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RESEARCH ARTICLE

HPLC aided optimization and standardization of potent intracellular anti-oxidant Heraclenin from *Aeglemarmelos* fruit pulp extracts

[1,2]Ramasamy Mohankumar
[3]Vinnie Cheeran
[2,3]Munuswamy-Ramanujam Ganesh

[1]Phytochemistry Division, IIISM, SRM IST, Kattankulathur, Tamil Nadu, India
[2]Department of Chemistry, SRM IST, Kattankulathur, Tamil Nadu, India
[3]Immunobiology Division, IIISM, SRM IST, Kattankulathur, Tamil Nadu, India

Corresponding author:
Ramasamy Mohan Kumar
rmk.2005@gmail.com

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ABSTRACT

Heraclenin, a furanocoumarin was isolated by column chromatography from chloroform extract of *Aegle marmelos* fruit pulp (AMFP) and characterized using spectroscopic techniques. Bioassay guided fractionation of the AMFP extracts led to the identification of the compound. Heraclenin, showed potent ability to reduce intracellular ROS levels in human monocytic cell lines (THP-1) compared to the AMFP extracts. DPPH based assays with heraclenin also confirmed the antioxidant potential of the compound. However, heraclenin did not show significant anti-proliferative activity against the THP-1 cell line. Further, HPLC based quantification of heraclenin in hexane, chloroform and ethanol AMFP extracts showed that the chloroform extract had significantly higher quantities of the compound compared to the other two extracts. The results suggest that chloroform might be the most suitable solvent for extracting heraclenin from AMFP. Since, the compound showed reduced cytotoxicity combined with potent ability to reduce intracellular ROS levels, heraclenin could be a promising candidate for anti-inflammatory therapeutic agent. Simlarly, the heraclenin rich chloroform extract could also be used as therapeutic formulation

Keywords- : Heraclenin, *Aegle marmelos*, HPLC quantification, Intracellular antioxidant, THP-I, Anti-inflammatory

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INTRODUCTION

Plants have always been a rich source of bioactive molecules and have repeatedly been used as therapeutic options for modulating inflammatory conditions. Typically plant based medicines depend on using whole extracts of plant parts like leaves, flowers, fruits, seeds, bark etc. However, with advancement in chromatographic and screening techniques focus has shifted towards identifying the active ingredients present in plant extracts. Particularly plants used in traditional medicinal systems like, Siddha and folk medicine are under investigation using latest screening techniques. Since, these plants have been in human use for several years as treatment options for specific diseases.

Aeglemarmelos (L.) *Correa* (AM) is commonly known as bael, is a small tree that grows to a height of about 13-18feet. It is found widely in the Indian subcontinent as well as in countries like Bangladesh, Srilanka and Burma. The spinous tree belongs to the family of *Rutaceae* and known as vilvam in Tamil. The tree is indigenous to India and considered auspicious as its leaves are commonly used to worship Lord Shiva. Traditionally, in ayurvedic and siddha system of medicine, roots, stem, flowers and fruits of AM are utilized for ameliorating various disorders. The most common traditional application of AM includes the treatment of dysuria [1] and as andiuretic [2] Apart from this roots and leaves are also used in the treatment of swelling and inflammation [3]. AM fruit (AMF)are sour and bitter in taste and are used for treating stomach related ailments like diarrhea, peptic ulcers and stomach irritation [4]. Earlier investigations have further revealed the therapeutic potential of AMFP (both raw and ripe) along with identifying several phyto constituents present in the AMF extracts [5].

Phytoconstituents like marmelosin, umbelliferone, scopoletin, aegeline, imperatorin, and several other furanocoumarins have been identified from the fruit and seed extracts of AM [6, 7]. Extensive studies in different *in vitro* and *in vivo* models have been carried out both for the AMFP extracts and some of its isolated compounds. These studies have shown the ability of the AMFP and its phytochemicals, to modulate cell function [8], protectagainst myocardial infarction[9], reducing insulin resistance [10] and cytotoxic activity against various cancer cell lines [11].One of the most commonly studied aspects of the AMFP is its antioxidant potential. Studies aimed at identifying antioxidant potential of the AMFPextract have shown that the extract reduces free radicals in, induced experimental colitis model [12], wound models [13], induced oxidative stress models [14, 15, 16] and inflammatory bowel disease [17]. These studies along with the cytotoxicity studies clearly highlight anti-oxidant potential of the AMFP extract. However, information related to the effect of different solvent extractions on the anti-oxidant and cytotoxicity of AMFP is not available. Since polarity of the solvent greatly influences the extraction process and nature of the compounds extracted. It is expected that extraction with solvents of varying polarity will alter the phytochemical profile of the AMFP extracts. This variationin the phytochemical profile will impact the antioxidant and cytotoxicity of the extracts. Hence, bioactivity comparison studies between HPLC standardized AMFP

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extracts were generated using solvents of different polaritywith the objective of identifying the most suitable solvent for enhancing the bioactivity of AMFP.

