

Isolation and Characterization of Chemopreventive Agent from *Sphaeranthus amaranthoides* Burm F

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ABSTRACT

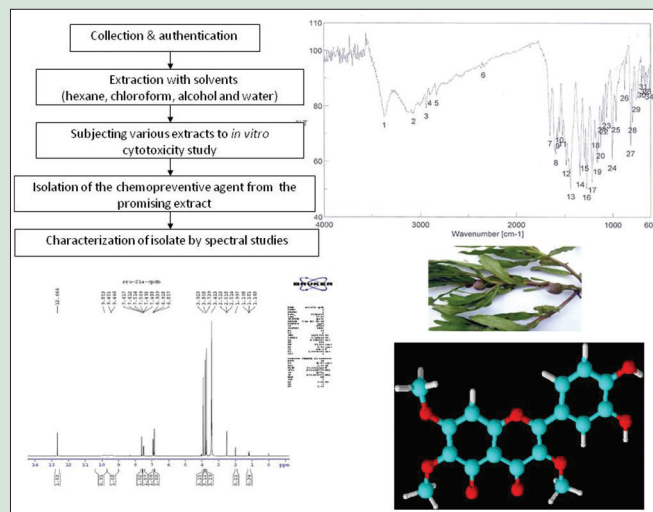
Objective: To investigate the *in vitro* cytotoxic effect and to isolate and characterize a chemopreventive secondary metabolite from *Sphaeranthus amaranthoides* Burm F (sivakaranthai).

Materials and Methods: *In vitro* cytotoxic effect was carried out by 3 (4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide assay. Different concentrations of the extracts were tested on three different cell lines namely A549, HT29, and MCF7. The chloroform extract was subjected to column chromatography, and the isolated compound was characterized by various spectral methods and by single crystal X-ray crystallography. **Results:** The concentration that cause 50% growth inhibition value of chloroform extract was found to be 0.9 and 19 µg/mL against MCF7 and A549 cell lines, respectively. Chloroform extract was subjected to column chromatography for the isolation of phytoconstituent. The structure of the isolated compound was identified by spectroscopic techniques such as infrared, nuclear magnetic resonance, XRD, and mass spectroscopy. On comparison of complete spectral detail of the compound, the proposed structure was identified as chryso-splenol D (a flavonoid). Chryso-splenol D was isolated for the first time from this plant. **Conclusion:** The chloroform extract had higher cytotoxic effect, and the isolated chryso-splenol D may be responsible for the anti-proliferative effect of the plant.

Key words: Characterization, Chemopreventive agent, Chryso-splenol D, Isolation, 3 (4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide assay, *Sphaeranthus amaranthoides*

SUMMARY

- The plant *Sphaeranthus amaranthoides* Burm F was extracted with solvents of increasing polarity. The chloroform extract was found to have cell inhibition towards MCF 7 and HT 29 cell lines. This was subjected to fractionation. Chryso-splenol D was isolated from the chloroform extract



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DOI : 10.4103/0974-8490.171101

INTRODUCTION

Cancer is one of the dreadful diseases in human, and nowadays there is extensive scientific and commercial interest for the discovery of new anticancer agents from natural sources. For the isolation of anticancer lead molecules from the medicinal plants, it is necessary to test the safety and efficacy of the plant. Some preliminary cytotoxic screening models are used for the selection of active plant extracts for the treatment of cancer.^[1] Plants are used traditionally for the treatment of various diseases. Therefore, it is necessary to isolate the component responsible for their activity. Asteraceae family is the largest family with a number of plants having different pharmacological activity. *Sphaeranthus amaranthoides* Burm F is one such plant that is unexplored for its active constituent. From the phytochemical review, it was inferred that only gas chromatography-mass spectroscopy (GC-MS) analysis of the methanolic extract of the plant was reported. Detailed literature survey revealed that there were no components isolated from this plant until now. Preliminary phytochemical analysis indicated the presence of steroids, flavonoids, alkaloids, terpenoids, etc., in the plant. Therefore, an attempt was made to isolate a bioactive component from this species.

