WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 5.990
Volume 5, Issue 02, 1346-1359. Research Article ISSN 2277– 7105

EXTRACTION, ISOLATION AND ANALYTICAL CHARACTERISATION OF AEGELINE ENANTIOMERS FROM *AEGLE MARMELOS*

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Article Received on
12 Dec 2015,

Revised on 02 Jan 2016,
Accepted on 26 Jan 2016

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ABSTRACT

Aegeline or N-[2-hydroxy-2(4-methoxyphenyl)-ethyl]-3-phenyl-2-propenamide is a main alkaloid isolated from the leaves of *Aegle marmelos* (bael). The plant is extensively used in the Indian traditional system of medicine viz. Ayurveda . It soothes impaired kapha, vata, body pain, poison, diarrhea, dysentery, vomiting and intermittent fever. Its leaves are known as a cure for asthma, inflammation, cough and diabetes. The leaves are trifoliate and aromatic. They have a shape, intermediate between ovate and lanceolate with reticulate pinnate venation. The present work was undertaken to provide an easy and reliable method for the extraction and isolation of Aegeline (N-[2-hydroxy-2-(4-methoxyphenyl)ethyl]-3-phenyl-2-propenamide) using high performance liquid chromatography (HPLC). The study also included the separation of the two enantiomers of Aegeline using chiral HPLC, its crystallographic structure, activity of the two enantiomers on cervical cancer and a nuclear magnetic resonance (NMR) method for the enantiomeric purity determination of collected fractions. Linear calibration curves were obtained over the range of 0.7 – 5mM for enantiomer-2 (R^2 = 0.998). This study was undertaken with the intention to develop a fast and reliable method for the enantiomeric purity determination of Aegeline and related compounds.

KEYWORDS: *Aegle marmelos,* Chiral HPLC, crystallography, NMR.

INTRODUCTION

Aegeline[1] is a main alkaloid isolated from the leaves of *Aegle Marmelos* (L.) Correa. It is an ancient tree and is considered sacred in Hinduism and is found commonly in India. It is also called as golden apple or wood apple (English), Bael (Hindi) and Koovalam or Vilvam (Malayalam).[2,3] In Ayurveda Bael leaves are used in dyspepsia, gastric indigestion, cold and sinusitis. There are many research reports suggesting potent hypoglycemic activity of Bael leaves[4,5] and is hence used in Ayurveda for treatment of diabetes. Biochemical compounds of bael leaves, fruits and seeds have been used in several diseases like diabetes, cardiovascular and anti- inflammatory.[6-12] Aegeline had shown antihyperglycemic and antidyslipidemic activities in validated animal models of type 2 diabetes mellitus.[13] The bael leaf contain seven monotorpene hydrocarbons (90.7%), three oxygenated monoterpenes (2.9%), four sesquiterpene hydrocarbons (3.1%) and one phenolic compound (0.2%).[14,15]

Fig.1. *Aegle Marmelos*

In this work the extraction of the *Aegeline* is taken up along with the separation of its enantiomers, its crystal structure and activity of individual isomers on cervical cancer, followed by an NMR estimation method for enantiomeric purity using chiral shift lanthanide reagents.

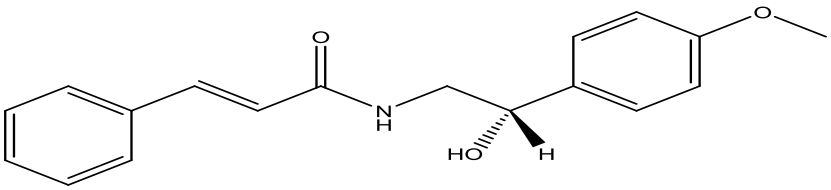


Fig.2. Structure of Aegeline [N-(2-hydroxy-2-(4-methoxyphenyl)ethyl)cinnamamide]

MATERIALS AND METHODS

Samples, chemicals and reagents

The leaves were collected from Vazhakulam in Ernakulam District, Kerala, India.

