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CYTOTOXICITY AND APOPTOTIC INDUCIBILITY OF METHANOL EXTRACTS OF *Bruguiera gymnorrhiza* AND *Aegialitis rotundifolia* AGAINST HepG2 AND MCF-7 CELL LINES

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ABSTRACT

The purpose of the present study was to investigate the methanolic extract of *Bruguiera gymnorrhiza* and *Aegialitis rotundifolia* for their cytotoxic and apoptotic induction activities. The cytotoxic activity was evaluated by MTT assay against hepG2 and MCF7 Cell lines. Induction of Apoptosis was carried hepG2 cell line. Morphological changes and DNA fragmentation were found up on incubation with extract. Both the plant extracts showed significant cytotoxic activity against hepG2 and MCF7 cell lines and the concentration required for 50% cell death was 77.29 µg/ml, and 99 µg/ml for *Bruguiera gymnorrhiza*; 97.77 µg/ml and 188.45 µg/ml for *Aegialitis rotundifolia* against hepG2 and MCF7 cell lines respectively. Thus from the present investigation it can be concluded that the methanolic extract of *Bruguiera gymnorrhiza* and *Aegialitis rotundifolia* exhibit both Apoptosis induction and Cytotoxic activities.

KEY WORDS: ***Apoptosis, MTT assay, Cytotoxicity, DNA Fragmentation, Mangrove.***

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INTRODUCTION

Cancer is the loss of control over the mechanisms that governs cell survival, proliferation and differentiation of cell which leads to qualitative and quantitative chromosomal abnormalities. Cells undergo neoplastic transformation to tumor cells.[1] In spite of good advancements for diagnosis and treatment, cancer is still a big threat to our society.[2] Cancer is second most common disease after cardiovascular disorder for maximum deaths in the world. It accounts for about 23% and 7% in USA and India respectively. The world's population is expected to be 7.5 billion by 2020 and approximations predict that about 15.0 million new cancer cases will be diagnosed, with deaths of about 12.0 million cancer patients.[3] It is believed that in near future, the number of cancer patients will increase in the developing and under developed countries which may rise up to 70%; a serious issue for all of us. The magnitude of cancer problem in the Indian subcontinent is increasing due to poor to moderate living standards and inadequate medical facilities. Most frequently observed cancers in Indian population are lungs, breast, colon, rectum, stomach and the liver.[4-5] The distinguished feature of cancer cell is its ability to circumvent apoptosis or programmed cell death, a highly organized cellular process to eliminate damaged or abnormal cells.[6] It is involved in maintaining homeostasis in multi cellular organisms through balance between cell proliferation and cell death. Apoptosis act like suicide programme by which cell dies without harming adjacent cells as the apoptotic bodies are readily phagocytosed by macrophages without triggering immune response.[7] The cells that are undergoing apoptosis exhibit distinct morphological characteristics such as chromatin condensation, membrane blebbing, reduction in cellular volume and nuclear fragmentation are similar in all cells with irrespective of their cell type and physiological or pathological conditions.[8-10] Although morphological characteristics of apoptosis were initially described, it is now clear that there is a highly complex molecular process that involves the convergence of various events resulting in the activation of cellular machinery responsible for apoptosis. The nuclear DNA of apoptotic cells shows a characteristic laddering pattern of oligo nucleosomal fragments. This results from inter-nucleosomal chromatin cleavage by an endonuclease in multiples of 180 base pairs[11] this fragmentation is regarded as hall mark of apoptosis. The accepted modality for cancer treatment involves surgery, radiation and drugs, singly or in combination. Cancer chemotherapeutic agents can often provide temporary relief from symptoms, prolongation of life and occasionally, cures. A successful anticancer drug should kill or in capacitate cancer cells without causing excessive damage to normal cells. This ideal solution is achievable by inducing apoptosis in cancer cells. The life span of both normal and cancer cell is significantly affected by the rate of apoptosis. Thus modulating apoptosis may be useful in the management and therapy or prevention of cancer. Synthesis or modification of known drugs continues as an important aspect of research. There is a continued need for new prototypes-new templates to use in the design of potential chemotherapeutic agents. Significantly, natural products are providing such templates. Recent studies on tumor inhibitory compounds of plant origin have yielded an impressive array of novel structures. Besides, epidemiological studies suggest that consumption of diets containing fruits, and vegetables, major sources of phytochemicals and micronutrients, may reduce the risk of developing cancer.[12-13] Certain products from plants are known to induce apoptosis in neoplastic cells but not in normal cells[14-16]. During the past decade, however, the evidence is gradually being shown that many cancer chemotherapeutic agents are not totally free from side effects. It is thus considered important to screen apoptotic inducers from plants, either in the form of crude extracts or as active components isolated from them. Mangroves are salt tolerant evergreen forests found along sheltered coastlines, shallow-water lagoons, estuaries, rivers or deltas in 124 tropical and sub-tropical countries of the world. Out of the total 15.2 million hectares global mangrove habitat, India is known to contribute about 448000 hectares.[17,18] Mangroves have a variety of economical and ecological uses. Many species of them have also been used traditionally in folklore medicine to treat various diseases since centuries. Recent research evidences suggest Indian mangrove plant species have anti bacterial and anti cancer activities. The potential of mangrove plants as a source of new bio active principles is still unexplored. Further there have been no detailed *invitro* studies on anticancer properties of leaves of mangrove plants from Corangi reserve forest, East Godavari district, Andhra Pradesh, India. Hence the current study is aimed on the evaluation of anticancer activity and their ability to induce apoptosis of two selected mangrove species of Corangi reserve forest.