MATERIALS AND METHODS

Collection of plant material

S. amaranthoides Burm F occurs as a weed in paddy field. The whole plant was collected from Thoothukudi District of Tamil Nadu in the year 2010. Identification of the plant and taxonomical authentication were done by Mr. V. Chelladurai, retired research officer, CCRAS, Tirunelveli. A voucher specimen was deposited at the herbarium of our department.

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Cite this article as: Gayatri S, Suresh R, Reddy CU, Chitra K. Isolation and Characterization of Chemopreventive agent from *Sphaeranthus amaranthoides* Burm F. *Phcog Res* 2016;8:61-5.

Preparation of extracts

The fresh plant was dried under shade and coarsely powdered. The powdered plant material was packed in a soxhlet apparatus and extracted with petroleum ether. The extract was removed, and the marc was pressed and dried. The marc was then extracted with chloroform for 72 h. Similar procedure was followed for ethyl acetate and methanol. All the extracts were concentrated and dried to get a constant weight.

Cell culture

The cell lines procured from American type culture collection, USA was cultured in Dulbecco's Modified Eagle Medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 mg/mL). It was then subcultured in cell culture flask and incubated at 37°C in a humidified 5% CO₂ air atmosphere.

Cytotoxicity assay

In microtiter plate, cells in exponential growth phase were cultured. Once the cell density reaches 70-80% confluence, they were trypsinized and seeded in 96-well plates at a concentration of $5-10 \times 10^3$ cells/100 μ L/well. It was then incubated in CO₂ incubator for 24 h. Extracts with the concentration ranging from 100, 10, 1, 0.1, and 0.01 μ g/mL in 100 μ L were added to the plates and incubated in CO₂ incubator for 48 h. Fifty microliters of 3 (4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) (5 mg/mL in phosphate buffered saline) were added to each well and further incubated for 2 h 30 min. The medium was carefully decanted. The air dried formazan crystals were dissolved in 100 μ L of dimethyl sulfoxide (DMSO), the plates were mildly shaken at room temperature and the optical density was measured at 570 nm using Synergy H4 microplate reader.^[2]

From the optical densities, the percentage growths were calculated using the following formulae:

If $T \geq T_0$, percentage growth = $100 \times ([T-T_0]/[C-T_0])$

and if T is less than T_0 , percentage growth = $100 \times ([T-T_0]/T_0)$

where T is optical density of test, C is the optical density of control, and T_0 is the optical density at time zero.

A dose response curve was generated using the percentage growth and concentration. The concentration that cause 50% growth inhibition (GI_{50}) value was calculated by interpolation.

Isolation of flavonoid

Silica gel of 60–120 mesh was packed in a glass column. Around 10 g of chloroform extract was mixed with silica gel 60–120 mesh and packed in the column. It was eluted with mixtures of solvents of increasing polarity. First, it was eluted with n-hexane, then with a mixture of n-hexane and chloroform of different ratios (5–100%). Following this, a mixture of chloroform and ethyl acetate (10–100%) was used for elution. It was followed by a mixture of ethyl acetate and methanol of various percentages such as 2, 4, 6, 8, 10, 50, and 100%. Around 100 fractions of 100 mL eluent were collected. Based on the thin layer chromatography profile, similar fractions were mixed together. A single spot was observed for fraction 42–43 which was eluted with chloroform and ethyl acetate in the ratio 1:1. Upon repeated washing with ethyl acetate and chloroform, a yellow amorphous powder was obtained. This compound gave a pink color upon reaction with magnesium and hydrochloric acid, indicating the presence of flavonoid.

Characterization of the isolated flavonoid

A GC-MS was recorded on Agilent MSD VL mass spectrometer. The sample was dissolved in deuterated chloroform and injected through

direct probe inlet. An infrared spectrum was recorded on a Jasco FT-infrared (IR) spectrophotometer. Sample preparation was done by KBr pellet method, and it was scanned from 600 to 4000/cm. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker 200 MHz instrument using Tetramethylsilane as an internal reference for both ¹H and ¹³C NMR experiments. Deuterated DMSO was used as the solvent.

In Single crystal XRD, a total of 1,307 frames were collected. The total exposure time was 3.63 h. The frames were integrated with the Bruker SAINT Software (Sheldrick, 2013) package using a narrow-frame algorithm.