The collected plant species was identified by –Dr. A.K.Pradeep (Asst. Professor, Dept. of Botany, Calicut University, Kerala, India) and authenticated with Calicut University Herbarium. (Herbarium Catalogue no. 86970)

LR grade Acetone, Hexane, Absolute ethanol and Methanol was purchased from S.D.Fine Chem (Bangalore, India). HPLC grade Acetonitrile, Sodium bicarbonate AR grade and Dichloromethane LR grade was purchased from Rankem (Bangalore, India), and Trifluoroacetic acid (TFA) (for IR and NMR Spectroscopy) was purchased from Spectrochem (Bangalore, India). Water used for the preparation of mobile phase was purified using Merck Millipore Milli-Q® Ultrapure Water (Merck KGaA, Darmstadt, Germany). Solvents for NMR, DMSO-d6, (99.80%D, with 0.03% TMS, v/v) was purchased from Eurisotop (St-Aubin Cedex, France), CDCl3, (99.80%D, with 0.03% TMS, v/v) and Europium(III)tris[3-(heptafluoropropylhydroxymethylene)-d-camphorate] from Sigma-Aldrich Chemicals Pvt. Ltd., (Bangalore, India) and CD3CN, (99.80%D, with 0.03% TMS, v/v) from Aldrich (Steinheim, Germany).

Extraction and isolation of Aegeline

The leaves were plucked and dried in the sun and was made into a powder form by grinding. About 500g (as 50g packs) of the powdered leaves was used for soxhlet extraction with a 2:1 combination of hexane and acetone as solvent. The leaves (each pack) were extracted for 12 hours. Another portion of approximately 100g (50g packs) was extracted with methanol as solvent. Both the extracts was then concentrated to obtain around 19g of hexane: acetone extract and 4.5 g of methanol extract.

Analytical and preparative high-performance liquid chromatography

A Agilent 1100 series HPLC (Agilent Technologies, Santa Clara, CA, USA.) equipped with binary pump, autosampler and photodiode array detector was used. The analysis was carried out on a Agilent Zorbax Eclipse C18, 150 mm × 4.6 mm, and 5µ particle size (Agilent Technologies) using 0.1% trifluoroacetic acid in water and 0.1% trifluoroacetic acid in Acetonitrile (9:1) as mobile phases with a gradient elution at a flow rate of 1ml/min at the temperature 25◦C. UV detection was carried out at 220 nm. Data acquisition time was 50 min. The data was recorded using Chemstation software (Agilent Technologies).

Hexane/Acetone extract and Methanol extracts were separately injected and the chromatograms was obtained. The major component was observed at a retention time of 27minute. Both the chromatograms showed the same percent composition (approx.. 17% and 15% respectively) for the major peak.

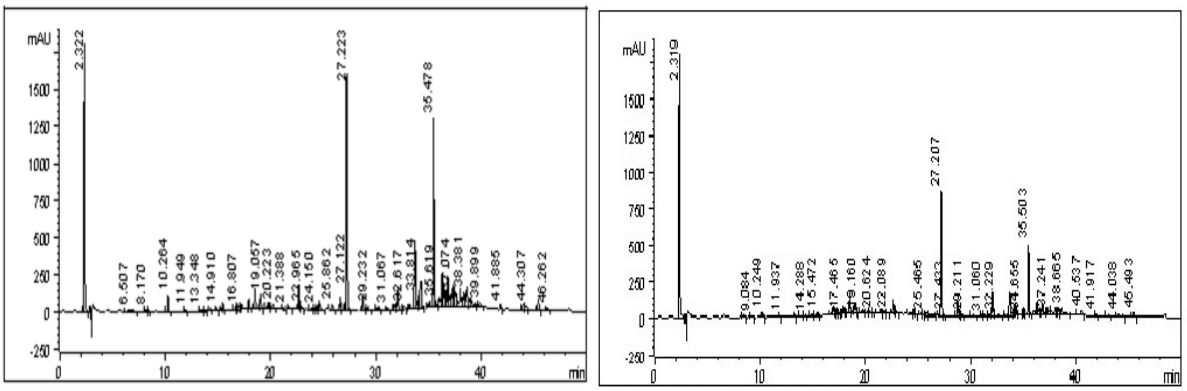


Fig.3. Hexane and Methanol extract

The purification of the crude extract was performed on a Agilent 1200 series Prep HPLC (Agilent Technologies) equipped with binary pump, autosampler and photodiode array detector using acetonitrile and 0.1% trifluoroacetic acid in water as mobile phase. PrepHT Zorbax C18, 150 mm × 21.2 mm, and 5µ particle size column (Agilent Technologies) was used. A total of five peaks were isolated using gradient elution. All the five were individually injected into HPLC and LCMS. Peak-1 which was the major component (***Aegeline***) gave a purity of 94% . This was purified again to achieve a 97% purity using the above mentioned preparative methodology. The preparative effluent was then concentrated on a Buchi rotovap. A yield of 1.2g was thus collected which showed a purity of 97% in 0.1%TFA: 0.1% TFA in acetonitrile (98:2) mobile phase. The compound was then treated with saturated sodium bicarbonate solution and extracted using dichloromethane and concentrated. The resultant free base gave a purity of 99% with a yield of 900mg.

