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Suman Joshi DSD
Department of Biotechnology, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur, Andhra Preadesh-522510, India

Chander Amgoth
School of Engineering Sciences and Technology, University of Hyderabad, Hyderabad, Telangana-500046, India

Surya Narayana S
Department of Biotechnology, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur, Andhra Preadesh-522510, India

Padmavathi CH
Department of Biotechnology, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur, Andhra Preadesh-522510, India

Madhavi J
Department of Microbiology, Acharya Nagarjuna University, Nagarjuna Nagar, Gunturn Andhra Pradesh-522510, India

Krishna Satya A
Department of Biotechnology, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur, Andhra Preadesh-522510, India

Correspondence:
Krishna Satya A
Department of Biotechnology, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur, Andhra Preadesh-522510, India
Email: akrishnasatya78[at]gmail.com

Antioxidant and anticancer activities of an Aporphine alkaloid isolated from *Alphonsea sclerocarpa*

Suman Joshi DSD, Chander Amgoth, Surya Narayana S, Padmavathi CH, Madhavi J, Krishna Satya A*

ABSTRACT

Herein, we report on the anticancer and antioxidant activity of an Aporphine alkaloid isolated from medicinal plant *Alphonsea sclerocarpa*. Conventional column chromatography and preparative HPLC methods were used to isolate and purify the alkaloid. Based on NMR, FTIR and MS/MS spectroscopic techniques the compound was identified as Crebanine. This is the first report on the presence of Crebanine from the leaves of *Alphonsea sclerocarpa*. Antioxidant activity was evaluated using DPPH and FRAP assay. The antioxidant activity was dose-dependent and activity increased with the increase in the concentration. The cytotoxicity assay was carried out using MTT assay. The results showed remarkable cytotoxic activity against K562 (CML-chronic myeloid Leukaemia blood cancer) cells with a % inhibition of 37.545 at 500µm concentration after an incubation of 24h.

Keywords: *Alphonsea sclerocarpa*, antioxidant activity, anticancer activity, MTT assay, DPPH assay, FRAP assay, Crebanine.

INTRODUCTION

Cancer, the second leading cause of mortality worldwide with more than 277 different types, a majority of them was named based on the type of cell or organ in which they invade [1]. Abnormal proliferation of blood cells in the bone marrow and blood-forming organs lead to a malignant condition commonly called as leukaemia. These abnormally proliferated cells are immature white blood cells. The beginning of leukemia may be sudden (acute) or slow and gradual (chronic). Chronic Myelogenous Leukaemia (CML) is a common type of leukemia in the Asian countries. It affects more males than females [2]. The characteristic features of CML are that CML is hemato proliferative neoplasm causing an uncontrolled growth of immature cells that make a certain type of white blood cell called myeloid cells [3]. The diseased cells build up in the bone marrow and blood. The annual incidence of CML in India was originally reported to be 0.8 to 2.2 per 100,000 populations [4]. The exposure to radiations can increase the risk of CML. As plants are well known for radioprotective efficacy [5] and well known for having considerable compounds that can combat cancer, the plant *Alphonsea sclerocarpa* was selected to screen for the presence of phytochemicals capable of combating cancer cells. The anticancer activity was tested against the K562 (Chronic Myeloid Leukaemia) cells.

Antioxidants play a pivotal role in the maintenance of good health by providing protection against oxidative stress induced by free radicals by scavenging them. As free radicals are responsible for the aging, inflammation, mutation, carcinogenesis, ischemia-reperfusion injury, atherosclerosis, and neurodegenerative disorders *etc.* [6], the screening for the presence of antioxidant property in plants occupy a predominant position in pharmacognosy. The potential antioxidant activity can be evaluated using DPPH and FRAP methods and Ascorbic acid as a standard.

Alphonsea sclerocarpa belongs to Annonaceae family and is endemic to South India and Sri Lanka well known for its potential medicinal properties. The medicinal property of *Alphonsea sclerocarpa* can be attributed to the presence of various phytochemicals especially alkaloids. The previous studies have reported on antimicrobial and antioxidant activities of *Alphonsea sclerocarpa* using crude extracts of its leaves [7]. An antioxidant can be defined as “any substance that delays or inhibits oxidative damage to a target molecule” [8].