Intracellular (Reactive Oxygen Species) ROS are implicated in the pathology of cancer, atherosclerosis and diabetes as an aggravator. Therapeutic molecules and extracts that ameliorate these diseases are known to possess strong ROS inhibitory activity. Earlier studies on AMFP have not attempted to identify the effect of AMFP extracts on intracellular ROS levels. Investigating the effect of different AMFP extracts have on the intracellular ROS levels could provide insight into the mode of action of the extract. Moreover, the extract showing the most ROS reducing activity could be further analyzed to yield antioxidant molecule(s) with potential therapeutic activity in cancer, atherosclerosis and other inflammatory disorders.

The current study compares the effect; different solvent extractions have on the cytotoxicity and anti-oxidant effect of AMFP extracts. The study investigates effect of AMFP extraction the intracellular ROS in human monocytic cell lines (THP-1).These cell lines, derived from blood of a patient suffering from acute monocytic leukemia, are commonly used in cytotoxic studies to investigate the anti-cancer potential of extracts and compounds. Also, being a monocytic cell line the cells are useful to study inflammatory disorders like atherosclerosis. This makes the cell line ideal for antioxidant and cytotoxic screening studies. Extracts that is efficient in reducing Intracellular ROS levels in THP-1 cell lines will be further fractionated using chromatographic techniques to identify any novel phytochemical(s) present in the AMFP extracts. The identified phytochemical(s) will be further tested for intracellular ROS activity and the results compared with standardized extract activity.

1. MATERIALS AND METHODS

1.1 Reagents and Standards

RPMI-1640, Penicillin and Streptomycin were purchased from Lonza. Fetal Bovine Serum was purchased from HiMedia (HiMedia, India). (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (MTT), Propidium Iodide (PI), 2, 2-diphenyl-2-picrylhydrazyl hydrate (DPPH) and 2',7'-dichlorofluorescein-diacetate (DCFH-DA) was purchased from sigma (Sigma-Aldrich Co., USA).

1.2 Cell line maintenance

THP-1 cell line was purchased from NCCS, Pune. The cell line was cultured in RPMI-1640 medium supplemented with 10% Fetal Bovine Serum (FBS) and 100 U/ml penicillin and 0.1 mg/mL streptomycin in a humidified atmosphere containing 5% CO_2 at 37°C. The cells were sub-cultured at appropriate intervals of time and used in the assays.

1.3 Plant Sample Collection and extraction method

Fresh fruits of AM were collected from in and around Chennai, Tamil Nadu, India, during the month of March-April and the plant authentication was performed by Dr. Jayaraman, Director of Plant and Anatomy Research Centre, Tambaram, Chennai. Herbarium was prepared and stored at herbarium repository, SRM Institute of Science and Technology, Kattankulathur.

Fruit pulp and rind from the collected AM were separated and shade dried at room temperature. The dried fruit pulp material was powdered coarsely and subjected for solvent extraction. The AMFP powder

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(500g) was simultaneously extracted with hexane, chloroform and ethanol (2000mL) by cold maceration technique with occasional shaking for 72hrs and process repeated thrice for completion of extraction. The extracts were concentrated to remove solvents using rotary evaporator under vacuum. The percentage (%) yield of the extract **(Table: 1)** was calculated using the formula**:**

Extract Yield = [Wt. of the extract (in gms) / Wt of the raw powder (in gms)] X 100

The prepared hexane, chloroform and ethanol extracts were further used for the bioactivity studies.