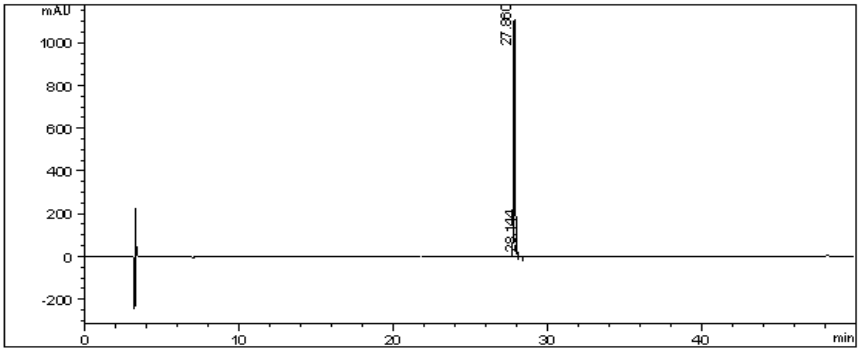


Fig.4. HPLC chromatogram

Aegeline showed a chiral centre at the –OH carbon and hence an enantiomer separation was attempted using a Agilent 1100 series HPLC (Agilent Technologies) and Lux Amylose-2, 250 mm × 4.6 mm, 5µ particle size chiral column (Phenomenex, Inc. Torrance, CA, USA) and Chiralpak-IA, 250 mm × 4.6 mm, 5µ particle size chiral column (Daicel Corporation, Japan). The two enantiomers were separated with a retention time difference of 2minutes on Amylose-2 column and less than one minute difference on IA column .

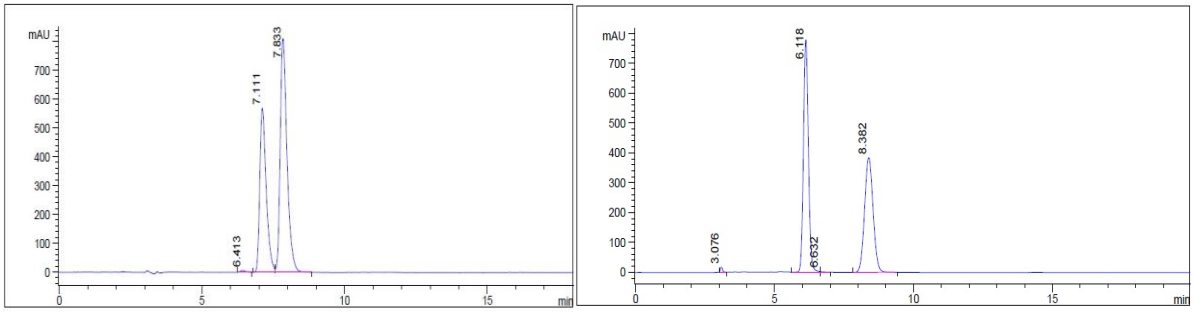


Fig.5. Analytical chiral separation using Chiralpak IA and Amylose-2 columns

A preparative chiral separation was performed on the pure compound of interest using a Agilent 1200 series Prep HPLC (Agilent Technologies) and Lux Amylose-2, AXIA Packed, 250 mm × 21.2 mm, and 5µ particle size column (Phenomenex Inc.). Hexane and Ethanol in the ratio 37:63 was used as mobile phase and the individual enantiomers were separated and concentrated. 600mg each of individual enantiomers was obtained.