The present study is aimed at identifying the structurally elucidated phytochemicals which are having the potential anticancer and antioxidant activities.

MATERIALS AND METHODS

Collection of plant material

The plant material was collected from Seshachalam forest region of Andhra Pradesh, identified by taxonomist and a voucher specimen with No. 871 was deposited at Sri Venkateswara University. The collected plant material was washed thoroughly to remove dust and shade dried. The well dried plant leaves were grinded and stored in dark, dry place until further use.

Phytochemical extraction

The 1000gms of dried plant leaf powder was taken in 1 liter capacity Soxhlet extractor, extracted in successive extraction method with different non polar to polar solvents such as n-Hexane, Chloroform, Ethyl acetate and Methanol. The obtained crude was rotary evaporated, dried at room temperature, weight was measured and % yield was calculated as per the formula [9].

$$\%yield = \frac{\text{Weight of the extract (gms)X100}}{\text{Weight of the sample used for extraction (gms)}}$$

Purification of Phytochemicals

The crude extracts were dissolved in respective solvents, checked with TLC and subjected to conventional column chromatography. For this purpose Silica gel of 100-200 mesh size was taken and loaded into the column. To this, the crude extract was added, run with different solvents such as n-Hexane, Chloroform, Ethyl acetate and Methanol. The fractions were collected, tested with TLC. The MeOH fraction was subjected to further purification using preparative HPLC. The HPLC system Shimadzu (Kyoto, Japan) was equipped with dual pump LC-20AD binary system. Photodiode array (PDA) detector SPD-M20A was used for detection and Merck C_{18} column was used as stationary phase. The separation of phytochemicals was achieved with a two pump linear gradient program for pump A [Water containing 0.1 % formic acid (HCOOH)] and pump B [Acetonitrile (ACN)]. The purified fraction was subjected to structural and functional analysis.

Structural elucidation

The purified compound was subjected to various spectroscopy methods such as FTIR, ^{1}H-NMR, ^{13}C-NMR and MS/MS for identifying the structure and molecular weight of the compound.

NMR Spectroscopy

The ^{1}H-NMR and ^{13}C-NMR were carried out using Bruker 400MHz advance NMR spectroscopy. For this purpose, the purified compound was dissolved in DMSO and subjected to instrumental analysis.

FTIR Spectroscopy

The FTIR was carried out using Bruker FTIR spectroscopy. The KBr pelleting method was used to analyze the compound. The FTIR spectra were measured from 4000 to 500 cm^{-1}.

Antioxidant activity

The potential antioxidant activity of compound was evaluated by using the free radical scavenging assay by 2, 2-Di Phenyl-1-Picryl Hydrazyl (DPPH) method and Ion reducing power method using Ferric Reducing Antioxidant Power (FRAP) assay.

DPPH assay

For estimating the antioxidant activity using DPPH, different concentrations of the compound such as 12.5, 25, 50, 75 and 100 µg/mL were dissolved in DMSO followed by addition of 4mL of the 0.004% (w/v) DPPH dissolved in methanol. The reaction mixture was kept for incubation in dark for 30 minutes. Ascorbic acid was used as a standard. The absorbance was measured at 517nm using Thermo scientific UV-Visible spectrophotometer. Ascorbic acid was used as the standard [9]. The DPPH scavenging activity (%) was calculated as follows:

$$\text{DPPH scavenging activity \%} = \frac{(A0-As)}{A0} X100$$

Where, (A0) is the absorbance of the control, (As) is the absorbance of the plant sample

FRAP assay

Each concentration of 1mL was prepared by dissolving12.5µg/mL, 25µg/mL, 50µg/mL, 75µg/mL and 100µg/mL concentrated sample by dissolving in DMSO. From this, 200µL of sample was mixed with 3.8mL of FRAP reagent and incubated in the dark at room temperature for 30 minutes. After incubation the optical density was measured at 593nm, reagent alone was used as a blank [11]. All the experiments were conducted in triplicates and mean values were taken.