Plant material

In our present study, the fresh leaves of *Bruguiera gymnorrhiza*, and *Aegialitis rotundifolia* were collected from Corangi reserve forest, Kakinada, East Godavari district, Andhra Pradesh, India. Geographical location of Corangi reserve forest is between 16°39'Nlongitude – 17°N longitude and 82°14'E latitude – 82°23'E latitude. The collected leaves were washed thoroughly under running tap water in the laboratory to remove the surface dust particles present on the surface and shade dried in a well – ventilated place at room temperature. The dried leaves were ground to a coarse powder and subjected to solvent extraction.

Solvent extraction

Methanol was used as solvent to prepare the crude leaf extracts. The powered material was first soaked for 12 hrs in 500 ml of methanol and then subjected to extraction by refluxing for 6 to 8 hrs below the boiling point of the solvent. The extract was concentrated by evaporating at a reduced pressure using rotary evaporator. The concentrated extract was further dried at 37°C for 3 to 4 days in order to facilitate complete evaporation of the solvent

Cell lines and culture conditions

HepG2 and MCF7 cell lines were purchased from the National Centre for Cell Science (NCCS), Pune, India. The cell lines were cultured in Dulbecco Modified Eagle's Medium (DMEM). Culture medium was supplemented

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with 10% fetal bovine serum, antibiotic and antimycotic solution in conditions of 5% CO_2 and 95% air at 37°C.

Invitro cytotoxic studies

Common basic steps that are present in *Invitro* Cytotoxic activity include: 1) Isolation of cells, 2) Incubation of cell lines with drug for appropriate period of time, 3) Assessment of cell survival and 4) Interpretation of results.[19] Colorimetric assay (MTT) is mainly useful in the determination of cellular proliferation, viability and activation. The need for sensitive, quantitative, reliable and automated methods led to the development of standard assays. Cell proliferation and viability assays are of particular importance for routine applications. These techniques are considered to be fast and economical for the evolution of anticancer compounds. [20] Cytotoxicity of sample on tumor cell was measured Micro culture tetrazolium assay (MTT assay). [21] The MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) tetrazolium reduction assay was the first colorimetric assay for assessing cell metabolic activity and cell viability developed for a 96 well format that was suitable for high throughput screening (HTS). The MTT substrate is prepared in physiologically balanced solution, added to cells in culture, usually at a final concentration of 0.2- 0.5 mg/ml, and incubated for appropriate period of time. The enzymes of the cells are capable of reducing the tetrazolium dye to its insoluble formazon, which has purple colour. The quantity of formazon is measured by recording changes in absorbance at 570 nm using a plate reading spectrophotometer. Viable cells with active metabolism convert MTT in to purple coloured formazon product with an absorption maximum near 570 nm. When cells die, they lose the ability to convert MTT in to formazon, thus colour formation serves as a useful and convenient marker of only viable cells. The exact cellular mechanism of MTT reduction in to formazon is not well understood, but likely involves reaction with NADH or similar reducing molecules that transfer electrons to MTT. In this method first, Cells were seeded in to individual 96-well plates and incubated under the above conditions. After a day of incubation, cells were treated with various concentrations of the four selected mangrove plant extracts ranging from 10 µg to 200 µg/ml. In order to obtain IC50 values absorbance was measured at 570 nm in an ELISA multiplate reader. The percentage of inhibition of growth was calculated using the formula

