

Characterization of Anticancer Principles of *Celosia argentea* (Amaranthaceae)

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ABSTRACT

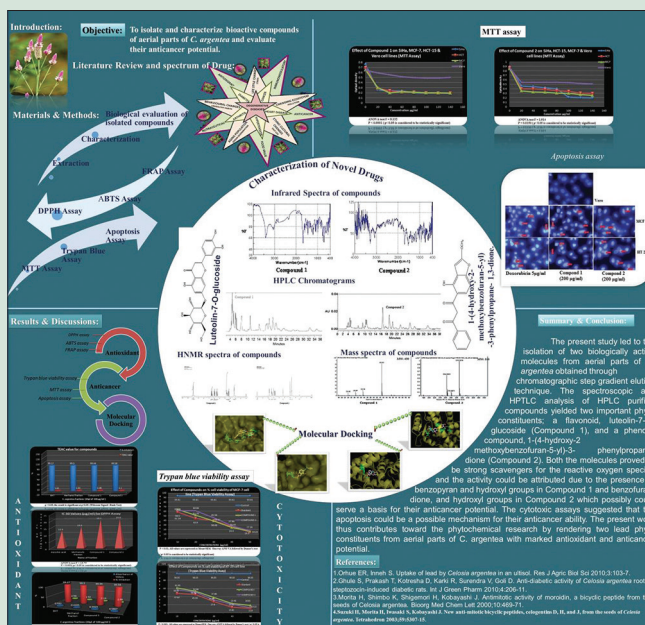
Background: An Indian origin, *Celosia argentea* is a weed growing during rainy season traditionally claimed for treating several ailments. Early researches on *C. argentea* were focused on the anti-cancer screening of seeds, with few reports on aerial parts. **Objective:** To isolate and characterize bioactive compounds of aerial parts of *C. argentea* and evaluate their anticancer potential. **Materials and Methods:** The methanolic aerial part extract was fractionated on column chromatography using chloroform: methanol mixture. The fractions; 80:20 and 95:5 were purified on MCI-HP20 HPLC column. Chromatographically pure compounds were pooled, concentrated and characterized spectroscopically. The compounds were further screened for anti-oxidant and cytotoxic potential. **Results:** Isolated compounds were confirmed as: (1) Luteolin-7-O-glucoside and (2) phenolic, 1-(4-hydroxy-2-methoxybenzofuran-5-yl)-3-phenylpropane-1,3-dione. Both exhibited significant antioxidant potential with IC₅₀ values of 20.80 and 21.30 µg/ml for 2,2-diphenyl-1-picrylhydrazyl assay (***P* < 0.001) and significant Trolox equivalent antioxidant capacity (TEAC) values for 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (**P* < 0.05) and ferric reducing antioxidant potential assay (*****P* < 0.0001). In 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide assay, Compound 1 and 2 showed potent cytotoxicity against SiHa, HCT, MCF-7 cancer cell lines at 20 µg/ml (*****P* < 0.0001) and 18 µg/ml (***P* < 0.01), respectively, without affecting the normal Vero cells. Both compounds enabled maximum reduction in cell viability at 50 µg/ml against HT-29 (***P* < 0.001) and MCF-7 cell lines (***P* < 0.01) in trypan blue viability assay. Apoptosis occurred at concentrations of 47.33 ± 0.8 µg/ml and 56.28 ± 1.2 µg/ml for Compound 1 and 35.15 ± 0.4 µg/ml and 28.05 ± 0.3 µg/ml for Compound 2 for HT-29 and MCF-7 respectively. **Conclusion:** A novel anticancer phenolic compound; (1-(4-hydroxy-2-methoxybenzofuran-5-yl)-3-phenylpropane-1,3-dione), isolated from aerial parts of *C. argentea* was a valuable finding of the research.

Key words: 2,2-diphenyl-1-picrylhydrazyl (DPPH), 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT), anticancer, *Celosia argentea*, anti-oxidant

SUMMARY

- The present study validated the potential of the plant *C. argentea* as an antioxidant, and anticancer remedy with two valuable isolations. Although one of them is a known compound: Luteolin 7-O glycoside, the other

isolated phenolic compound; (1-(4-hydroxy-2-methoxybenzofuran-5-yl)-3-phenylpropane-1,3-dione), is the first to be reported and thus can be considered as a valuable outcome of this research work



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INTRODUCTION

Celosia argentea Linn. [Figure 1] commonly named as semen celosiae, cock's comb, quail grass, the wool flower is a herbaceous annual plant widely grown in many regions of tropical Africa especially Nigeria, Benin and Congo. In India and other tropical regions of the world such as Sri Lanka, South Asia, and America, it grows as a post monsoon weed. It is highly consumed as a leafy vegetable because of its high nutritive value.^[1]

The whole plant of *C. argentea* is well known as a traditional medicine in India for the treatment of diarrhea, piles, bleeding nose, disinfectant, inflammation, hematological and gynaecologic disorders.^[2] The plant is enriched with primary and secondary metabolites such as carbohydrates,

lipids, amino acids, peptides, phenols, phenolic acids, flavonoids, terpenes, and alkaloids; the important being bicyclic peptides, celogenamide-A, celogentin-A-D, H, J, and K, moroidin, celosian,