Cell culture and cytotoxicity studies

K562 (Chronic Myeloid Leukaemia) cells were grown in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 IU/mL penicillin, 100mg/mL streptomycin and 2mM L-glutamine. Cells were grown in a 5% CO_2 incubator at 37°C and cell viability was determined by the trypan blue dye exclusion method. Cells were sub-cultured twice a week, seeding at a density of about 2×10^{3} cells/mL.

MTT Assay

For the determination of cell viability, cells were seeded into 24-well culture plates at a density of approximately $5x10^{3}$ cells per well Cells were allowed to attach for 24h in a humidified 5% CO_2 incubator at 37°C. Test compound was solubilized in DMSO before further dilution with growth medium. The final concentration of DMSO in the wells never exceeded 0.25%. Cisplatin (positive control), test compound at concentrations ranging between 0.1 and 1000mM, were added to test wells whereas no compound was added to the control cells. Cells were exposed to the test compounds for 48h. Immediately after the 48h incubation period, cell numbers were determined using

the MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay as previously described [12]. Briefly, cells were incubated with 200mL MTT (Sigma) (0.5mg/mL in growth medium) for 4h at 37°C. The formazan product was then dissolved in DMSO, and plates were agitated on a shaker for 5min, before the absorbance was read at 540nm on a multi well scanning spectrophotometer. The values obtained were used to determine the percentage inhibition of cell growth caused by the compounds [13].

RESULTS AND DISCUSSION

% Yield Figure-1 depicts the pie chart of % yield. The Hexane extract showed 2% of yield, Chloroform extract showed 3%, Ethyl acetate showed 4%, Methanol extract showed 6% whereas Aqueous extract showed 8% of yield. The Debris accounted for 77%.

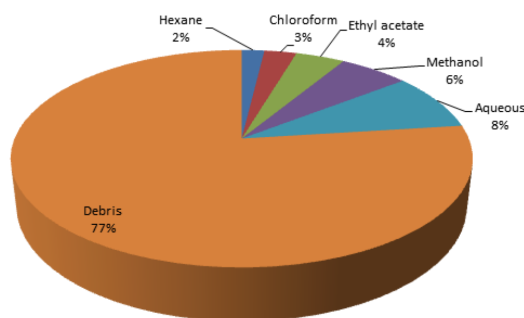
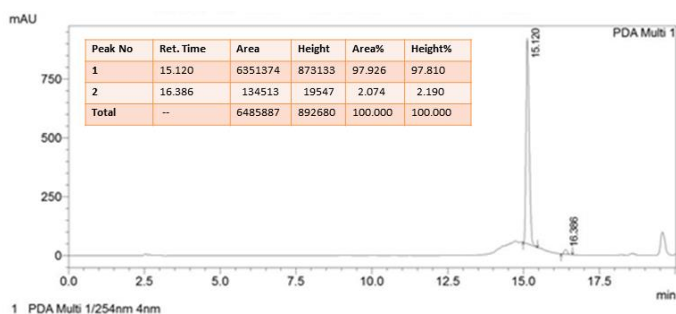


Figure 1: % Yield of *Alphonsea sclerocarpa* leaf extract.

Compound purification

The HPLC purified compound showed a purity of 97.8%. The image of HPLC chromatogram was represented in figure-2.



Peak No	Ret. Time	Area	Height	Area%	Height%
1	15.120	6351374	873133	97.926	97.810
2	16.386	134513	19547	2.074	2.190
Total	--	6485887	892680	100.000	100.000

Figure 2: Chromatogram of HPLC purified fraction. The figure shows peak of compound and table present in the in-set depicts the results of HPLC run.