$$\% \text{ of Cell viability} = 100 - \{100\text{x } (A_c - A_t)/A_c\}$$

Where, A_t = Absorbance value of test compound
A_c = Absorbance value of control

Ao/eb staining

The cells were treated with test compound at IC50 concentration and incubated for 24 hr in CO_2 incubator at 37°C. The cells were removed by trypsination and collected by centrifugation including the non adherent cells. The cell pellet was resuspended in medium and cell suspensions (25 µl) were transferred to glass slides. Dual fluorescent staining solution (1 µl) containing 100 µg/ml AO and 100 µg/ml EB (AO/EB, Sigma) was added to each suspension and then covered with a cover slip. The morphology of apoptotic cells was examined and counts the cells within 20 min using a fluorescent microscope. [22] Acridine orange is a vital dye and will stain both live and dead cells. Ethidium bromide will stain only cells that have lost membrane integrity. Live cells will appear uniformly green. Early apoptotic cells will stain green and contain bright green dots in the nuclei as a consequence of chromatin condensation and nuclear fragmentation. Late apoptotic cells will also incorporate ethidium bromide and therefore stain orange, but, in contrast to necrotic cells, the late apoptotic cells will show condensed and often fragmented nuclei. Necrotic cells stain orange, but have a nuclear morphology resembling that of viable cells, with no condensed chromatin.

Dna fragmentation assay

The inter nucleosomal cleavage of DNA was analyzed by DNA fragmentation assay as described by Kim *et al.,* 2001; Zuo *et al*., 2006. [23, 24] In this approach, the cells were treated with the appropriate concentration of test solution for 24 hr. After incubation, the cells were harvested, washed thrice with Phosphate buffer saline (PBS)and lysed by incubating with 500Ul of lysis buffer (100 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl (PH-8.0), 0.5% SDS, and 1 mg/ml Protinase-K) at 54°C with gentle agitation for 2 hr or until all solid material was digested. DNA was extracted with Phenol:Chloroform (24:1) followed by ethanol precipitation and the DNA pellet was air dried. The DNA was resuspended in 20 µl of Tris-EDTA (TE) buffer containing 50 µg/ml of RNase-A, incubated for 30 min at 37°C, and analyzed on a 1.5% agarose gel for DNA fragmentation. The gels were stained with 0.5 µg/ml of Ethidium bromide (EB) and documented under UV transilluminator.

RESULTS

The plant products serve as vital sources of blocking and suppressing agents that interfere with the carcinogenic process. Since mechanism relating to cell death and cancer attenuation by plant products has received limited attention, the present work was carried out to illustrate apoptotic and anti proliferative effect of *Bruguiera gymnorrhiza* and *Aegialitis rotundifolia.*

Cytotoxic activity

Percentage of cytotoxic activity on hepg2 cell line

The Cytotoxic activity and subsequent induction of cell death by methanolic extract of *Bruguiera gymnorrhiza* and *Aegialitis rotundifolia* were demonstrated on human cancer cell lines such as hepG2 by performing MTT assay. Different concentrations of plant extracts such as 10 µg/ml, 25 µg/ml, 50 µg/ml, 100 µg/ml and 200 µg/ml were used to treat the cells. The methanolic extract of *Bruguiera gymnorrhiza* showed significant cytotoxic activity on hepG2 cell line in a dose dependent manner. The percentage of cytotoxic activity or inhibition showed by *Bruguiera gymnorrhiza* was 15.15 for 10 µg/ml, 24.74 for 25 µg/ml, 39.51 for 50 µg/ml, 52.62 for 100 µg/ml and

78.18 for 200 μg/ml extract. The percentage of cytotoxic activity showed by *Aegialitis rotundifolia* was 24.47 for 10 μg/ml, 27.76 for 25 μg/ml, 49.02 for 50 μg/ml, 56.13 for 100 μg/ml and 67.85 for 200 μg/ml .But however; The Cytotoxic activity of *Bruguiera gymnorrhiza* was found to be more effective than *Aegialitis rotundifolia* on hepG2 with IC50 value of 77.29 μg/ml and 97.77 μg/ml respectively.