1.4 MTT Assay to find IC_{50}

The IC_{50} value for the three AMFP extracts was evaluated by MTT reduction assay. The assay was carried out as per earlier reports (18) with small modifications to the protocol.THP-1 cells (1 X 10^6cells/mL) were treated with different concentrations (0.07-10mg/mL) of AMFP extracts. After 24 hr exposure, 10 µl of MTT solution (5 mg/mL stock solution) was added in each well and re-incubated for 3 hr at 37°C. The absorbance was recorded at 540 nm after dissolving the formazan crystals using DMSO. The cell viability was calculated using the formula:

% cell viability = (OD of treated cells / OD of Control cells) X 100.

The IC_{50} was calculated and the concentration identified as most suitable was used for the downstream bioassays (Fig: 1). The experiment was later repeated with heraclenin isolated from AMFP Chloroform extract (Fig: 4).

1.5 Measurement of intracellular reactive oxygen species generation using FACS

Intracellular ROS production was detected by flow cytometry using DCFH-DA. THP-1 cells treated with 3 AMFP extracts at IC_{50} concentrations determined by MTT assay. The cells were then labeled with 1 µL of H_2DCFDA (13 mM) a fluorescence probe and incubated at 37˚C for 30 min in the dark. Cells were then resuspended in FBS-PBS buffer and analyzed immediately using Becton–Dickinson FACS-Calibur flow cytometer (Becton Dickinson, NJ) with the excitation and emission wavelengths of 480nm and 530 nm respectively. The histogram plot obtained for the individual AMFP extracts treated cells was compared with the H_2O_2 controls (Fig: 2). The data was analyzed using cell quest pro software from BD. The experiment was later repeated with heraclenin(Fig: 4b) isolated from AMFP Chloroform extract.

1.6 Isolation and characterization of Heraclenin

Among the three AMFP extracts, chloroform extract showed the most potency to reduce the intracellular antioxidant levels in the THP-1 cell lines. Preliminary screening of the AMFP chloroform extract with TLC also indicated the presence of a one major compound. Hence, chloroform AMFP extract was taken up for further fractionation using conventional gravity based Silica Gel (60 – 120 mesh) column chromatography. The column was packed with silica, loaded with AMFP chloroform extract in the form of admixture and eluted with increasing polarity of ethyl acetate in hexane. The fractions were monitored with TLC and fractions containing the pure compound were pooled and concentrated under vacuum. The pooled fractions yielded a white solid. The white solid was subjected to structural characterization with ^{1}H &^{13}C NMR and other analytical spectroscopic techniques like FT-IR, MS and identified as furanocoumarin, heraclenin (Fig: 3).

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1.7 FACS Mediated apoptotic activity

The apoptotic activity of the AMFP extracts and heraclenin on THP-1 cell lines was determined using flow cytometer (BD FACS (Calibur) by PI staining. THP-1 cells (1×10^5 cells/mL) were treated with AMFP extracts and heraclenin in a 12-well plate and incubated for 24hr, followed by incubation with PI for 10 min in dark. The cells were then analyzed using flow cytometer (Fig: 6).

1.8 Radical Scavenging Activity

Radical scavenging activity of the AMFP extracts and heraclenin was examined using DPPH as the reagent. Different concentrations (0.07 – 10 mg/mL) of AMFP extracts and heraclenin (0.007 – 1 mg/mL) were added to methanolic solution of DPPH (0.1 mM). The reaction mixture was incubated for 30 min at room temperature; the absorbance was recorded at 517 nm. DPPH solution and ascorbic acid were used as control and reference respectively (Fig: 5).

1.9 HPLC Analysis

Estimation of heraclenin in AMFP non-polar to polar solvent like hexane, chloroform and ethanol extracts was carried out using RP-HPLC.The samples were analyzed using HPLC Shimadzu Prominence model comprising of LC20AD binary solvent delivery module, SPD M20A PDA detector, a Rheodyne injector (model 7125, USA) valve fitted with a 20µl loop, CT0-20A Column oven. The system was controlled with the controller module equipped with CBM-20A Communications Bus Module and the data acquisition was set using the Lab solutions software (7.1 Version). The chromatographic separation was performed on a Phenomenex-Luna (Tor-rence, USA) RP–C18 column (250 × 4.6 mm, 5u particle size) was used as stationary phase. The resolution of compounds was performed by linear gradient elution using Water (A) and Methanol (B) with a flow rate of 0.5 mL/min was followed as 90 - 92% (B, 0.01 - 2.00 min), 92 – 95% (B, 2.00 – 5.00 min), 95 – 98% (B, 5.00 – 6.00 min and hold for 2min), 98 – 100% (B, 8.00 – 9.00 min and hold for 2min), and finally 90% (B, 11.00 – 20.00 min and hold for 10min), This was followed by a 5min equilibration period prior to the injection of each sample, and the column was set at ambient temperature. The extract samples and isolated heracleninwas injected using hamilton syringe (Switzerland) into injection loop, and were detected at 215 nm (Fig:6).Theamount of heraclenin (Retention time =6.4 min) present in the extract was estimated using isolated heraclenin (Table 2).