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Figure 1: *Celosia argentea* plant

citrusin C, cristatainetc.^[3,4] Hayakawa and co-workers reported the anti-metastatic effect of *C. argentea* seed extract based on its immunomodulation properties including induction of cytokines such as interleukin (IL)-12, IL-2, and interferon-gamma that led to a Th1 dominant immune state activating macrophages to tumoricidal state causing basis for the inhibition of cancer metastasis.^[5]

The plant is also reported to contain flavonoids like 5-methoxy-6,7-methylene dioxy-2'-hydroxyisoflavone and its 2'-methoxy derivative: Tlatlanquayin. Flavonoids play an important role in a plant as a defense and signaling compounds in reproduction, pathogenesis and symbiosis, by inhibition of proliferation of peripheral blood mononuclear cells, two-way MLR and natural killer cell, as well as no production. Further inhibition of production of IL-2 and tumor necrosis factor- α production in human peripheral blood mononuclear cells, which further blocked the binding of DNA to various factors responsible for synthesis of IL-2 and IL-2R genes, which are necessary for T-cell activation and proliferation.^[6] Several classes of flavonoids were also reported for their antiproliferative activity against various human cancer cells.^[7]

Owing to the fact that the seeds of *C. argentea* have been screened effectively for various biological activities viz anti-oxidant, immuno modulatory, and anti-mitotic, and the aerial parts though reported with rich flavonoid content are not yet screened for their anticancer potential, this research work is principally aimed at isolation and characterization of the bioactive components of the aerial parts and screening them for the possible antioxidant and anticancer potential.

MATERIAL AND METHODS

Extraction

Coarse, dried powder of aerial parts of *C. argentea* was passed through 180 mesh and was subjected to successive solvent extraction with solvents in ascending order of polarity. Phytochemical evaluation detected methanolic fraction to be enriched in flavonoids. Hence, methanolic fraction was concentrated and loaded on a column of Sephadex using a step gradient of chloroform: methanol mixture (95:5, 90:10, 80:20,....100:0).^[8]

The two fractions 95:5 and 80:20 that gave distinct single bands with the selected solvent system were further purified on HPLC column of MCI HP20. Fractions were collected using an automatic fraction collector and monitored by HPLC. Chromatographically pure compounds were pooled, concentrated, and freeze-dried. The protocol followed was as below:

- Sample preparation: Each dried fraction was dissolved in HPLC grade methanol. Following centrifugation supernatant was injected
- Column: MCI HP20; 20 × 6 cm
- Mobile phase gradient: Methanol: water (35:65) followed by methanol:water:acetic acid (30:70:1)
- Flow rate: 7.48 ml/min
- Injection volume: 2 mL
- Ultra violet (UV) detection: 280 nm
- Fraction collection: 0.5 min/fraction with 6 ml/tube fraction
- Yield: Compound 1 - 17 mg; Compound 2 - 21 mg.

Characterization

The isolated compounds were further subjected to various spectroscopic studies like:

UV (V-530; Perkin Elmer Lambda-35), fourier transform infrared spectroscopy (FT-IR) (FTIR 460 Plus; Jasco Corporation), proton nuclear magnetic resonance (¹H-NMR) (FTNMR - R-20B; Perkin Elmer, Mumbai), and mass spectra (MS) (Perkin Elmer Auto system; Perkin Elmer, Mumbai). Confirmation of identity was performed by comparing with the marker compounds on HPTLC (Linomat 5-140435; CAMAG).^[9,10]

Biological evaluation of isolated compounds

In-vitro antioxidant activity: 2,2-diphenyl-1-picrylhydrazyl assay^[11]

A solution of 3.3 mg 2,2-diphenyl-1-picrylhydrazyl (DPPH) in 100 ml methanol was prepared and 1.0 ml of this solution was mixed with 1.0 ml of extract in methanol containing 0.02–0.1 mg of the extract. The reaction mixture was vortexed thoroughly and left in the dark at 25°C for 30 min. The anti-oxidant on interaction with DPPH, transfers electron or hydrogen atom to DPPH, thus neutralizing the free radical character and converting it to 1,1-diphenyl-2-picryl-hydrazine. The absorbance of the mixture was measured at 517 nm. BHT was used as reference. The ability to scavenge DPPH radical was calculated by the following equation:

$$\% \text{Radical scavenging activity} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100$$

Where $\text{Abs}_{\text{control}}$ is the absorbance of DPPH radical + methanol;

$\text{Abs}_{\text{sample}}$ is the absorbance of DPPH radical + sample extract/standard.

2, 2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) scavenging activity

The stock solutions included 7 mM 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) solution and 2.4 mM potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 ml ABTS+ solution with 60 ml methanol to obtain an absorbance of 0.706 ± 0.001 units at 734 nm using the spectrophotometer. Fresh ABTS solution was prepared for each assay. Plant extracts (1 ml) were allowed to react with 1 ml of the ABTS solution, and the absorbance was taken at 734 nm after 7 min using the spectrophotometer. The ABTS+ scavenging capacity of the extract was compared with that of BHT and percentage inhibition was calculated using the same equation as that for DPPH method. Results are expressed by comparison with standard amounts of the synthetic anti-oxidant Trolox to give rise to the TEAC which is equal to the millimolar concentration of a Trolox solution having the antioxidant capacity equivalent to a 1.0 mM solution of the substance under investigation. The TEAC value reflects the relative ability of

hydrogen or electron-donating anti-oxidants to scavenge the ABTS radical cation compared with that of Trolox.^[11]