Percentage of cell viability on mcf -7 cell line

The methanolic extracts of *Bruguiera gymnorrhiza* and *Aegialitis rotundifolia* were checked for the percentage of cytotoxic activity using different concentrations of the extracts such as 10 μg/ml, 25 μg/ml, 50 μg/ml, 100 μg/ml and 200 μg/ml on MCF-7 cell line. . The percentage of cytotoxic activity showed 0.41 for 10 μg/ml, 27.56 for 25 μg/ml, 40.71 for 50 μg/ml 50.74 for 100 μg/ml, 54.70 for 200 μg/ml while using the *Bruguiera gymnorrhiza* extract on MCF7 cell line. But the percentage of cytotoxic activity was 3.26 for 10 μg/ml, 21.19 for 25 μg/ml, 33.37 for 50 μg/ml, 51.53 for 100 μg/ml and 58.25 for 200 μg/ml while using the *Aegialitis rotundifolia* extract on MCF7 cell line. The IC50 values of *Bruguiera gymnorrhiza* and *Aegialitis rotundifolia* on MCF7 cell line are 99 μg/ml and 188.45 μg/ml respectively. The results were shown in table no 1 and 2 and it clearly indicates that the extracts of *Bruguiera gymnorrhiza* and *Aegialitis rotundifolia* showed much significant anticancer activity on hepG2 and MCF7 cell lines.

Table 1
Percentage of cytotoxic activity of Bruguiera gymnorrhiza and AegialitisRotundifolia on HepG2 Cell line

S. No	Extract	10 μg/ml	25 μg/ml	50 μg/ml	100 μg/ml	200 μg/ml
1	*Bruguiera gymnorrhiza*	15.15	24.74	39.51	52.62	78.18
2	*Aegialitis rotundifolia*	24.47	27.76	49.02	56.13	67.85

Table 2
Percentage of cytotoxic activity of Bruguiera gymnorrhiza and AegialitisRotundifolia on MCF-7 Cell line

S. No	Extract	10 μg/ml	25 μg/ml	50 μg/ml	100 μg/ml	200 μg/ml
1	*Bruguiera gymnorrhiza*	0.41	27.56	40.71	50.74	54.70
2	*Aegialitis rotundifolia*	3.26	21.19	33.37	51.53	58.25

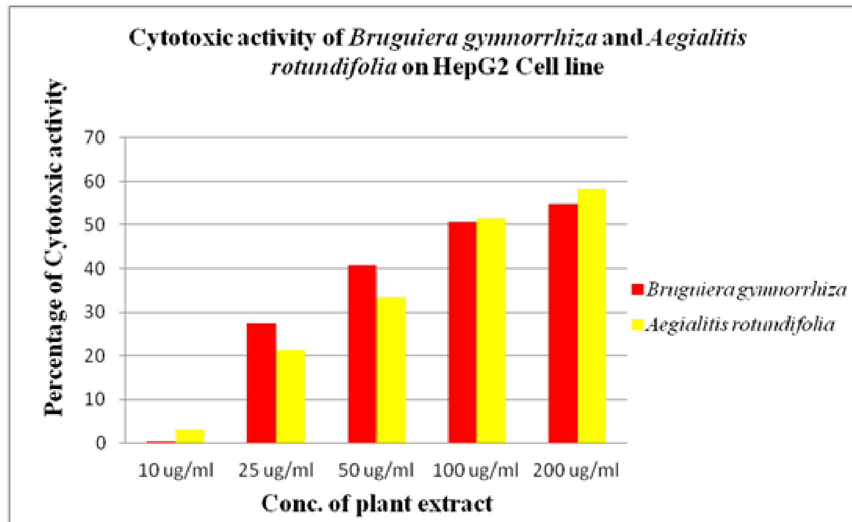


Figure 1
Cytotoxic activity of Bruguiera gymnorrhiza and Aegialitis rotundifoliaon HepG2 Cell line