1.10 Microbial Biotransformation of Heraclenin

Biotransformation of heraclenin was attempted utilizing phytopathogenic fungi, *Botrytis cinerea*, *Curvularialunata*, *Aspergillusniger* in order to obtain novel Heraclenin derivatives with possible enhancement in the activity of the parent compound. The biotransformation process was carried out according to the previous reports [19]. Aliquots of the organic layers were analyzed by TLC. Blank assays without substrates and without fungi were carried out in parallel as controls.

2. RESULTS AND DISCUSSION

2.1 Extraction of *Aeglemarmelos* with various solvents:

Simultaneous extraction of AMFP extracts with equal volume of different solvents was employed.The percentage (%) yield of extraction varied with the polarity of solvent used. As per weight, least polar solvent

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hexane yielded the lowest amount of extract followed by mid-polar chloroform and high polar solvent ethanol **(Table: 1).**

Extract	Extract yield (% w/w)
Hexane	0.7128
Chloroform	1.4422
Ethanol	5.2637

Table 1: Amount of extracts obtained in solvents with different polarities

2.2 IC_{50} Determination for the AMFP extracts

MTT assay with the THP-I cells was used to identify the IC_{50} value for all the AMFP extracts. The IC_{50} value of the hexane, chloroform and the ethanolic extracts were calculated as 2.06 mg/mL, 2.54 mg/mL and 3.18 mg/mL respectively **(Fig:1)**.The IC_{50} values obtained from this experiment helped in carrying out intracellular ROS activity of the extracts by using flow cytometry.

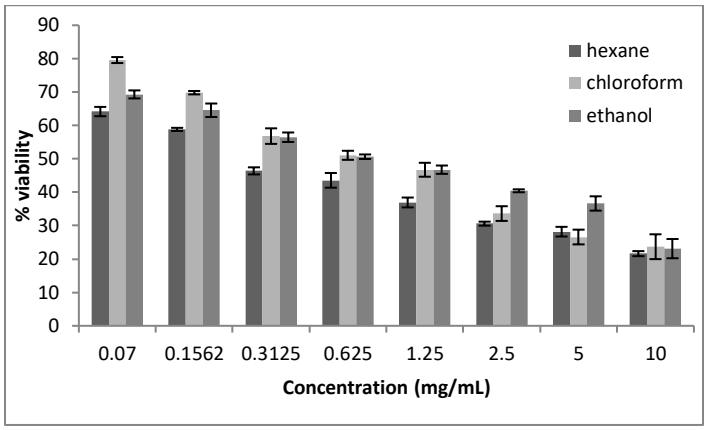

Fig: 1. *In-vitro* activity of the extracts against THP-1 cell line (A) the percent cell viability of THP-1 cells against the three AMFP extracts measured by MTT assay at `24 hr`.

2.3 Screening the AMFP extracts for intracellular antioxidant activity in THP-1 cells

Excessive intracellular ROS has been associated with inflammatory disorders like atherosclerosis and cancer. Ability of compounds and extracts to modulate and reduce the ROS has long been accepted as an indicator of a compound's ability to therapeutically alter the inflammatory process. The three extracts i.e., hexane, chloroform and the ethanolic extract of AMFP were screened to identify their effect on intracellular ROS in THP-1 cell lines. The objective of the screening was to identify the extract with most potent intracellular anti-oxidant activity for further downstream investigation and characterization. All the three AMFP extracts, showed ability to reduce the intracellular ROS levels compared to the H_2O_2 control group. However, AMFP chloroform extract showed significant

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higher ability to reduce the intracellular ROS activity in THP-1 cell lines compared to hexane and ethanolic AMFP extracts**(Fig: 2).** Hence further investigations of chloroform AMFP extract was carried out to identify the presence of any active molecule with ability to modulate intracellular ROS.