Ferric reducing antioxidant potential assay

The stock solutions included 300 mM acetate buffer (3.1 g sodium acetate and 16 ml acetic acid), pH 3.6, 10 mM 2, 4, 6-tripyridyl-S-triazine (TPTZ) solution in 40 mM HCl, and 20 mM FeCl₃ solution. The fresh working solution was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ, and 2.5 ml FeCl₃. The temperature of the solution was raised to 37°C before using. Plant extracts (150 µl) were allowed to react with 2850 µl of the ferric reducing antioxidant potential (FRAP) solution for 30 min in the dark condition. Readings of the colored product (ferrous tripyridyltriazine complex) were taken at 593 nm. The standard curve was linear between 200 and 1000 µM of Trolox. The FRAP scavenging capacity of the extract was compared with that of BHT and the percentage inhibition was calculated using the same equation as that for DPPH method.^[12]

Cytotoxicity evaluation

3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide assay

The 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT) -assay is based on the conversion of the tetrazolium salt MTT to blue formazan which depicts cell viability. Color development in terms of optical density was measured spectrophotometrically at 540 nm and documented by ELISA reader at IRSHA (Bharati Vidyapeeth University, Pune). Optical density values were converted to % cell viability to get the respective IC₅₀ values for SiHa, HCT-15, MCF-7 and Normal monkey kidney Vero cells.^[13]

Trypan blue dye exclusion assay

The assay is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue, whereas dead cells do not. Trypan blue is a blue acid dye that has two azochromophores group. The percentage of viable and nonviable cells were determined, using trypan blue exclusivity stain. The cell lines HT-29 and MCF-7 were grown at 37°C at humidified 5% CO₂ in the RPMI-1640 medium. The RPMI-1640 medium was filtered using 0.45 µm membrane filter. Various concentrations that are, 10, 20, 30, 40, and 50 µg/ml were prepared by dissolving known the weight of Compound 1 and 2 in dimethyl sulfoxide. Similar solutions containing the same concentrations of cyclophosphamide were also prepared and served as standard solutions. Control cells were incubated in a culture medium that is, RPMI-1640 medium only. Cancer cells were seeded at a density of 2 × 10⁴ cells/well and they were treated with different concentrations of Compound 1 and 2 for 48 h at 37°C in the presence of 5% CO₂. After 48 h, 20 µl of the medium and equal volume of 0.4% trypan blue were mixed, incubated for 5 min at 37°C in the presence of 5% CO₂. Viable and dead cells were examined and counted under a light microscope at ×100 by Neubauer hemacytometer. The percent cell viability determined by the following formula:

$$\% \text{ Cell survival} = \frac{(At - Ab)}{(Ac - Ab)} \times 100$$

At = Absorbance of Test, Ab = Absorbance of Blank (media), Ac = Absorbance of control (cells).^[14]

In-vitro apoptosis study by 4',6-diamidino-2-phenylindole staining

To characterize cell-specific apoptotic process in HT29 and MCF-7 cells, analysis of chromatin condensation and nuclear

fragmentation was performed by 4',6-diamidino-2-phenylindole (DAPI) staining using fluorescence microscopy. After treatment of 70–80% confluent HT29 and MCF-7 cells with varying concentration of samples Compound 1 and 2 for 24 h, cells were quickly washed with ice-cold PBS and fixed in ice-chilled acetone: methanol (1:1) mixture for 10 min at 4°C in the dark. The cells were washed twice with ice-cold PBS and then incubated for 30 min with the DNA-specific fluorochrome, DAPI. The excess DAPI was removed with ice-cold PBS wash, and the cells were observed and photographed using fluorescence microscope.^[15]

RESULTS AND DISCUSSION

Isolation

Figures 2 and 3 represents the isolation of Compound 1 and 2 respectively by HPLC using MCI HP 20 column.

Characterization

Figures 4-8 represents the characterization of isolated compounds by UV, IR, NMR MS spectroscopy and HPTLC.

Compound 1

Compound 1 was obtained as a yellow amorphous solid with a melting point in the range of 266-268°C. Its UV spectrum in methanol showed peaks at λ_{max} 344 and 263 nm [Figure 4].

FT-IR spectra of the compound showed bands at ν_{max} 3389 cm⁻¹ (strong and broad) suggesting –OH group; 2991 cm⁻¹, strong (aliphatic –C-H stretch); 3150 cm⁻¹ strong (aromatic –C-H stretch); 1715 cm⁻¹, very strong (aromatic –C=O); 1624 cm⁻¹, strong (C=C stretch) and 1370 cm⁻¹ strong suggests –C-O stretch of pyran ring; 1523 cm⁻¹, strong (–C=C stretch of aromatic ring); 1446 and 722 cm⁻¹ (medium) suggest aliphatic and aromatic C-H bend, respectively [Figure 5].