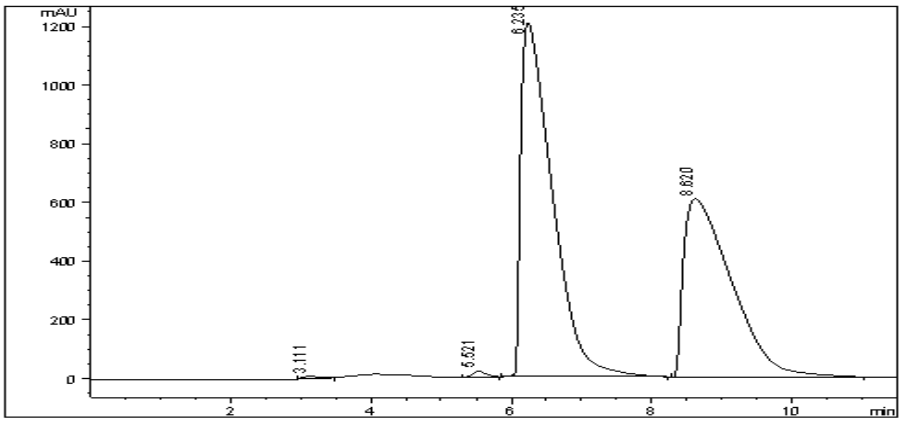


Fig.6. Preparative Chiral Separation of Aegeline enantiomers

Mass Spectroscopy

Mass spectral analysis was performed on a single quadruple mass spectrometer (Shimadzu Corporation, Kyoto, Japan) and a triple quadrupole mass spectrometer MDS Sciex model API2000 (AB Sciex Pte. Ltd.,Foster, CA, USA). Detection of ions was performed in

electrospray ionization, positive and negative ion mode. The negative mass of Aegeline (296) was confirmed on Shimadzu single quadrupole and the positive mass (298) was confirmed on triple quadrupole. The positive ionization came with a fragment mass of 280 which was later confirmed as neutral loss of one molecule of water through tandem mass studies.

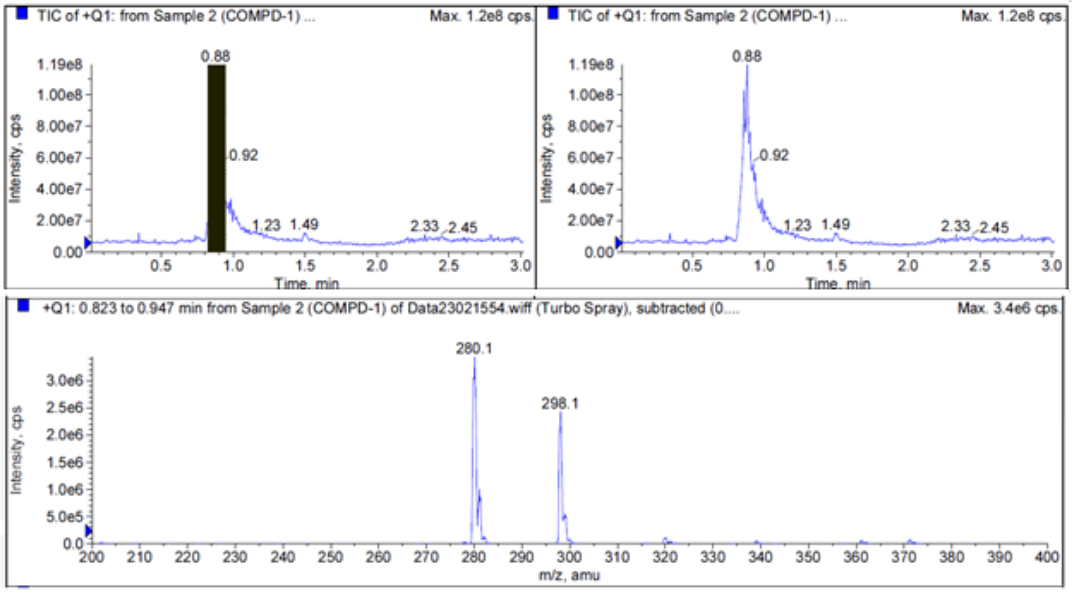


Fig.7. Mass chromatograms (with neutral loss fragment mass 280)

NMR spectroscopy

The ^{1}H, ^{13}C, DEPT 135 and two dimensional (2D) NMR experiments such as COSY, Heteronuclear Single Quantum Coherence Spectroscopy (HSQC) and Heteronuclear Multibond Coherence Spectroscopy (HMBC) were performed on Varian Mercury plus 400 MHz NMR instrument (Varian Medical Systems Inc., Palo Alto, CA, USA) at 25°C in DMSO-d6.

Compound was assigned the molecular formula of $C_{18}H_{19}NO_3$ by positive electrospray ionization technique (298.1) calculated for 297.136 Da. The ^{13}C NMR and DEPT spectra displayed 14 carbon signals, consisting of one methyl, eight methine, one methylene and four quaternary. The proton correlations were established using multiplicity edited HSQC spectrum. It also provides information on number of carbons corresponding to the proton signals. A portion of proton connectivities was established using ^{1}H-^{1}H COSY. HMBC Spectra were used to establish the ^{1}H and ^{13}C long range connectivities and assigning quaternary carbons.