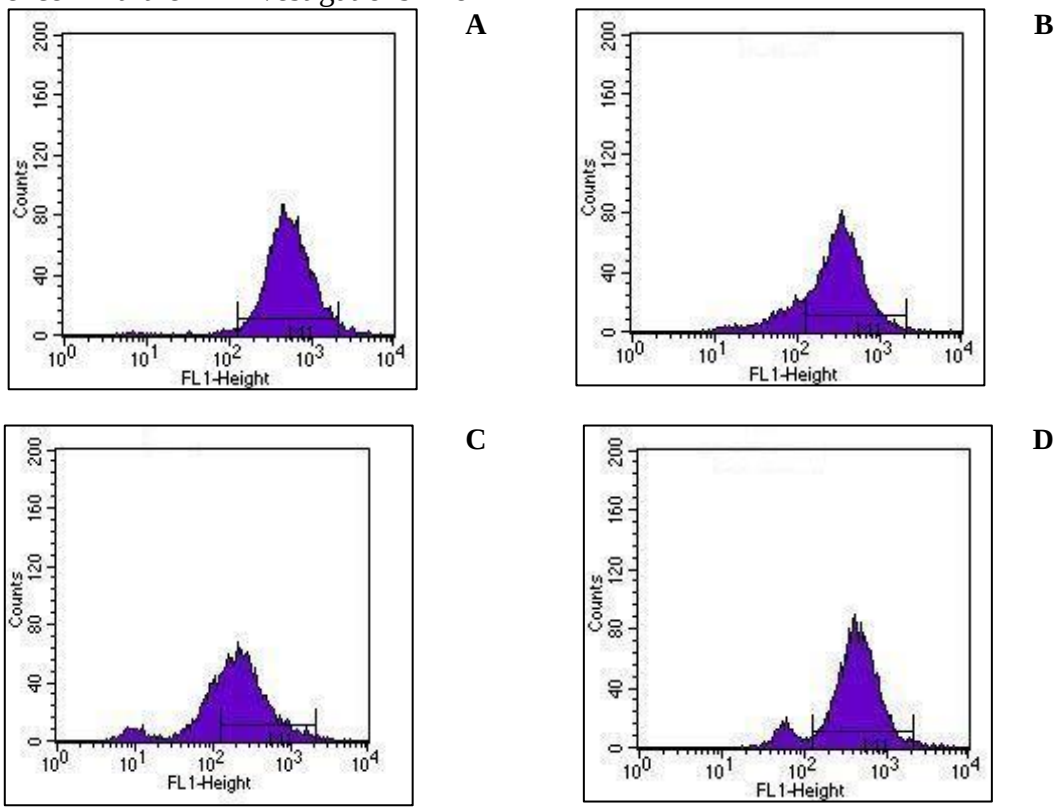

Fig: 2. Histogram plots obtained from BD FACS **for different solvent extracts of AMFP showed varying degree of intracellular ROS inhibition in THP-1 cells compared to the positive H_2O_2 treated cells stained with DCFH-DA (A) H_2O_2 treated (B) THP-1 and $CHCl_3$ (C) THP-1 and Hexane (D) THP-1 and Ethanol**

2.4 Isolation and characterization of Heraclenin:

Chloroform AMFP extract showed the most potent intracellular antioxidant reducing ability in THP-I cell line. Further, TLC investigation of the chloroform extract revealed significant presence of at least one compound. Hence the AMFP Chloroform extract was subjected to conventional column chromatography to isolate any compound that could alter intracellular ROS. The column chromatography of AMFP Chloroform extract resulted in a white solid. The isolated pure compound was characterized using ^{1}H&^{13}C NMR, Mass and FT-IR spectroscopic analysis. The ^{1}H&^{13}CNMR, spectral data of the compound matched with existing literature [20]report and was identified as Heraclenin. **(Fig: 3)**

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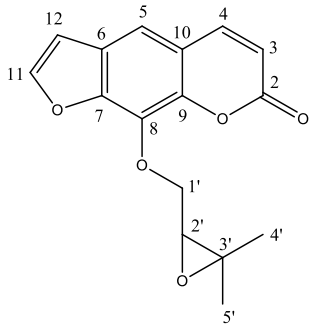