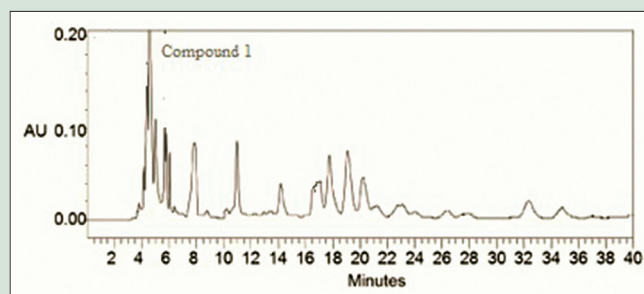


Figure 2: Chromatogram showing Compound 1 on MCI HP 20 HPLC column

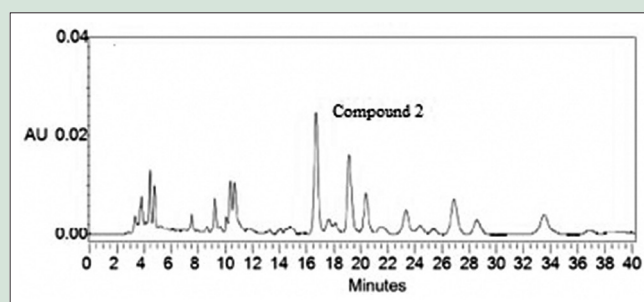
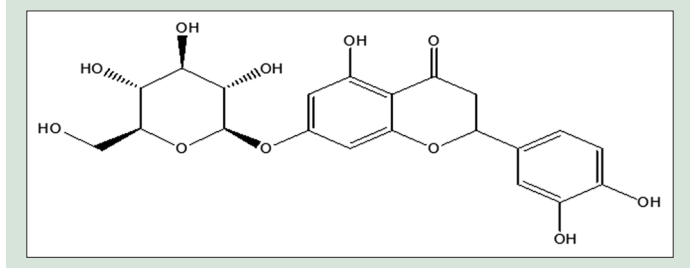


Figure 3: Chromatogram showing Compound 2 on MCI HP 20 HPLC column

$^1\text{H-NMR}$ data, δ 2.2183 (s)-OH; δ 3.234-3.3851-(d)- CH_2 ; δ 3.412-3.764; 6 (m)-6 H; δ 5.422 1 (m)-CH; δ 5.628; 1 (d)-CH; δ 5.934-6.032-(d)-ArH; δ 6.325-6.616-3 (m)-H; δ 10.014-4 (s)-OH shows the presence of aromatic and benzopyran ring that suggests possibility of a flavonoid structure [Figure 6].

Gas chromatography-mass spectroscopy (GC-MS) exhibited the $[\text{M} + \text{H}]^+$ at m/z 450.09 dissociated primarily by the loss of glucose residue (-162) resulting in the peak at 287.9871 [Figure 7].

The results confirmed that the isolated Compound 1 is luteolin-7-O-glucoside ($\text{C}_{21}\text{H}_{24}\text{O}_{11}$).



Compound 1: Luteolin-7-O-glucoside.

Compound 2

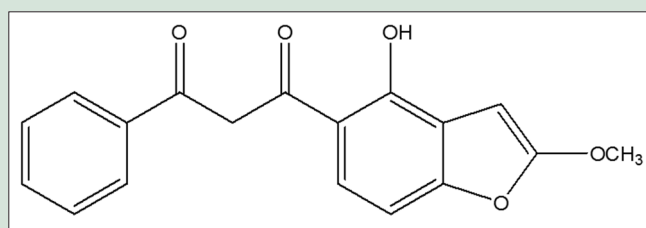
Compound 2 was obtained as yellowish brown amorphous solid having a melting point in the range of 187–189°C. Its UV spectrum in methanol showed a peak at λ_{max} 427 nm [Figure 4].

As indicated in Figure 5; FT-IR spectra of the compound showed bands at 3504 cm^{-1} (strong and broad) suggesting phenolic -OH group;

2889 cm^{-1} , strong (aliphatic -C-H stretch); 3150 cm^{-1} strong (aromatic -C-H stretch); 1640 cm^{-1} , very strong suggesting aliphatic -C=O; 1610 cm^{-1} , weak (aromatic -C=C stretch); peaks between 1050 cm^{-1} and 1250 cm^{-1} (strong) suggests -C-O stretch of furan ring; -C-O stretch of phenolic ether is predicted between 1080 and 1120; bands at 1446 and 722 cm^{-1} (medium) suggest aliphatic and aromatic C-H bend, respectively.

The results of $^1\text{H-NMR}$ data summarized as; δ 3.687-3 (s)-O- CH_3 ; δ 4.042; 2 (s)- CH_2 ; δ 5.280; 1 (s)-H; δ 6.810-6.972-1 (d)-2H; δ 6.978-7.145-1 (d)-2H; δ 7.213-7.858-5 (m)-6H; δ 10.102; 1 (s)-OH, showed resemblance to curcumin with the addition of benzofuran ring indicated by δ 6.810 and δ 6.972, that suggests possibility of a novel phenolic compound with benzofuran ring [Figure 6]. GC-MS exhibited the $[\text{M} + \text{H}]^+$ at m/z 310.3216 [Figure 7].

The results confirmed that the isolated compound is a novel phenolic compound; 1-(4-hydroxy-2-methoxybenzofuran-5-yl)-3-phenylpropane-1, 3-dione ($\text{C}_{18}\text{H}_{16}\text{O}_5$).



Compound 2: 1-(4-hydroxy-2-methoxybenzofuran-5-yl)-3-phenylpropane-1,3-dione.