The NMR data are provided below which was found in accordance with Aegeline structure.

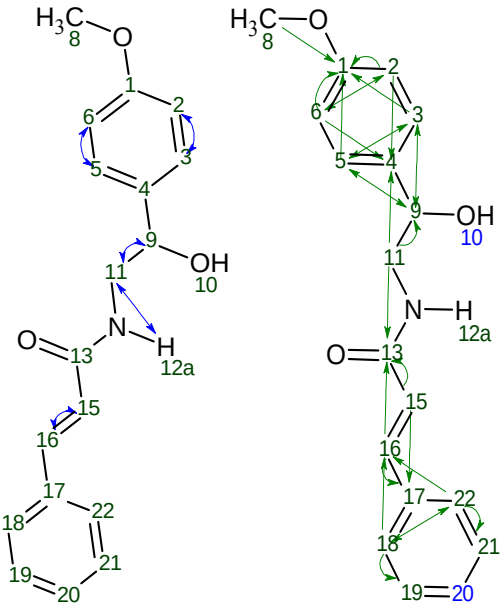


Fig.8. ^{1}H-^{1}H COSY and ^{1}H-^{13}C HMBC correlations of compound.

Table 1: ^{13}C and ^{1}H NMR Spectroscopic data (400MHz, DMSO) for Aegeline

Position	∂C Type	DEPT	∂H	Multiplicity	COSY	C HMBC	H HMBC
1	158.317	C					H8,H2,H6,H5,H3
2, 6	113.39	CH	6.897	br d (8.33)	H5,H 3	C4, C1	
3,5	127.1	CH	7.274	br d (8.33)	H2,H 6	C1, C9	H9
4	135.679	C					H11, H2, H6
8	54.977	CH3	3.732	s		C1	
9	70.917	CH	4.61	br d (5.10)	H11	C5,C3	H11, H5, H3
10		OH	5.445	br s			
11	46.998	CH2	3.228	ddd (13.16, 7.66, 5.24)	H11,H 9	C9, C13, C4	
11	46.998	CH2	3.411	br d (5.37)	H11,H12a		
12a		NH	8.157	br t (5.51, 5.51)	H11	C13	
13	165.035	C					H11,H15,H16,H12a
15	122.341	CH	6.724	d (15.85)	H16	C17,C13	
16	138.448	CH	7.397	br d (13.97)	H15	C17,C13	H22,H18
17	134.928	C					H15,H6
18,22	127.406	CH	7.547	br d (6.98)		C16,C21,C19	
19,21	129.295	CH	7.366	m			H22,H18
20	128.846	CH	7.407	br d (9.67)			

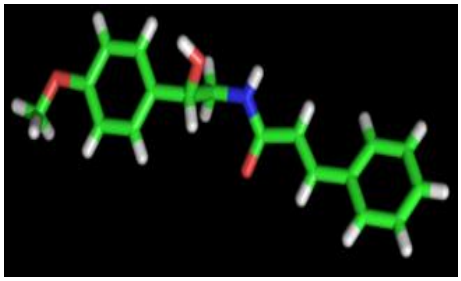
X-ray Diffraction Data collection

A suitable single crystal of each enantiomer was carefully selected under a polarizing microscope and the single crystal data were collected on a Bruker Kappa Apex2 CCD diffractometer at 293(2) K. The X-ray generator was operated at 40 kV and 30 mA using Mo Kα(λ = 0.71073 A˚) radiation. Data were collected with ω scan width of 0.5. The data

reduction followed by Empirical absorption corrections were applied with the various modules within the Apex2 software suite. The structures were solved by direct methods using the SHELXTL package and refined by full-matrix least-squares on F2 from the same. All Non-hydrogen atoms were refined anisotropically and hydrogen atoms were refined with a riding model. Structure was drawn using Mercury 3.1 and Pymol.