Fig: 3. Heraclenin

2.5 Structural characterization of isolated compound

^{1}H-NMR (Bruker, 500MHz, $CDCl_3$, δ ppm): 6.38(1H, d, J=9.5 Hz, H-3), 7.78(1H, d, J=9.5Hz, H-4), 7.41(1H, S H-5), 7.70(1H, d, J=2.5Hz, H-11), 6.83(1H, d, J= 2.0Hz, H-12),4.59 (1H, d, J = 5.5 Hz , H-1'), 4.60 (1H, d, J = 5.5 Hz, H-1'),3.32 (1H, t, J = 5.5Hz , H-2'), 1.29 (3H, S, H-4'), 1.35 (3H, S,H-5')

^{13}C-NMR (Bruker, 500MHz, $CDCl_3$, δ ppm): 160.32(C-2), 114.84(C-3), 144.30(C-4), 113.87(C-5), 126.00(C-6), 148.35(C-7), 131.49(C-8), 143.65(C-9), 116.53(C-10), 146.82(C-11), 106.82(C-12), 72.48(C-1'), 61.33(C-2'), 58.17(C-3'), 24.56(C-4'), 18.87(C-5'),

IR(cm^{-1}) : 3513(C-C-O), 3127(Aromatic-CH), 2970(CH), 1719(O-C=O), 1592, (-C=C-), 1403(CH_3), 1102(C-O).

Melting point: 113-114^{0}C

2.6 Evaluation of IC_{50} and ROS activity of Heraclenin:

The MTT assay earlier carried out for AMFP extracts was repeated with isolated Heraclenin and IC_{50} value was determine to be 0.223 mg/mL **(Fig: 4A).**To study the potential of Heracleninas an intracellular ROS reducing agent in THP-1 cells DCFDA study was performed with the IC_{50} values obtained from the MTT assay. Heraclenin showed very strong ability to reduce ROS in THP-1 cell lines compared to all the AMFP extracts and the H_2O_2 control samples **(Fig: 4B).**

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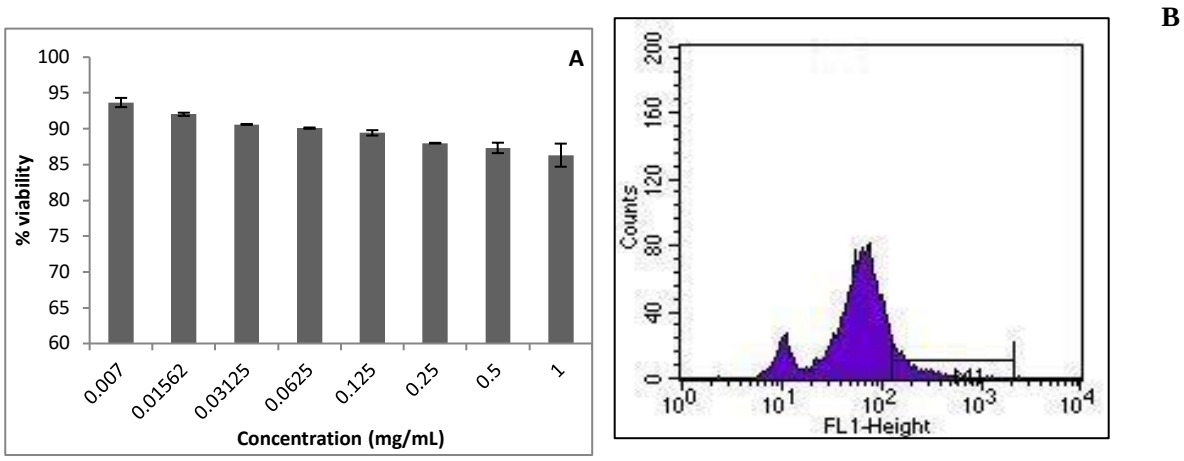

Fig: 4. *In-vitro* activity of the Heracleninagainst THP-1 cell line **(A)**the percent cell viability of THP-1 cells against the isolated compound Heraclenin.**(B)**Histogram plots obtained from BD FACS **for Heraclenin showed varying degree of intracellular ROS inhibition in THP-1 cells compared to the positive H_2O_2 treated cells stained with DCFH-DA.**

2.7 Free radical scavenging activity on DPPH

Based on the results obtained from intracellular antioxidant assay, DPPH assay was carried out to identify the free radical scavenging activity of the AMFP extracts and heraclenin. The AMFP extracts and heraclenin showed concentration dependent ability to scavenge free radicals**(Fig: 5).**Comparison of the IC_{50} values of all the AMFP extracts, indicated that AMFP hexane extract (IC_{50} = 2.034 mg/mL) had better radical scavenging activity compared to Chloroform (IC_{50}= 1.786mg/mL) and ethanolic extracts (IC_{50} = 1.128 mg/ml). **(Fig: 5A).** TheIC_{50} value of Heraclenin was calculated as 0.196 mg/ml **(Fig: 5B).**