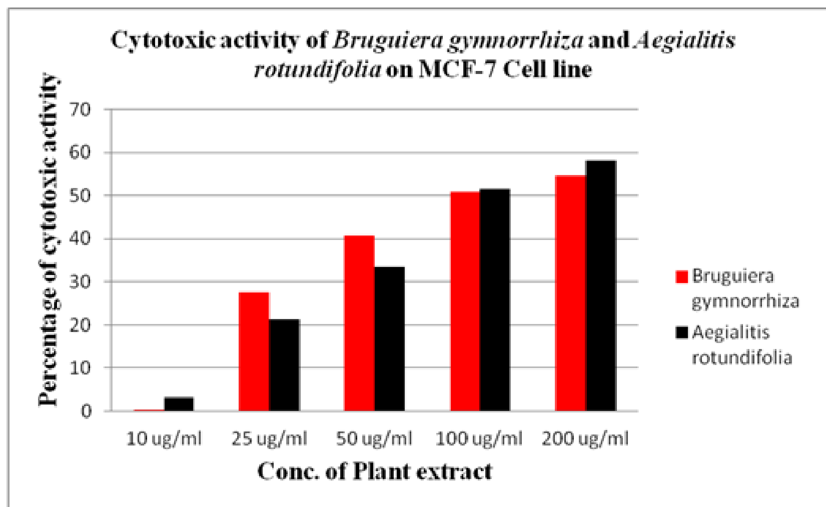


Figure 2
Cytotoxic activity of Bruguiera gymnorrhiza and Aegialitis rotundifoliaextractson MCF-7 Cell line

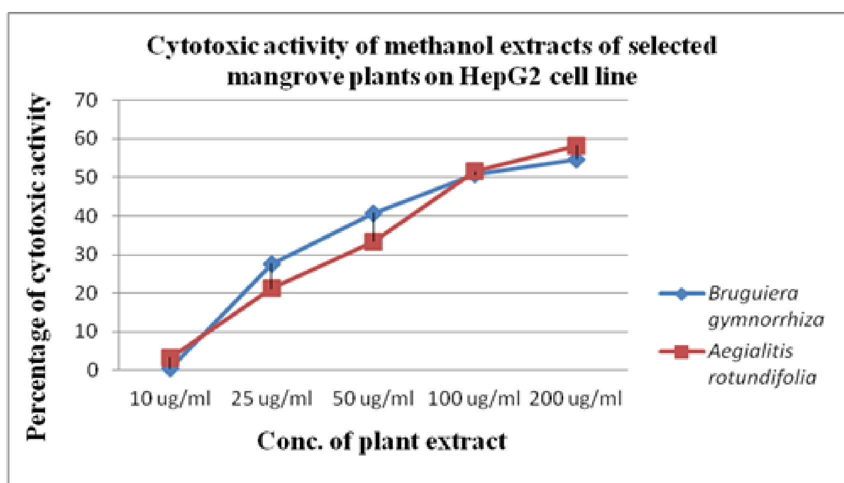


Figure 3
Graphical representation of cytotoxic activity of methanol extracts of selectedmangrove plants on HepG2 cell line

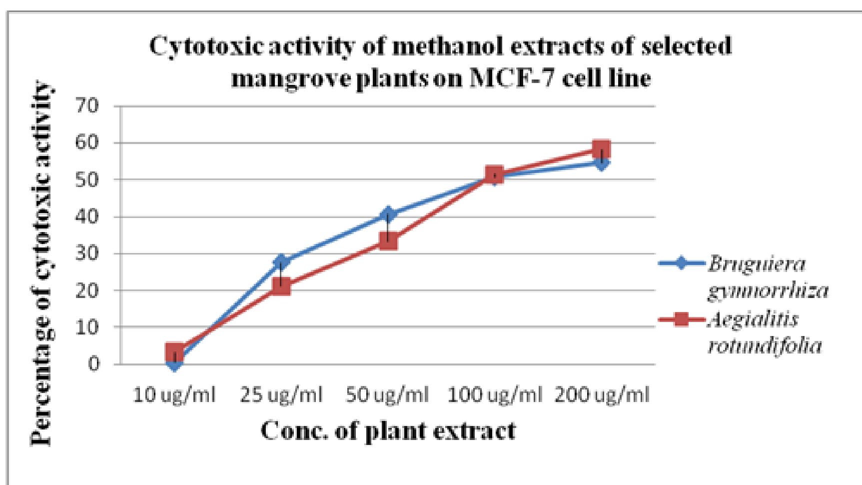
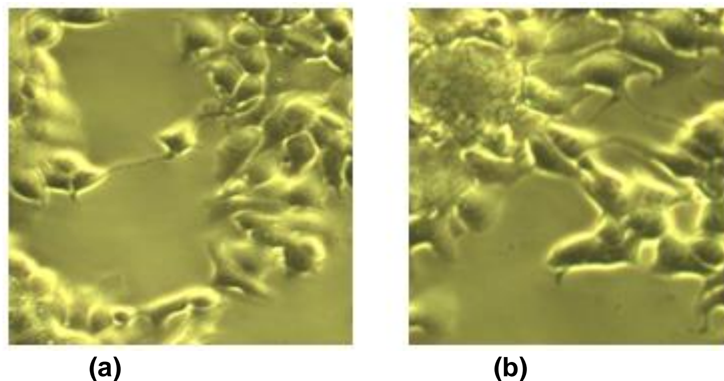
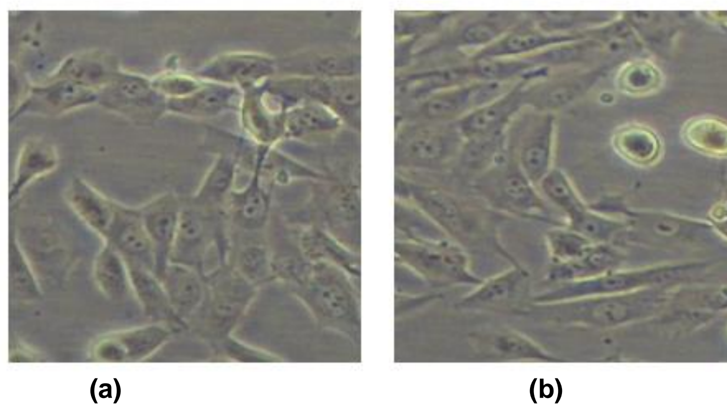