However, the purified compound was subjected to FT-IR spectroscopy to determine the functional groups. The FT-IR spectra from 4000 to 500cm^{-1} showed various peaks. Among these, the broad peak at 3370 corresponds to Alcohol/Phenol OH stretch. Usually, OH stretch present in Alcohols and Phenols will give a broad peak in the range of 3550 –3200. The 2925 Peak corresponds to the functional group having CH_3 stretch. The 1685 peak corresponds to α, β unsaturated ketone *i.e*., C=O stretch. The peak at 1455 corresponds to O-H stretch. The peak at 1050 corresponds to alkoxy C-O stretch. The peak at 724 corresponds to N stretch [14]. The IR spectrum was represented in figure 3 after processing the raw data obtained and replotted using Origin software version 8.0.

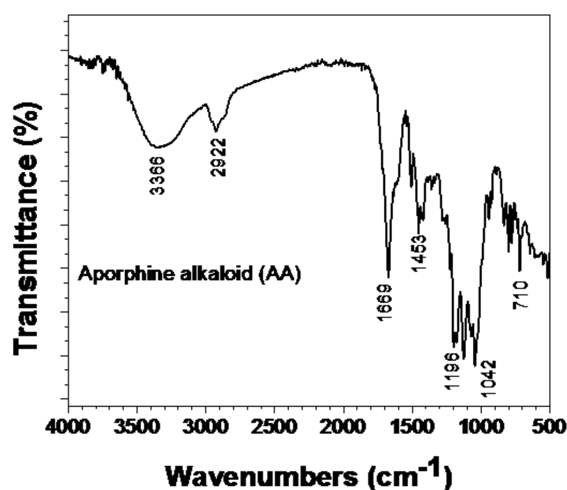


Figure 3: FTIR spectra of purified compound

NMR Spectroscopy

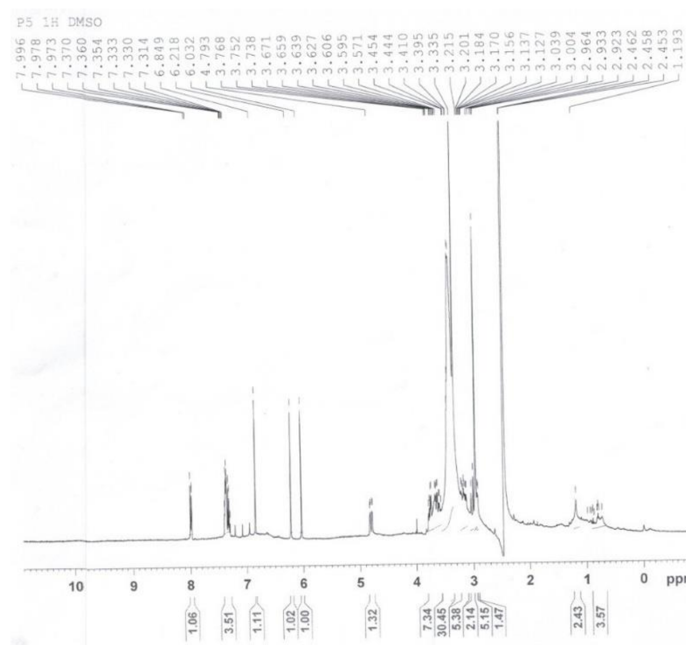


Figure 4: ^{1}H-NMR of Aporphine alkaloid

^{1}H NMR (d_6-DMSO, 400 MHz); δ 7.99 (1H, d, J = 7.2 Hz); 7.38-7.35 (2H, m), 7.31 (1H, dd, J= 8.4,1.6 Hz), 6.84 (1H, s), 6.21 (1H, s) 6.03 (1H, s), 4.80 (1H, dd, J= 4.0, 14.4 Hz), 3.76 (1H, δδ, J= 5.6,12.0 Hz), 3.65 (1H, dd, J=4.8, 12.8 Hz), 3.21-3.12 (2H,m), 3.03-2.92 (2H, m).