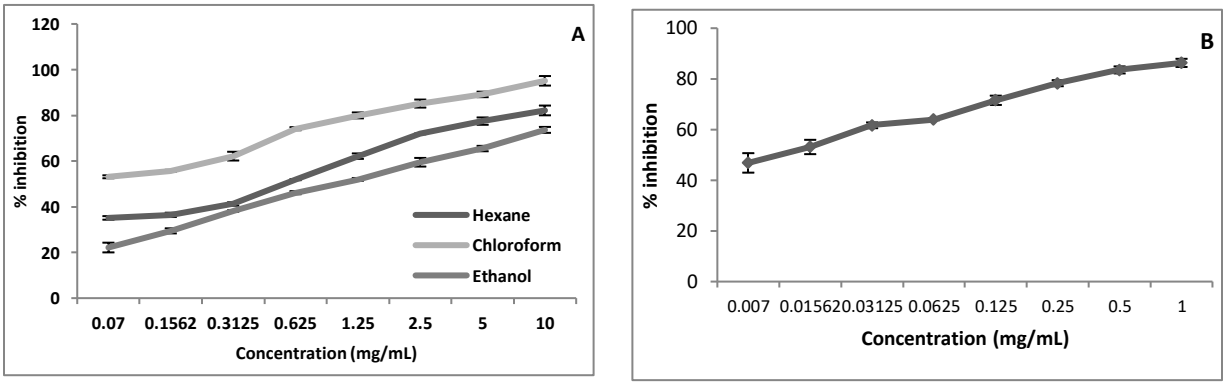

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Fig: 5. DPPH• radical scavenging activity of AMFP extracts **(A)** Concentration dependent radical scavenging effects of different concentrations of AMFP extracts **(B)** Radical scavenging effect of Heraclenin at different concentrations

2.8 Apoptotic activity of Heraclenin and AMFP extracts.

The cytotoxicity of a compound against cancerous cell lines is a strong indication of anticancer activity of the compound. PI based cytolysis assay using flow cytometer helps distinguish between living and dead cells. PI being an intercalating fluorophore binds to DNA of cells with compromised or lysed membrane, while live cells do not take up PI. AMFP extracts and heraclenin treated THP-1 cells were stained with PI and analyzed using flow cytometry. All three extracts showed significant cytotoxic activity. The AMFP hexane extracttreated cells showed the least PI positive cells (54.83%) while the chloroform and ethanolAMFPextracts showed a cell death percentage of 76.32% and 86.42% respectively. Interestingly the compound heraclenin isolated from Chloroform did not show much cytotoxicity at the concentration tested. Indicating that in spite of its ability to reduce intracellular ROS, heraclenin might not have strong cytotoxic activity against THP-1 cells. **(Fig: 6)**

