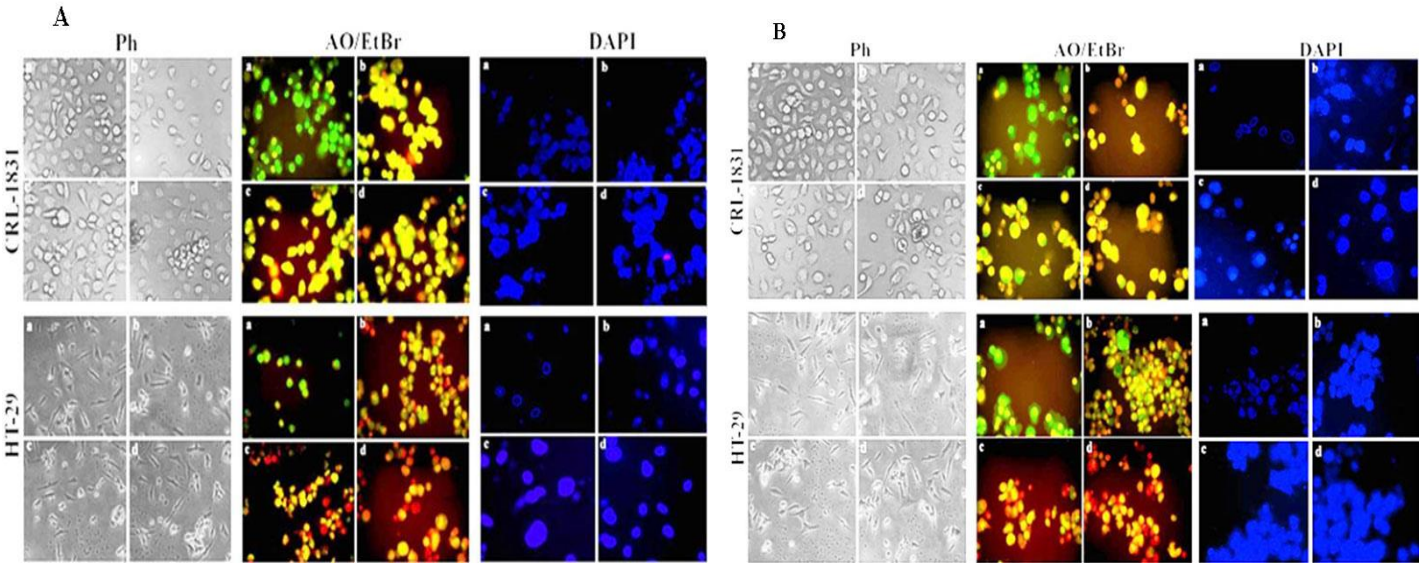
ACE treated *H. pylori* (A), pyrogallol treated *H. pylori* (B).Antibiotic resistant *H.pylori* treated with ACE (20 µg/ml) and pyrogallol (10 µg/ml) for 3h. *H. Pylori* stained with Annexin FITC-A and observed for distribution of cells for 3h with time intervals of 1h.

Figure 3: Cytotoxicity of ACE and pyrogallol on HT-29 cells and CRL-1831 cells



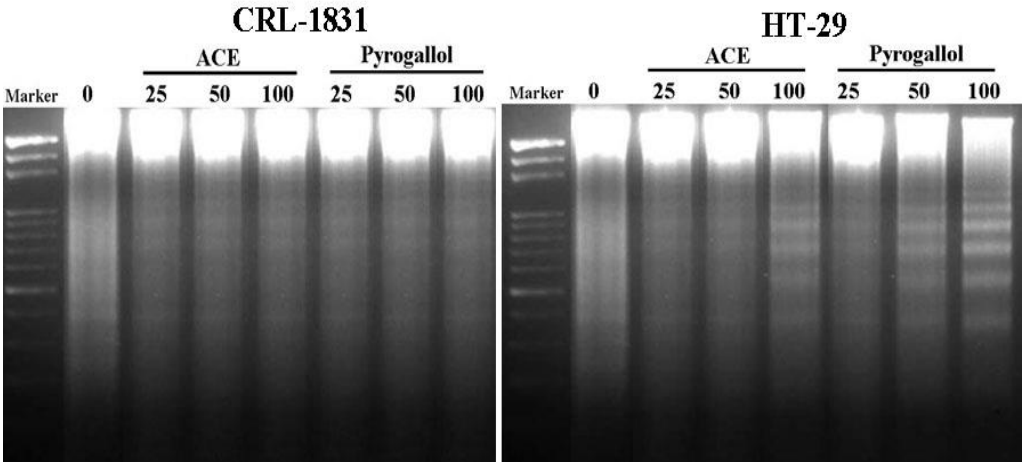
Data presented as mean ± STD of triplicates of three independent experiments. HT-29 and CRL-1831 cells treated with various concentration of ACE and pyrogallol for 24 h and cytotoxicity was determined by MTT assay. A. Normal colon epithelial cells (CRL 1831), B. Colon cancer cells (HT-29).