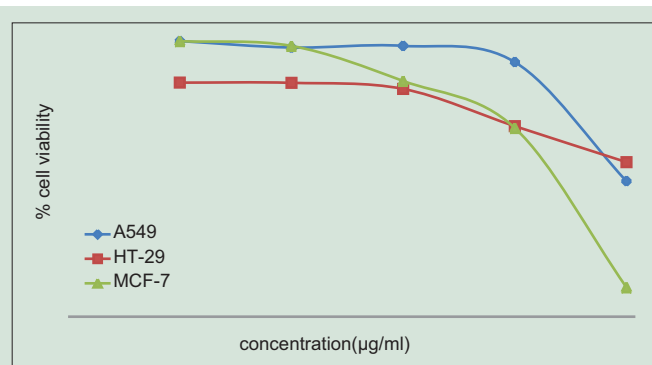


Figure 1: 3 (4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide dose response curve of petroleum ether extract on different cell lines

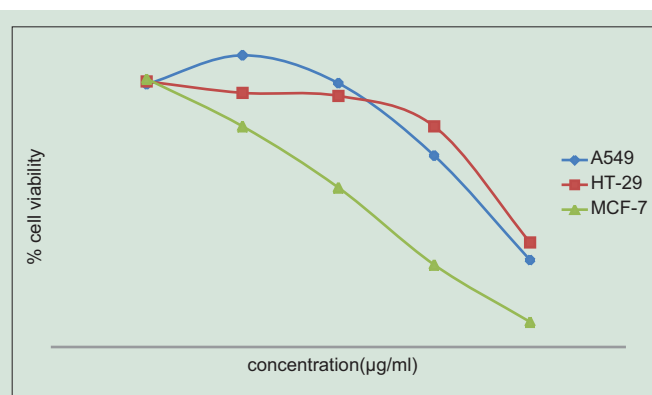


Figure 2: 3 (4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide dose response curve of chloroform extract on different cell lines

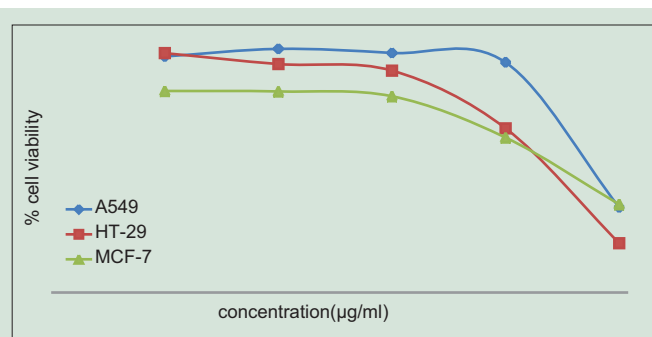


Figure 3: 3 (4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide dose response curve of ethyl acetate extract on different cell lines

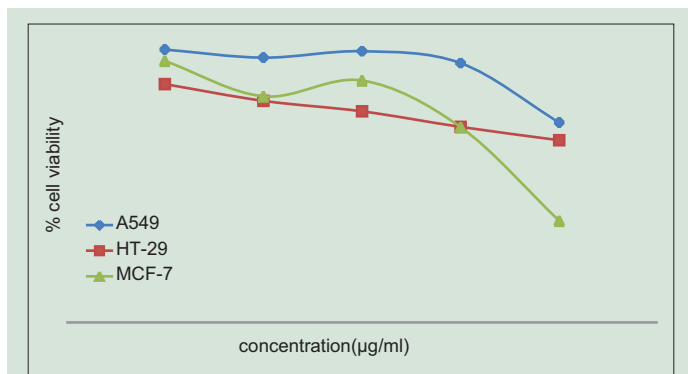


Figure 4: 3 (4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide dose response curve of methanol extract on different cell lines