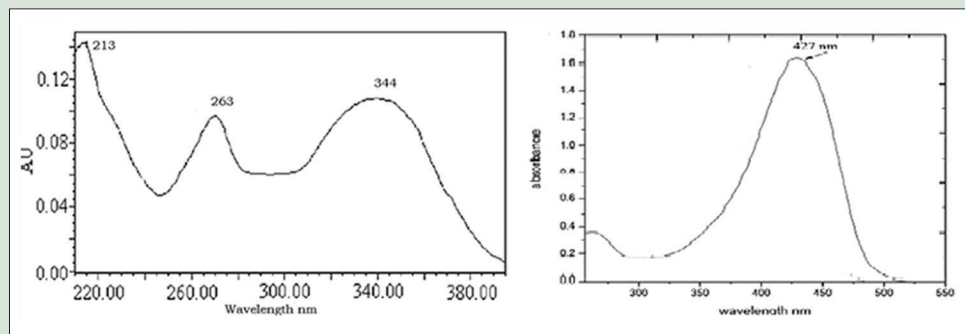


Figure 4: Ultra violet Spectrum of compounds

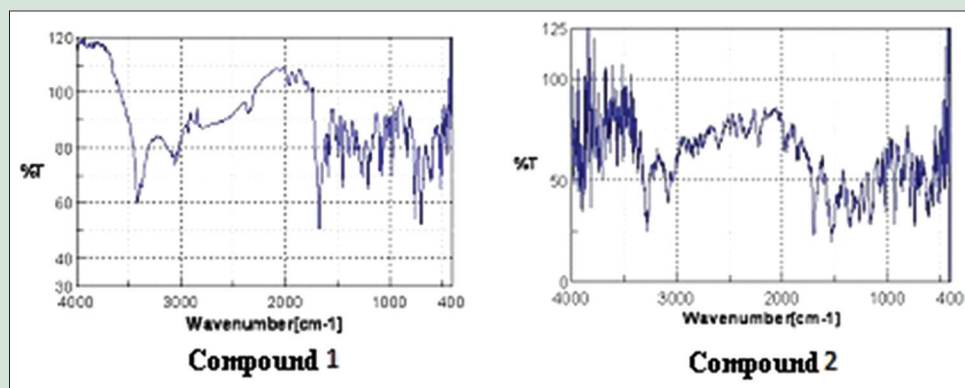


Figure 5: Infrared Spectra of compounds

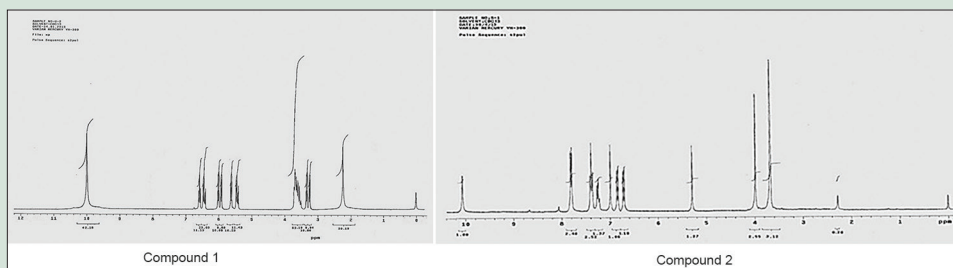


Figure 6: Proton nuclear magnetic resonance spectra of compounds

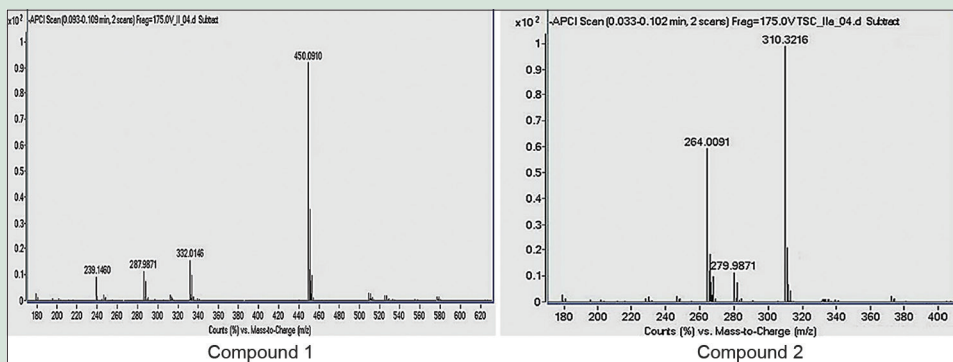


Figure 7: Mass spectra of compounds

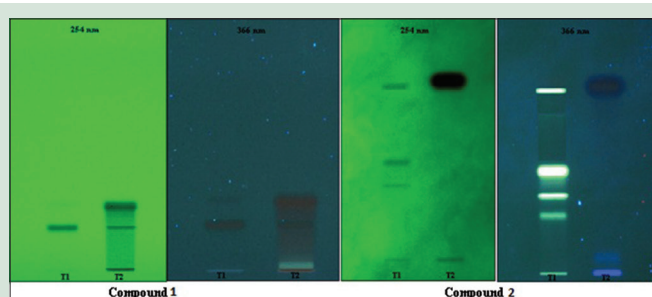


Figure 8: HPTLC profile of compounds along with markers

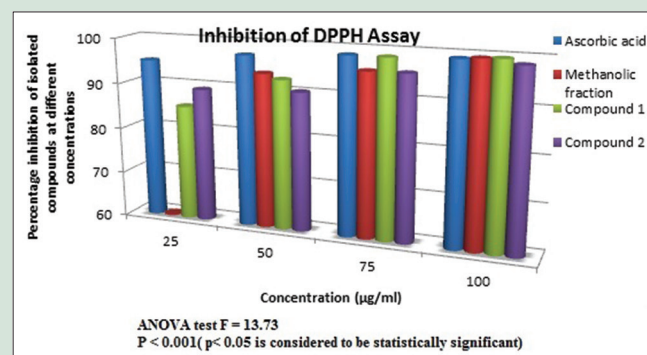


Figure 9: Percentage inhibition of isolated Compound 1 and 2 for 2,2-diphenyl-1-picrylhydrazyl assay