Figure 4: Apoptotic effect of ACE and pyrogallol on HT-29 cells and CRL-1831 cells



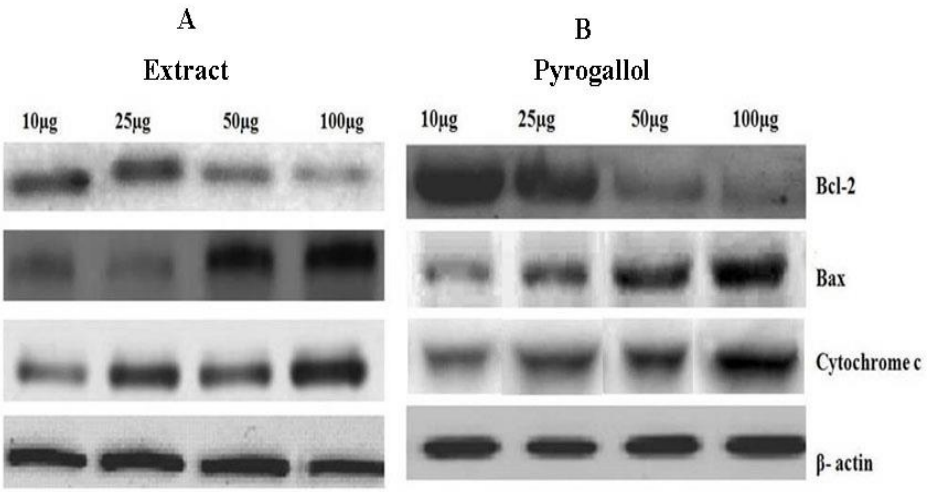
ACE treated cells (A), pyrogallol treated cells (B). Ph- Phase contrast image of a) Untreated control b) 25 µg/ml c) 50 µg/ml d) 100 µg/ml. Changes in cellular morphology was observed after 24 h of treatment. AO/EtBr fluorescent staining used to analyze the apoptotic morphology. Green: live cells, Yellow: necrotic cells, Red: dead cells. DAPI staining used to observe the nuclear fragmentation. Magnification at 20X.