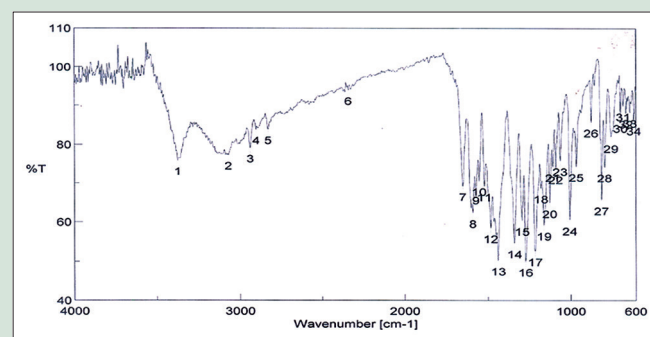


Figure 5: Infrared spectrum of the isolated compound

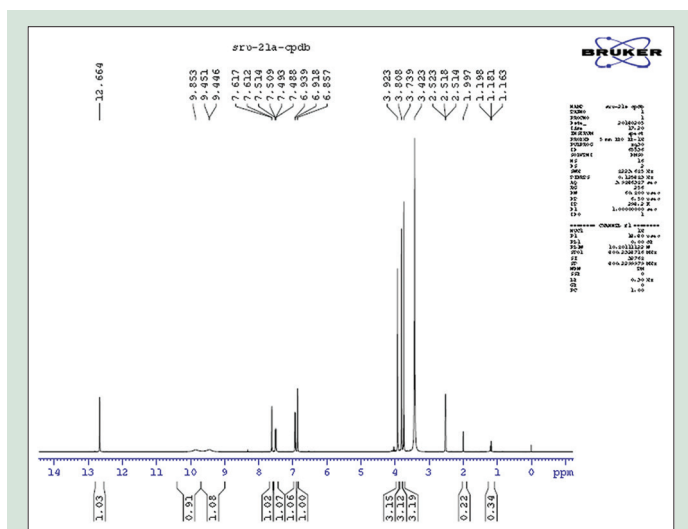


Figure 6: ¹H Nuclear magnetic resonance spectrum of the isolated compound

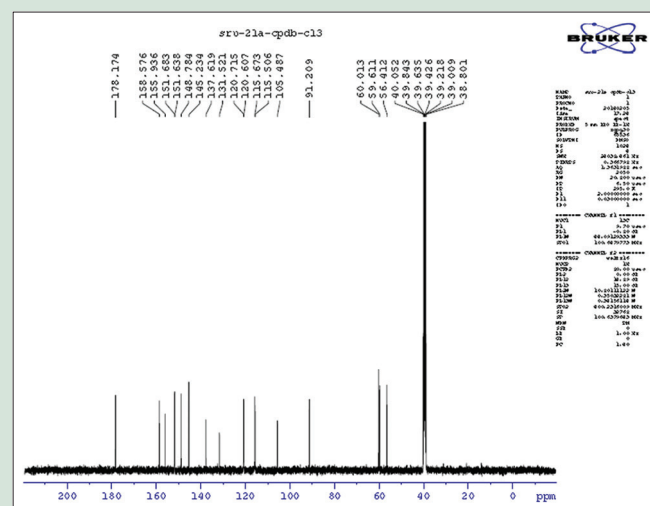


Figure 7: ¹³C Nuclear magnetic resonance spectrum of the isolated compound

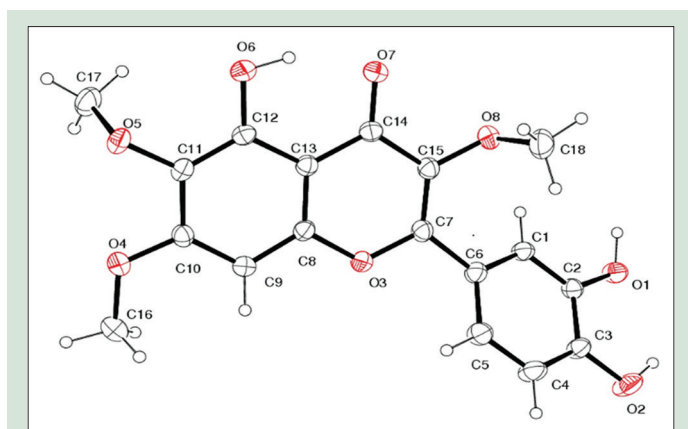


Figure 8: The molecular structure of isolated compound, showing the atom labeling