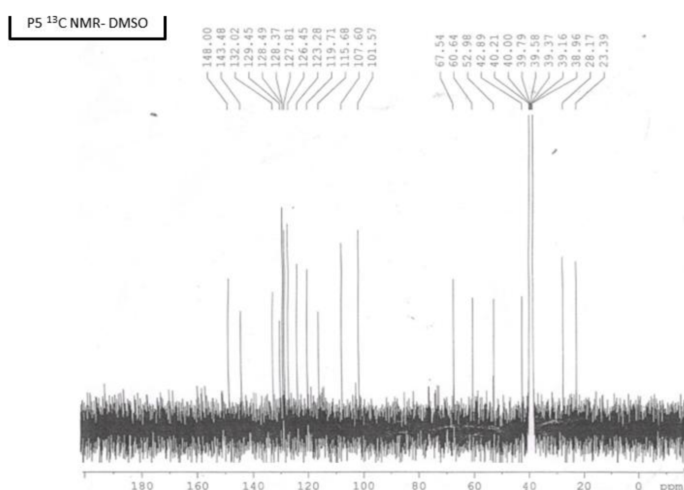


Figure 5: ^{13}C-NMR of purified Aporphine alkaloid.

^{13}C NMR (δ6 DMSO, 100 MHz); δ 148.0, 143.48, 132.02, 129.45, 128.49, 128.37, 127.81, 126.45, 123.28, 119.71, 115.68, 107.60, 101.57; 60.6, 42.89, 28.17, 23.39.

Mass spectrum

The mass spectrum was carried out to identify the mass of the compound. The m/z at 340 $(M+H)^+$ positive ion mode. The molar mass of the compound was found to be 339 based on the Mass spectroscopy (Figure-6).

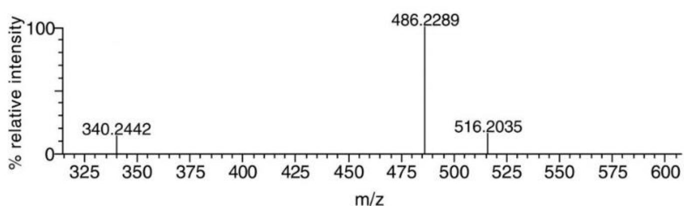


Figure 6: Mass spectra of compound (negative mode).

Structural elucidation of an Aporphine alkaloid

Based on the NMR and FTIR data the compound was identified as Crebanine. Crebanine is an Aporphine alkaloid [15]. The structure of Crebanine was reported in image-7. The molecular formula of the compound is $C_{20}H_{21}NO_4$ with a molecular weight of 339.

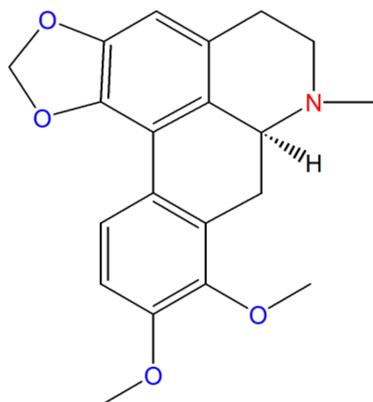
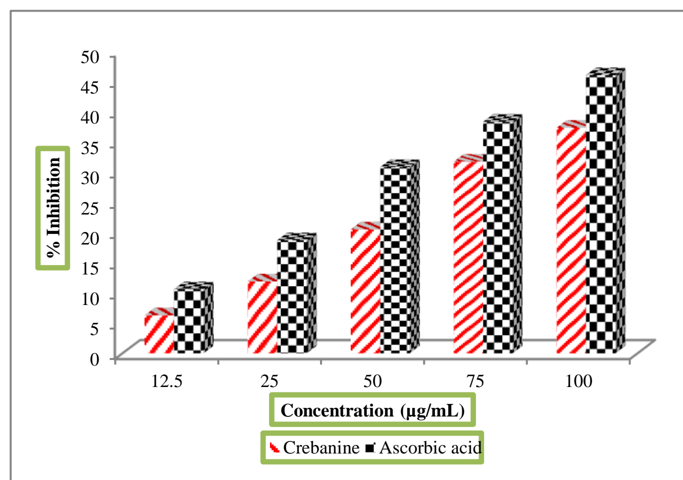