Figure 5 : Induction of DNA Fragmentation in CRL-1831 and HT-29 cells



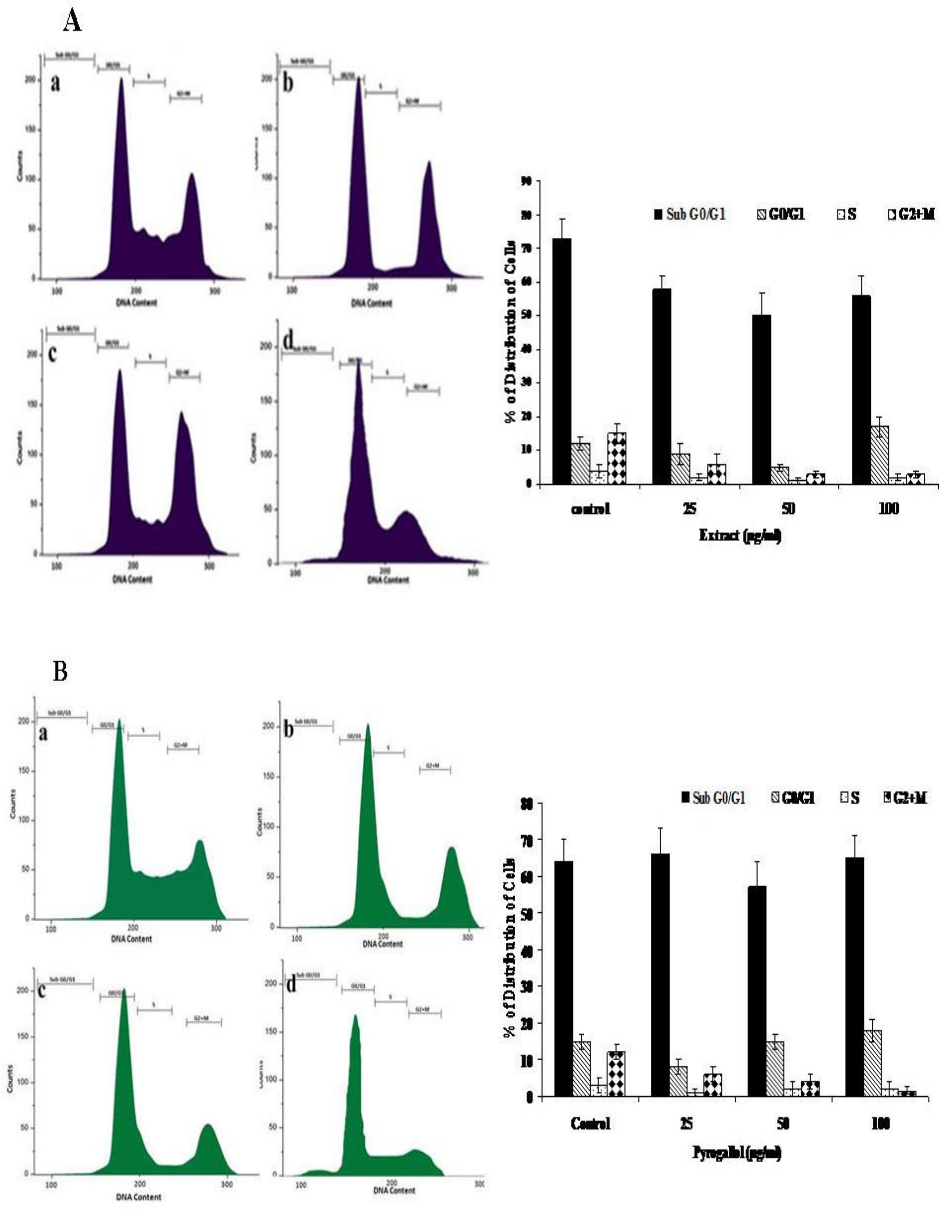
CRL-1831 and HT-29 cells were treated with ACE or Pyrogallol (0, 25, 50 and 100 μg/ml) for 24 h. DNA was isolated and resolved in agarose gel and examined by ethidium bromide staining.