Antioxidant activity

2,2-diphenyl-1-picrylhydrazyl assay

Percentage inhibition of DPPH radical by Compound 1 and 2 was found to be 99.65 and 98.65, respectively [Figure 9]. The free radical scavenging effect of both the compounds was almost equivalent to that of ascorbic acid at 100 µg/ml with IC_{50} Values (µg/ml) of 12.50, 19.10, and 19.80 for ascorbic acid, Compound 1 and 2, respectively [Figure 10]; thus indicating strong antioxidant activity of both the compounds though little lesser than ascorbic acid.

2, 2'-Azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) assay

The TEAC value reflects the relative ability of hydrogen or electron-donating antioxidants to scavenge the ABTS radical cation compared with that of Trolox [Figure 11]. As shown in Figure 12; both Compound 1 and 2 showed maximum percentage inhibition of ABTS radical (99.44; 99.58), with a TEAC value of 2.08 and 2.10, respectively, comparable with a standard antioxidant BHT (2.1). Thus, it can be clearly stated that both the compounds exhibit significant antioxidant activity.

Ferric reducing antioxidant potential assay

The ferric reducing antioxidant power of isolated compounds measured as an intensity of blue colour produced and expressed as mM of Trolox equivalent is expressed in Figure 13, where both compounds showed maximum free radical scavenging with percentage inhibition of 99.44 and 99.58 with TEAC value of 2.1 significantly comparable with standard antioxidant; BHT with percentage inhibition of 99.17 and TEAC value; 2.1.

Cytotoxicity evaluation

3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide assay

The potential anticancer effect of the isolated compounds from *C. argentea* aerial parts was investigated on the viability of SiHa, HCT, MCF-7, and Vero cell lines by MTT assay. It could be seen from

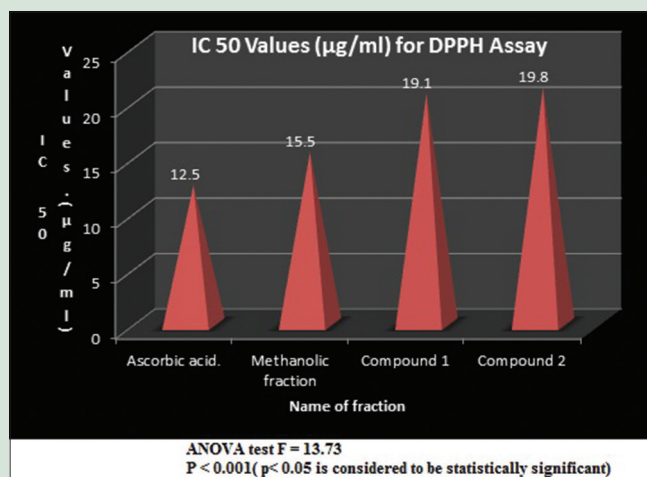


Figure 10: IC₅₀ values of isolated Compounds 1 and 2 for 2,2-Diphenyl-1-picrylhydrazyl Assay

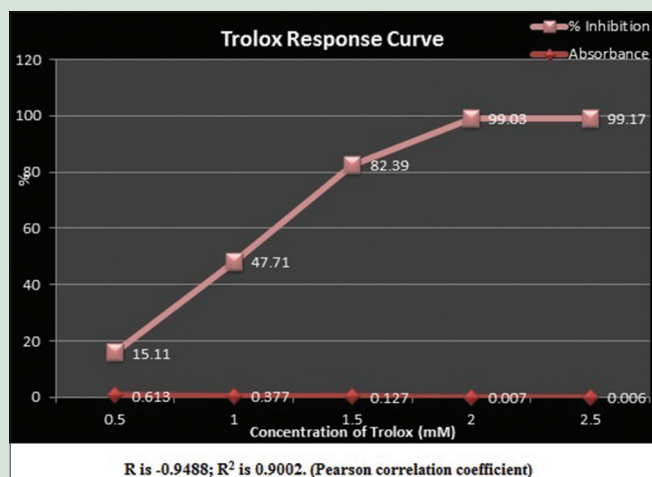


Figure 11: Concentration response curve of Trolox for 2, 2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) at 734 nm

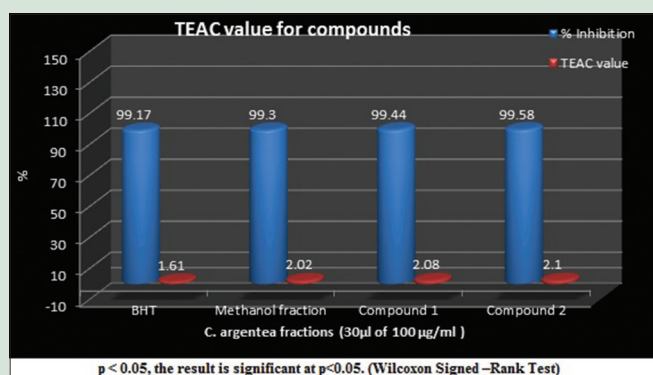


Figure 12: Percentage inhibition and TEAC values of isolated compounds for 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) assay