Table. 2: Crystallographic data of Aegeline enantiomers

Crystal data statistics	Enantiomer-I	Enantimer-II
Empirical formula	C18H19NO3	C18H19NO3
Molecular weight	297.35	297.35
Temperature/K	298	298
Crystal system	Monoclinic	Monoclinic
Space group	P2(1)	P2(1)
a/Å	6.8626(3)	6.8651(3)
b/Å	8.9642 (5)	8.9547 (5)
c/Å	12.9641(7)	12.952(6)
α/°	90	90
β/°	90.284	90.210
γ/°	90	90
Volume/Å^3	797.535	796.21
Z	2	2
ρ_{calc}g/cm^3	1.53	1.55
μ/mm^{-1}	1.288	0.892
Radiation	Mo Kα (λ = 0.71073)	Mo Kα (λ = 0.71073)
Max 2θ	129.04	129.04
Reflections collected	1658	3690
Independent reflections	1496[R_{int} = 0.0147, R_{sigma} = 0.0323]	1874[R_{int} = 0.0340, R_{sigma} = 0.0469]
Data/restraints/parameters	1496/1/199	1874/1/199
Index ranges	-4 =< h =< 7, -4 =< k =< 9, -15 =< l =< 11,	-7 =< h =< 7, -10 =< k =< 9, -15 =< l =< 14, Max. 2-theta = 128.93
Goodness-of-fit on F^2	1.0870	1.0210
Final R indexes [I>=2σ (I)]	R_1 = 0.0542 wR_2 = 0.1557	R_1 = 0.0473 wR_2 = 0.1317
Final R indexes [all data]	R_1 = 0.0550, wR_2 = 0.1557	R_1 = 0.0610, wR_2 = 0.1317
Largest diff. peak/hole / e Å^{-3}	0.226/-0.2300	0.215/-0.1200



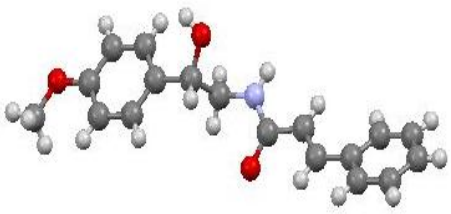


Fig.9. Enatiomer-I [(S,E)-N-(2-hydroxy-2-(4-methoxyphenyl)ethyl)cinnamamide]

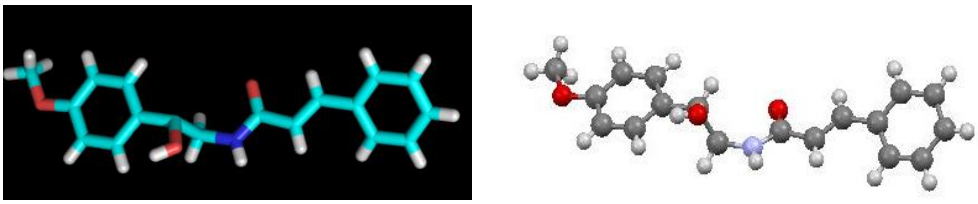


Fig.10. Enatiomer-II [(R,E)-N-(2-hydroxy-2-(4-methoxyphenyl)ethyl)cinnamamide]

Anticancer activity

All compounds were screened for their in vitro anticancer activity against representative human cancer cell line (HeLa cell line) by MTT assay. This is a colorimetric assay that measures the reduction of yellow 3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilised with an organic solvent (e.g. Dimethyl sulfoxide, Isopropanol) and then released solubilized formazan reagent is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of these cells.

The 3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) was made a solution in such a way that 10 mg was dissolved in 10 mL of Hank's balanced solution. The cell lines were maintained in 96 wells micro titre plate containing MEM media supplemented with 10 % heat inactivated fetal calf serum (FCS), containing 5 % of mixture of Gentamycin, Penicillin (100 Units/ mL) and Streptomycin (100 µg / mL) in presence of 5% CO_2 at 37ºC for 3 - 4 days. After 3 - 4 days, the supernatant was removed and MEM media was replaced with Hank's balanced solution and the cells were incubated overnight. The in-vitro growth inhibitions of test compounds were assessed by colorimetric or spectrophotometric method. This helps to determine the conversion of MTT into formazan blue by living cells. The supernatant was removed from the plate, added fresh Hank's balanced salt solution and was treated with different concentration of compound (approx. diluted with DMSO). The marketed anticancer drug Paclitaxel was tested as a reference compound in the assay. The control group contains only DMSO. After 24 hours of incubation at 37ºC in a humidified atmosphere of 5% CO_2, the medium was replaced with MTT solution (100 µL, 5 mg/mL in MEM medium) for further 4 hours. The supernatant was carefully aspirated and the precipitated crystals of Formazan blue was re-solubilised by adding DMSO (200 µL) and optical density was measured at wavelength of 570 nm using LISA microplate reader. The

results were represented out in triplicates for each concentration. Concentration at which the optical density (O.D) of treated cells was reduced by 50 % with respect to the untreated control. Calculation of the percentage of lyses of cells was done by comparing the O.D of sample to that of the control and also by microscopic analysis.