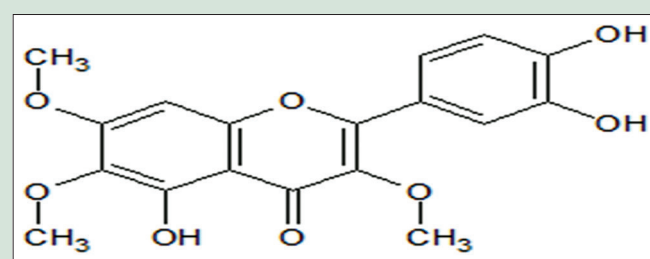


Figure 9: Chemical structure of isolated compound

RESULTS AND DISCUSSION

The effects of various extract of the plant on the growth of A549, HT29, and MCF7 cell lines were investigated by MTT assay. The extract which

caused at least 50 % of growth inhibition was accounted as cytotoxic. Figures 1-4 represent the MTT dose response curve of different extracts of *S. amaranthoides* Burm F on A549, HT29, and MCF7 cell lines. The cytotoxicity should be tested in more than one cell line because different cell lines might show different sensitivities when treated with different plant extracts. Thus, more than one cell lines seem to be essential for anti-cancer activity screening of the plant extract.^[3-5]

In the US NCI plant screening program, a crude extract is supposed to have *in vitro* cytotoxic activity following incubation between 48 h and 72 h if the GI₅₀ value in carcinoma cells is less than 20 µg/mL.^[6,7]

The chloroform extract exhibited a dose-dependent activity and possesses strong cytotoxicity against the cancerous cell lines [Table 1].

The chloroform extract was subjected to fractionation to isolate the chemopreventive component.

The isolated compound was found to be a yellow amorphous powder, with a percentage yield of about 0.7%. It was found to be soluble in ethyl acetate, alcohol, and DMSO while insoluble in water with a melting point of 230–232°C.

The molecular formula, $C_{18}H_{16}O_8$ was established by MS (EI+) $M + 1$ $m/z = 361.2$. IR spectrum showing the absorption at 3,371/cm indicated the presence of hydroxyl O–H group. There is a frequency shift of about 70/cm when compared to free hydroxyl group, which may be due to the multiple strong O–H...O hydrogen bonds. The carbonyl stretching vibration ($\nu[C=O]$) was indicated by the absorption vibration at 1,653/cm, which may be due to the conjugation interaction between the carbonyl group and aromatic ring. The bands at 1,443, 1,216, 1,183, and 1,095/cm were due to C–O vibrations of alcohols. The IR spectrum is presented in Figure 5.

In 1H NMR spectrum, the singlet proton at δ 12.68 indicated the presence of OH group, one proton doublet at δ 7.6 and δ 6.9 and a one proton double doublet at δ 7.5 indicated the aromatic proton present in the structure. The presence of three methoxyl group corresponded to δ 3.7–3.9. A one proton singlet at δ 6.8 denotes H-8 of chromene ring. The 1H NMR spectrum of the isolated compound is given in Figure 6.

The ^{13}C NMR spectra indicated the presence 18 carbon atoms in the compound. The carbon of carbonyl carbon appeared at δ 178.174. The methoxyl group attached to chromene ring appeared at δ 56.4, 59.6, and 60. The carbon at C-8 occurred at δ 91.2. The carbon atoms of catechol ring appeared at δ 120.7 (C-1'), 115.6 (C-2'), 145.2 (C-3'), 148.7 (C-4'), 115.5 (C-5'), and 120.6 (C-6'). The signal at δ 151.68 indicated the presence of C-2. The ^{13}C NMR spectrum of the isolated compound is given in Figure 7.

Nicely grown crystal was analyzed by single crystal X-ray diffraction to get indubitably the structure of the compound. The integration of the data using a monoclinic unit cell yielded a total of 12,675 reflections to a maximum θ angle of 25.00° (0.84 Å resolutions), of which 2888 were independent. Data were corrected for absorption effects using the multi-scan method (SADABS). The final anisotropic full-matrix least-squares refinement on F^2 with 254 variables converged at $R1 = 3.32\%$, for the observed data and $wR2 = 9.39\%$ for all data. The goodness-of-fit was 1.025. The largest peak in the final difference electron density synthesis was 0.161 $e/\text{Å}^3$ and the largest hole was $-0.161 e/\text{Å}^3$ with an RMS deviation of 0.033 $e/\text{Å}^3$. On the basis of the final model, the calculated density was 1.450 g/cm^3 and $F(000), 752 e$. The molecular backbone of the compound was considered as a three-ring system, including a phenyl ring and a benzopyrone-fused ring [Figure 4]. The C–O bond distances ranged from 1.252 to 1.378 Å, in which C14–O7 (1.252 Å) is typical for a carbonyl double bond. Selected bond length and bond angle are shown in Tables 2 and 3.