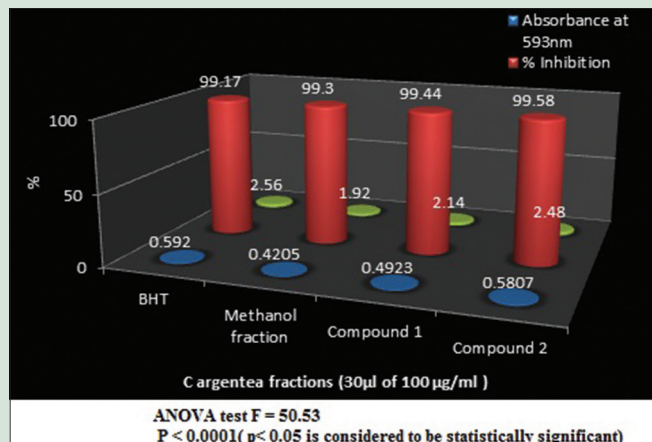


Figure 13: Absorbance, percentage inhibition and TEAC values for isolated compounds by ferric reducing antioxidant potential assay

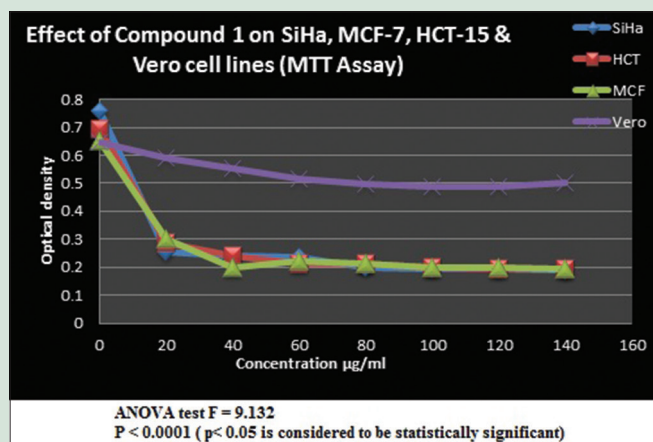


Figure 14: 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide assay of isolated Compound 1

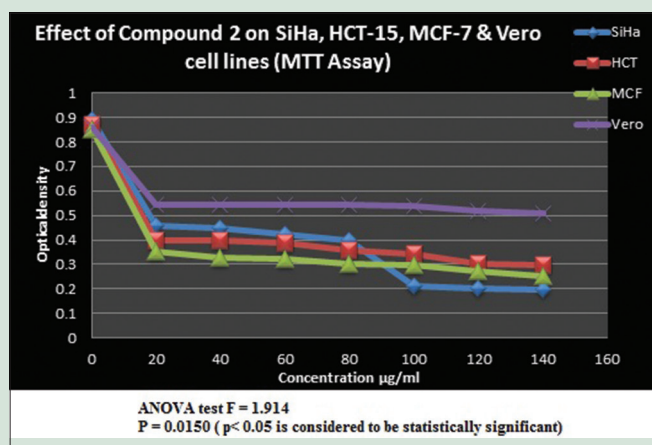


Figure 15: 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide assay of isolated Compound 2

Figure 14 that compound 1 showed strong cytotoxicity against all the three cell lines at concentration of 20 µg/ml similarly as seen in Figure 15; compound 2 showed potent cytotoxic activity against all the three cell lines at concentration of 18 µg/ml. Both the compounds

were less toxic on normal cells than on the investigated cancer cell lines.