Figure 6: Western Blot Analysis of Bcl-2, Bax and cytochrome c



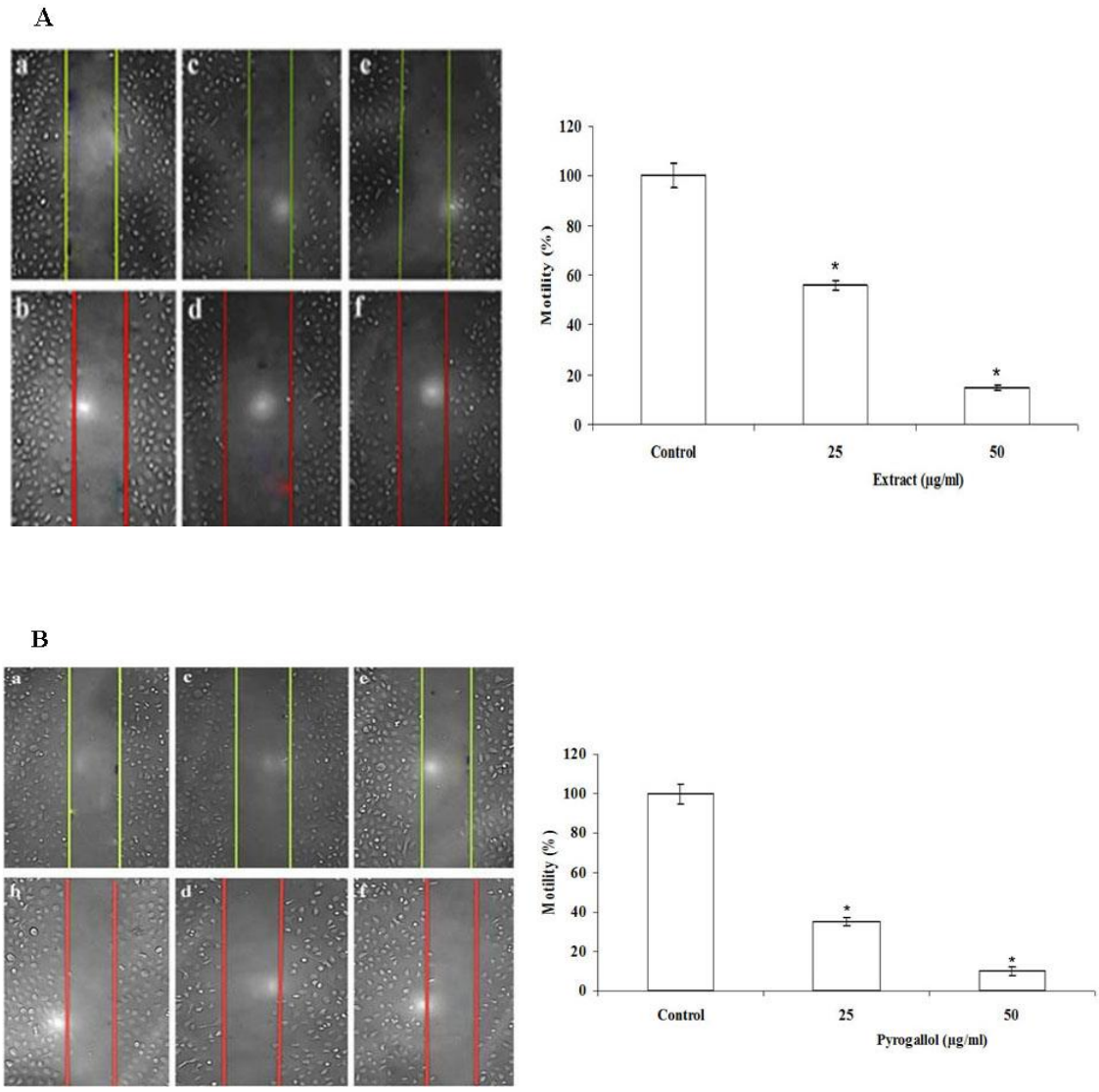
Whole cell lysate prepared from ACE or pyrogallol treated HT-29 cells. ACE and pyrogallol treatment resulted dose dependent decline in Bcl-2 expression and increase in BAX and cytochrome c expression. β-actin as a loading control.

Figure 7: Determination of Cell Cycle Arrest by ACE and Pyrogallol on HT-29 cells



ACE treated cells (A), pyrogallol treated cells (B). 2 X 10^5 HT-29 cells were treated with ACE and pyrogallol for 24 h. a) Untreated control b) 25 µg/ml c) 50 µg/ml d) 100 µg/ml. Cells were harvested and stained with propidium iodide as described. Experiment repeated thrice and the profile was similar.

Figure 8: Wound Healing Property of ACE and Pyrogallol on HT-29 cells



ACE treated cells (A), pyrogallol treated cells (B). HT29 cells were injured and cell migration assay with and without treatment of ACE or pyrogallol (25 and 50 µg/ml) was performed. ACE or pyrogallol treated HT29 cells lost the migration ability compare to untreated control. Magnification: 20X. Data presented as mean ± STD of triplicates of three independent experiments. * indicates the significant difference at $p<0.01$.