Figure 4
Graphical representation of cytotoxic activity of methanol extracts of selectedmangrove plants on MCF-7 Cell line



(a) (b)

Figure 5
Cytotoxic activity of selected plant extracts at 200 µg/ml concentrationon HepG2 Cell line
(a) Bruguiera gymnorrhiza (b) Aegialitis rotundifolia.

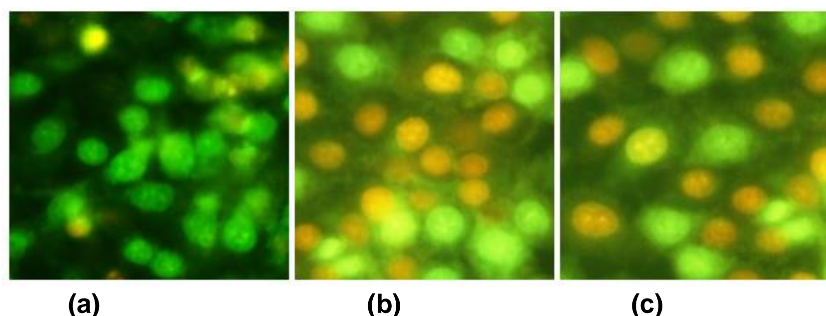


(a) (b)

Figure 6
Cytotoxic activity of selected plant extracts at 200 µg/ml concentration on MCF-7 Cell line
(a) Bruguiera gymnorrhiza and (b) Aegialitis rotundifolia

Ao/eb staining and dna fragmentation for apoptotic detection

Since both the plant extracts showed potent cytotoxic activity on hepG2 and MCF7 cell lines, the study was further extended to confirm the induction of apoptosis through AO/EB staining and DNA fragmentation analysis in hepG2 cell lines. To our confirmation, the AO/EB stain showed apoptosis induced morphological changes such as condensed cytoplasm and nucleus, aggregated chromatin and formation of membrane bound vesicles known as apoptotic bodies in the treated cells but these changes were not observed in untreated cells. AO/EB staining showed normal green nucleus in the control cells but the plant extract treated cells showed nuclear fragments and chromatin condensation with yellow and orange nucleus exhibiting early and late apoptosis respectively (fig-7). To verify whether the induced cell death is due to apoptosis the pattern of DNA bands on agarose gel were analyzed. Chromosomal DNA extracted from the cells treated with plant extracts for 24 hr exhibited inter nucleosomal DNA fragmentation which is a hallmark of cells undergoing apoptosis (Figure 8).