The molecular structure of the isolated compound, showing the atom labeling, is given in Figure 8. The final structure of the isolated compound chrysosplenol D is given in Figure 9.

From the literature, it was found that Fructus Viticis (*Vitex trifolia*), a traditional Chinese medicine, used in the treatment of human cancer contained chrysosplenol-D as one of the active constituents.^[8] In a study with HeLa cells, when the cells are stimulated by 12-otetradecanoyl phobol-13-acetate, chrysosplenol-D markedly inhibited the incorporation of ^{32}P into phospholipids.^[9] These were the evidence of chemopreventive nature of chrysosplenol D.

CONCLUSIONS

The present study demonstrated the anti-proliferative activity of the chloroform extract of the plant *S. amaranthoides* Burm F. From

Table 1: GI_{50} of various extracts of *Sphaeranthus amaranthoides*

Extract	GI_{50} ($\mu\text{g}/\text{mL}$)		
	A549	HT29	MCF7
Ethyl acetate	60.66	25.94	37.47
Petroleum ether	100.00	100.00	23.55
Methanol	100.00	100.00	45.74
Chloroform	19.03	31.89	0.90

GI_{50} : Concentration that cause 50% growth inhibition

Table 2: Bond lengths (Å)

Bond	Distance
C1–C2	1.375 (2)
C1–H1	0.93
C7–C15	1.358 (2)
C8–O3	1.3706 (17)
C8–C13	1.391 (2)
C9–H1	0.949 (17)
C10–C11	1.403 (2)
C11–O5	1.3783 (17)
C12–C13	1.419 (2)
C14–O7	1.2578 (17)
C1–C6	1.397 (2)
C2–O1	1.3547 (17)
C7–O3	1.3645 (17)
C8–C9	1.382 (2)
C9–C10	1.388 (2)
C10–O4	1.3460 (17)
C11–C12	1.370 (2)
C12–O6	1.3464 (17)
C13–C14	1.436 (2)
C14–C15	1.437 (2)

Table 3: Bond angles (°)

Angle	Degree
C2–C1–C6	120.40 (13)
C6–C1–H1	119.8
O3–C7–C6	112.08 (12)
C8–C9–C10	117.66 (13)
C10–C9–H1N	121.7 (10)
O4–C10–C11	114.20 (13)
C11–C12–C13	120.29 (13)
C8–C13–C12	117.70 (13)
C12–C13–C14	122.13 (13)
O8–C15–C14	117.42 (12)
O1–C2–C1	124.03 (13)
O2–C3–C2	121.13 (13)
O3–C8–C9	116.33 (12)
O4–C10–C9	124.55 (13)
C9–C10–C11	121.26 (13)
C12–C11–O5	120.92 (13)
O6–C12–C11	118.91 (13)
C8–C13–C14	120.15 (13)
O7–C14–C13	121.53 (13)
O4–C16–H16A	109.5

the spectral studies, it was identified that the isolated compound is chrysosplenol D. This is the first report for the occurrence of chrysosplenol D in the plant *Sphaeranthus amaranthoides* Burm F. The anti-proliferative activity shown by the chloroform extract may be due to the presence of flavonoid, chrysosplenol D. The effect of flavonoids in reducing cancer risk has been reported in various studies.^[10]

Acknowledgments

The authors would like to thank the Management of Sri Ramachandra University for the financial support to carry out this work under GATE project. The authors also thank IIT Madras for helping in the X-ray crystallographic studies.

Financial support and sponsorship

Management of Sri Ramachandra University for the financial support to carry out this work under GATE project.

Conflicts of interest

There are no conflicts of interest.

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