Table. 3: Anticancer activity of enantiomer-1 and enantiomer-2

Percentage growth inhibition in HeLa cervical cell line				
Compound	**Conc. (µg/mL)**	**O.D. at 570 nm**	**% of lysis as observed**	**IC50(µg/mL)**
Enantiomer 1	10	0.781	No lysis	No activity
	20	0.721	No lysis	
	30	0.681	No lysis	
Enantiomer 2	10	0.516	25	20 µg
	20	0.415	50	
	30	0.411	> 50	
Control	-	0.862	No lysis	-
Paclitaxel	10	0.512	No lysis	30 µg
	20	0.484	25	
	30	0.401	50	

IC50- Half maximal inhibitory concentration – It is the half maximal (50 %) inhibitory concentration (IC) of a Substance (50% IC, or IC50)

Cell line- HeLa – Human cervix

Enantiomeric purity determination of Aegeline isomers using NMR

Chiral derivatizing reagents or chiral shift reagents (CSRs) are usually employed for the detection and quantification of two enantiomers using NMR.[16-18] In this work, camphor based chiral lanthanide shift reagent (CLSR) was used for the investigation of signal separation of RS-Aegeline. Europium (III) tris[3-(heptafluoropropylhydroxymethylene)-d-camphorate] (Eu[hfc]3) was used as the CLSR. The Europium lanthanide was used, since it did not have any interfering peaks at the ppm region of interest (3.5- 4.5ppm). The O-CH_3 peak showed marked separation when a 10mM soln. of europium in $CDCl_3$ was added to a racemic mixture (prepared my mixing equal concentrations of Enantiomer -I and Enantiomer -II). Solvents like dimethyl sulfoxide (DMSO) and acetonitrile (ACN) were also used as diluents. $CDCl_3$ as the diluent for CLSR gave the maximum separation of the O-CH3 peak of the enantiomers. Hence the experiment was conducted using $CDCl_3$.

NMR Equipment

Instrument: Varian 400MHz NMR with ATB Probe.

Acquisition Parameters: SWH (Sweep width) of 6410.3Hz; ambient temperature; line broadening (LB) of 0.3Hz

General procedure

Eu[hfc]3 solution: 119.6mg of Eu[hfc]3 in 10ml $CDCl_3$.

Spinning tubes of 5mm internal diameter (i.d) containing 500µl of sample solutions were used.

Preparation of samples: 14.85mg each of Enantiomer –I and Enantiomer -II were weighed and dissolved in 1ml of $CDCl_3$. (Stock-I and Stock-II). 100µl of Stock-I , 100µl of Stock-II and 300µl of $CDCl_3$ was added to it. To this racemic mixture 500µl of Eu[hfc]3 solution was added (5mM).

A series of sample dilutions of one enantiomer, while maintaining the other enantiomer as constant, were thus prepared. The concentrations ranged from 5mM to 0.7mM for the NMR study.