(a) (b) (c)

Figure 7
AO/EB staining-
(a)- Control, (b) *Bruguiera gymnorrhiza*, (c)*Aegialitis rotundifolia*

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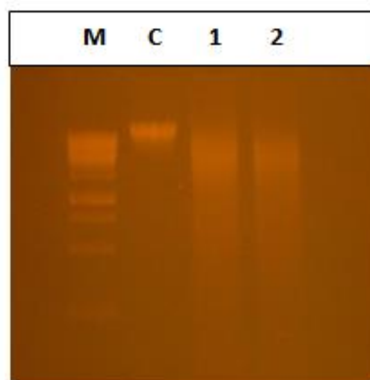

Figure 8
DNA Laddering Assay: M- GeneRuler 1 kb. DNA Ladder (Fermentas); C-control; 1-*Bruguiera gymnorrhiza*; 2- *Aegialitis rotundifolia*

DISCUSSION

Induction of apoptosis selectively in cancer cells is one of the prime aim of cancer therapy. Recently medicinal plants have become the source of alternative therapy for cancer treatment due to their safety and efficacy where they simultaneously influence different phages of disease through different mechanisms. Cytotoxicity induced cell death can be divided morphologically and biochemically in to apoptosis and necrosis. Apoptosis is the programmed cell death which is characterized by morphological changes such as membrane blebbing, cell shrinkage, chromatin condensation, and DNA degradation. It plays a vital role in regulating growth, development and in recent times it has been an important target for the development of effective anticancer drugs. The present study investigated the cytotoxic activity and subsequent induction of cell death by methanolic extract of indigenous medicinal plants *Bruguiera gymnorrhiza* and *Aegialitis rotundifolia* was demonstrated against two human cancer cell lines such as HepG2 and MCF7 by performing MTT assay, AO/EB staining and DNA fragmentation analysis. A methodical evaluation of Cytotoxicity effects revealed that the methanolic extracts of both *Bruguiera gymnorrhiza* and *Aegialitis rotundifolia* showed dose dependent cytotoxic activity against HepG2 and MCF7 cell lines. The IC50 values of methanolic extracts of *Bruguiera gymnorrhiza* and *Aegialitis rotundifolia* were found to be less than 100 µg/ml indicating potent cytotoxic activity and further potentials of these extracts for the isolation of biologically active Phytochemicals. Most anti-cancer drugs are designed to eliminate rapidly proliferating cancerous cells, and therefore, they typically show Cytotoxicity and induce apoptosis in cancer cells. Apoptosis is a highly organized cell death process characterized by loss of plasma membrane phospholipid asymmetry, enzymatic cleavage of the DNA in to oligo nucleosomal fragments, and segmentation of of the cells into membrane bound apoptotic bodies. The current study also investigated the induction of apoptosis in HepG2 cell lines upon treatment with methanolic extract of *Bruguiera gymnorrhiza* and *Aegialitis rotundifolia* Acridine orange – Ethidium bromide assay by fluorescent microscope revealed that the methanolic extracts of *Bruguiera gymnorrhiza* and *Aegialitis rotundifolia* induced apoptosis, but not necrosis, in HepG2 cells. DNA fragmentation is a hall mark of property of apoptosis and DNA fragmentation assay further corroborated the methanolic extracts *Bruguiera gymnorrhiza* and *Aegialitis rotundifolia* induced apoptosis in HepG2 cells. Anti cancer drugs with minimal side effects on normal cells are highly desirable for therapeutic purpose. Agents that are capable of inducing selective apoptosis of cancer cells, without causing much harm to normal cells, have received considerable interest in the development of novel cancer chemotherapeutic drugs The cancer activity of medicinal plants is mainly attributed to poly phenols including phenols, flavonoids, alkaloids, tannins etc., which also exhibit strong antioxidant activity.[26] The group of flavonoids consist more than 4000 varieties which makes 60% of total poly phenols found naturally in many plants.[27] Quercetin, Myricetin, Kaempherol are the members of Flavonoids which are found in many vegetables and fruits have been proven to be effective inhibitors of cancer cell growth.[28-30] The present study demonstrated the activity of methanolic extracts of *Bruguiera gymnorrhiza* and *Aegialitis rotundifolia* in inducing cytotoxicity and apoptosis in both hepG2 and MCF-7 cancer cell lines.

CONCLUSION

The preliminary report of present study clearly indicates that both the methanolic extracts of *Bruguiera gymnorrhiza* and *Aegialitis rotundifolia* effectively inhibit the proliferation of HepG2 and MCF-7 cell lines by the mechanism which involves the induction of apoptosis. According to these results, it is suggested that the *Bruguiera gymnorrhiza* and *Aegialitis rotundifolia* may be a considerable source for the development of anticancer drugs. Further, isolation of bioactive compounds from the extract will be a significant achievement in the cancer therapy and drug development.

CONFLICT OF INTEREST

Conflict of interest declared none.

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