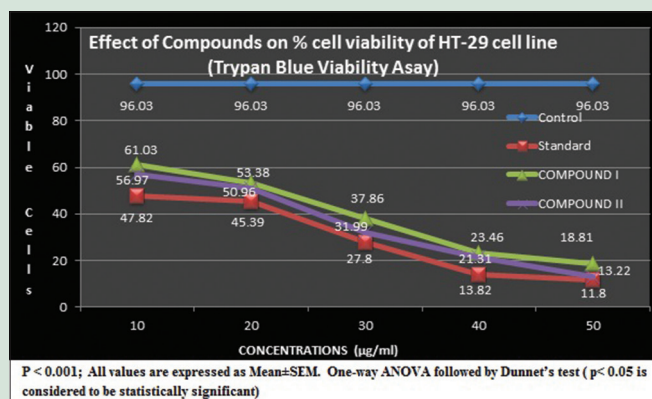


Figure 16: Effect of Compound 1 and 2 on cell viability of HT-29

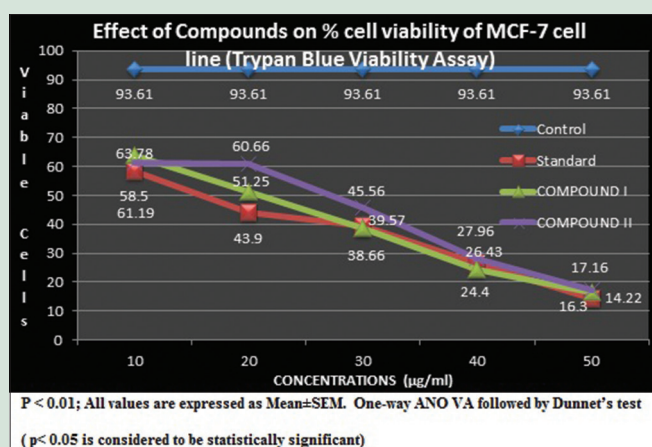


Figure 17: Effect of Compound 1 and 2 on cell viability of MCF-7

Trypan blue viability assay

The assay is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue, whereas dead cells do not. The intensity of the color is directly proportional to the number of nonviable cells. Treatment of Compound 1 and 2 against HT-29 and MCF-7 in a concentration range of 10, 20, 30, 40, and 50 µg/ml showed significant decrease in percent cell viability with increased concentrations: maximum activity being observed at concentration of 50 µg/ml comparable with standard drug cyclophosphamide exhibiting strong cell cytotoxicity [Figures 16 and 17].

Apoptosis assay

For the preliminary characterization of the cytotoxicity induced by the test compounds, Compound 1 and 2; in the cancer cells, the changes in cell morphology were examined under a phase contrast microscope. In both the cancer cell lines, cell rounding up, cell shrinkage, membrane blebbing, and loss of cell adhesion were induced by Compound I and II [Figure 18]. These cellular changes are the characteristics of the apoptotic induction of cell death.

To further characterize the cell death induced by the compounds, analysis of chromatin condensation and nuclear fragmentation was performed by DAPI staining using fluorescence microscopy. The results revealed that the test compounds induced the nuclear condensation and cell fragmentation into apoptotic bodies, the distinct characteristics of the apoptotic process, in all of the cancer cell types. Thus, it can be claimed that the mode of cell death triggered by test compounds might be the process of apoptosis, which is recognized as a novel strategy for identification of anti-cancer drugs. In contrast, no cell morphological abnormality and DNA fragmentation of normal cells treated with test compounds were observed suggesting a nontoxic effect of these compounds to normal cells.

CONCLUSION

The present study led to the isolation of two biologically active molecules from aerial parts of *C. argentea* obtained through chromatographic

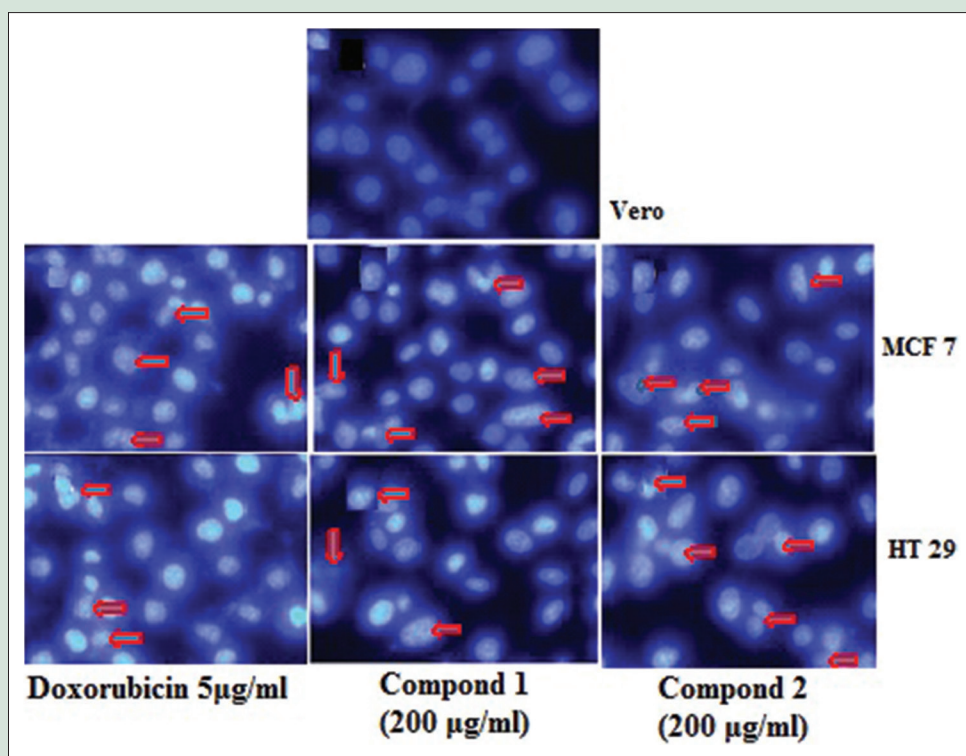


Figure 18: Cell morphology of apoptotic cells after test drugs treatment

step gradient elution technique. The spectroscopic and HPTLC analysis of HPLC purified compounds yielded two important phyto constituents; a flavonoid, luteolin-7-O-glucoside (Compound 1), and a phenolic compound, 1-(4-hydroxy-2-methoxybenzofuran-5-yl)-3-phenylpropane-1,3-dione (Compound 2).^[15] Both the molecules proved to be strong scavengers for the reactive oxygen species and the activity could be attributed due to the presence of benzopyran and hydroxyl groups in Compound 1 and benzofuran, dione, and hydroxyl groups in Compound 2 which possibly could serve a basis for their anticancer potential. The cytotoxic assays suggested that the apoptosis could be a possible mechanism for their anticancer ability. The present work thus contributes toward the phytochemical research by rendering two lead phyto constituents from aerial parts of *C. argentea* with marked anti-oxidant and anticancer potential.

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Conflicts of interest

There are no conflicts of interest.

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