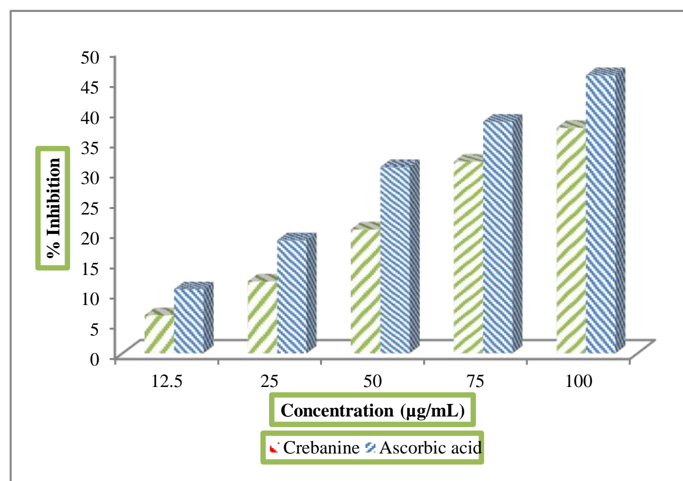
Figure 7: Chemical structure of Crebanine compound

Antioxidant activity

The purified and structurally analyzed compound Crebanine was tested for its potential antioxidant activity. The Crebanine showed dose dependent antioxidant activity *i.e.*, with the increase of concentration, there is increase in activity. The results of antioxidant activity were plotted in gtraph-2 for DPPH assay and graph-3 for FRAP assay respectively. The concentration of compound was taken on the X-axis where as % of inhibition was taken on the Y-axis for plotting the graphs.



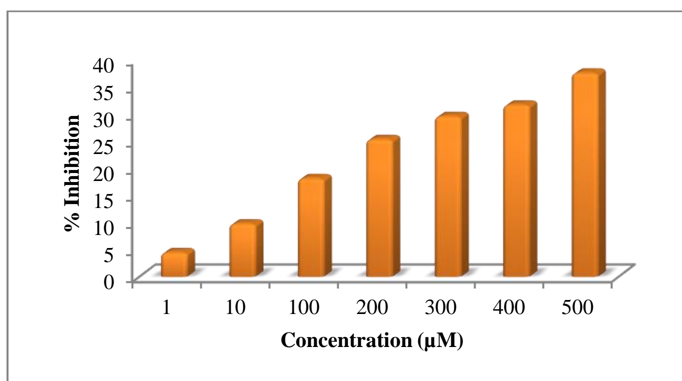
Graph 2: DPPH free radical scavenging assay.



Graph 3: FRAP ion reducing power assay

Anticancer activity

The isolated, structurally characterized and identified phytochemical Crebanine was subjected to evaluation of anticancer activity against K562 cell line (Myeloid Leukaemia Cancer cell line). Apart from cancer lines the activity of Crebanine was also tested against the HEK (Human Embryonic Kidney) cell line to determine the cytotoxicity. The anticancer activity results were represented in graph-1. The inhibition was calculated as % of inhibition and was plotted as graph by taking concentration of compound on the X-axis and % of inhibition on Y-axis.



Graph 4: Anticancer activity of Crebanine against K562 cell lines

The cytotoxicity assay clearly indicated that the extract was effective in inhibiting proliferation of K562 cells. The inhibition activity is concentration dependent and with the increase in the concentration it showed more efficiency in inhibition. The extract did not show any significant growth inhibition on HEK 293 cells. The IC_{50} of the extract in inhibiting K562 cells is determined to be 665 mg/mL with a % of inhibition of 37.545 at 500µm concentration after a period of 24 hours incubation.

CONCLUSSION

From our present research it is evident that the *Alphonsea sclerocarpa* is having potential anticancer activity. The Plant is having various phytochemicals. Among all the phytochemicals Alkaloids occupy the major part. The Aporphine alkaloid Crebanine is present largely in *Alphonsea sclerocarpa* which is conferring anticancer and antioxidant activities to the plant.

Conflict of interest: The authors declare conflict of interest as none.

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