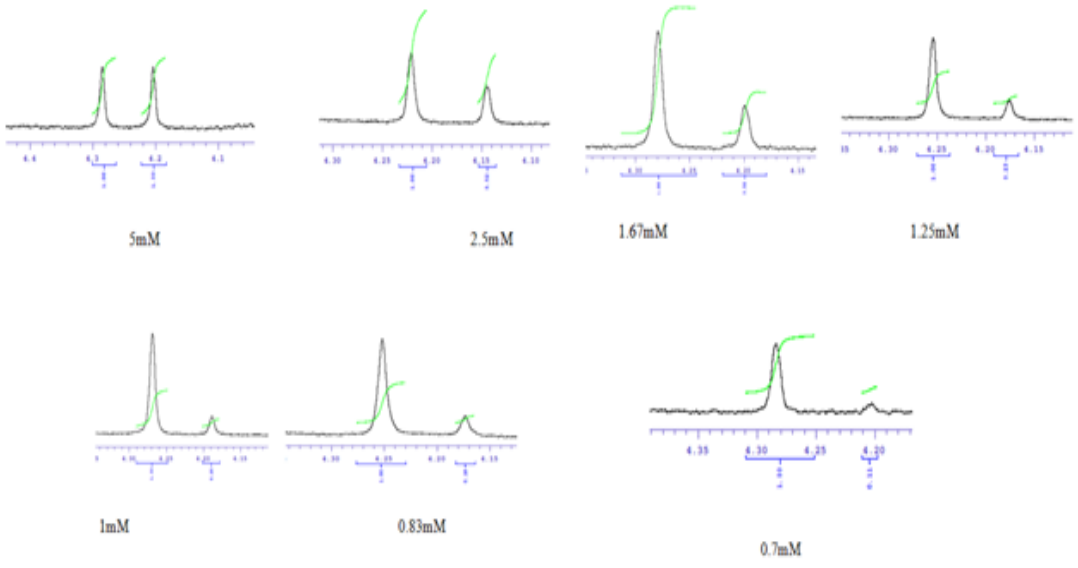


Fig. 11. ^{1}H NMR spectra of enantiomer-I and II and CLSR (Eu[hfc]3

The linearity was investigated through a signal intensity ratio by keeping Enantiomer-I concentration as the same and varying the concentrations of Enantiomer -II. The linearity was confirmed to be good in the method. (R^2 = 0.998).

Table. 4: Linearity of Enantiomer -II added to Enantiomer -I

Theoretical Values (integral)	Observed Values (integral)	Concentration
1	1	5
0.5	0.52	2.5
0.33	0.32	1.67
0.23	0.23	1.25
0.2	0.2	1
0.16	0.18	0.87
0.14	0.11	0.7

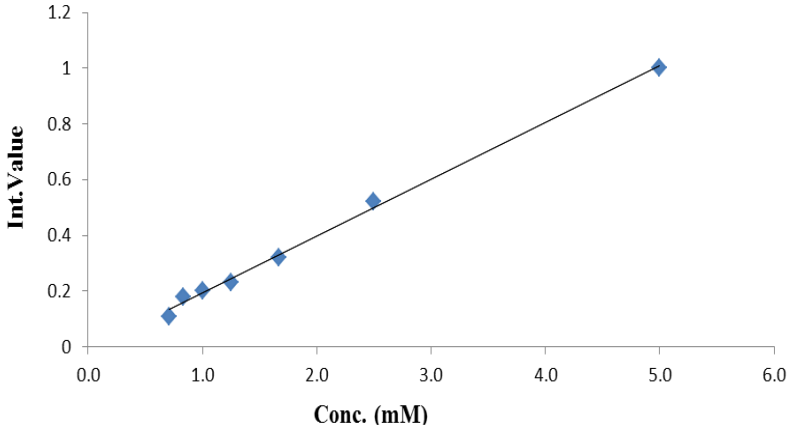


Fig.12.Linearity plot of Enantiomer -II added to Enantiomer -I

Using this method a 0.7mM concentration was detectable and this was found in agreement with the HPLC results using chiral stationary phase.

RESULTS AND DISCUSSION

The study gives an efficient method for the extraction of a major ingredient of *Aegle Marmelos* Correa and a reliable NMR method for the enantiomeric purity determination. Different combinations hexane: acetone and methanol was used as extraction solvents. It was observed that methanol gave the same percent yield as in the case of 1:1 ratio of hexane and acetone. For the separation of the isomers with good resolution different combinations of solvents like ethanol, isopropanol was used along with hexane onto immobilized IA column from Daicel Industries and Lux Amylose-2 column from Phenomenex with varying ratios. It was observed that hexane and ethanol in the ration 30: 70 gave a suitable separation on Amylose-2 column. This was then extrapolated to preparative separation and a ratio of 37:63 of hexane and acetone on Amylose-2 gave 99% pure individual enantiomers. The crystallographic study of the single crystal of enantiomer-I and II have shown to be

monoclinic. Both the enantiomers were screened for in-vitro anticancer activity against Paclitaxel. It was found that enantiomer-II is superior to marketed drug Paclitaxel for its in-vitro activity and enantiomer-I was not potent. The enantiomeric purity determination using CLSR have shown that a validated method can be developed using NMR technique which is both reliable and fast.

CONCLUSION

The extraction and identification of the major ingredient, Aegeline in *Aegle Marmelos* was taken up as it has shown to exhibit antidiabetic properties. This compound also shows an in-vitro anticancer activity and can be a potential raw material for further studies in the same therapeutic area.

ACKNOWLEDGEMENTS

The authors thank Dr. Narasimha Rao and Vinutha R (XRD team) of Aurigene Discovery technologies Ltd and Dr.Naveen of Mysore University for the crystallographic support and the molecular biology team of Maratha Mandal institute of technology, Belagavi, Karnataka, India, for executing the anti-proliferative studies by MTT assay.

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