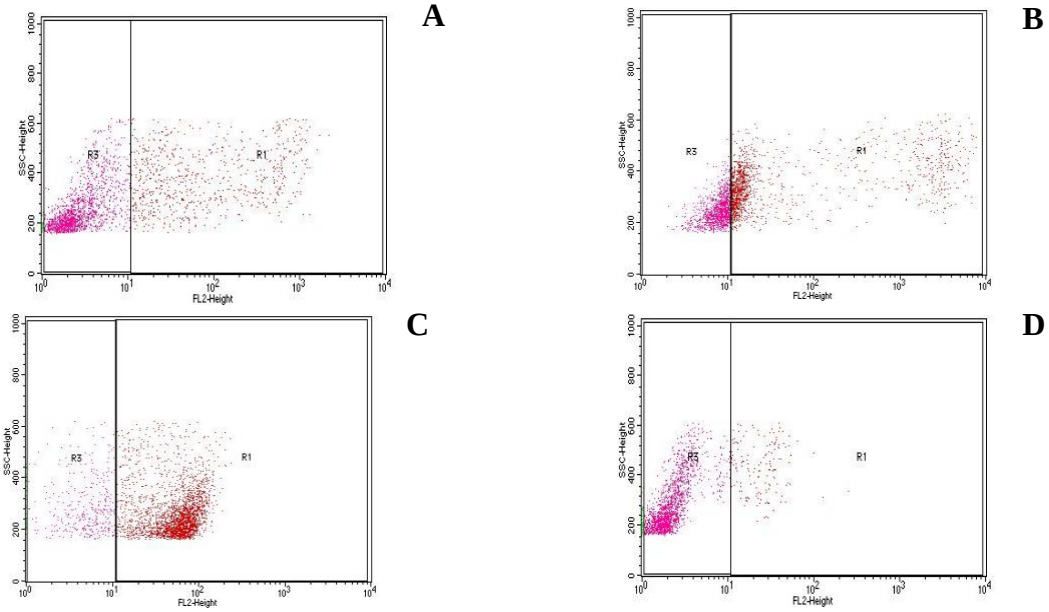

Fig: 6. FACS dot plot of PI stained THP-I cells treated with AMFP extracts and Heraclenin (A) Control, (B) AMFP hexane extract treated cells (2.5mg/mL), (C) AMFP chloroform extract treated cells(2.5mg/mL), (D) AMFP ethanol extract treated cells (2.5mg/mL) (E) THP-1 cells treated with Heraclenin (0.25mg/mL)

2.9 HPLC estimation of heraclenin in AMFP chloroform extract:

Intra cellular antioxidant studies conducted in THP-I cells and DPPH assay showed that heraclenin had a significant antioxidant potential. This antioxidant ability of heraclenin makes it a promising candidate for anti-inflammatory therapeutics. Further,

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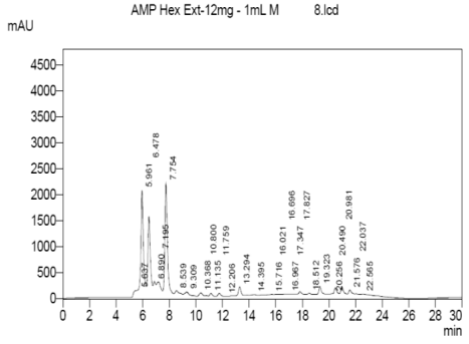
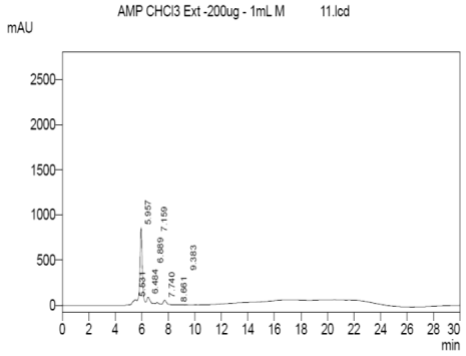
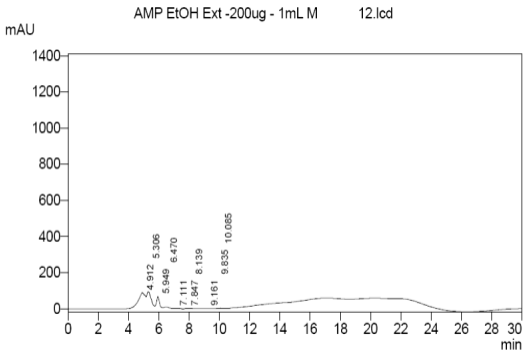
earlier literature reports (21) also highlight heraclenin has a promising therapeutic agent. Hence, it would be advantageous to identify the best method for enriching and isolating the compound from the AMFP. As mentioned earlier, the current study deals with solvent of three different polarities and their ability to efficiently extract phytoconstituents from AMFP. Considering the fact that one of the phytoconstituents of AMFP, heraclenin was identified in all three extracts through TLC, all the three extracts were subjected to RP-HPLC. The objective of subjecting the AMFP extracts to HPLC was to identify the extract with the highest amount of heraclenin. For this study heraclenin isolated from AMFP chloroform extract was used as a standard. The results were calculated from the HPLC chromatogram peak area and expressed in (% w/w) of the extract. Based on the HPLC results AMFP chloroform extract had the highest yield of heraclenin (4.837%) followed by hexane (1.078%) and ethanol extracts (0.09482%) respectively (**Fig: 7)**. The results clearly indicate that the extraction of AMFP extract with chloroform as solvent would be the best method for extraction and isolation of heraclenin compared to low polar hexane or highly polar solvent ethanol.



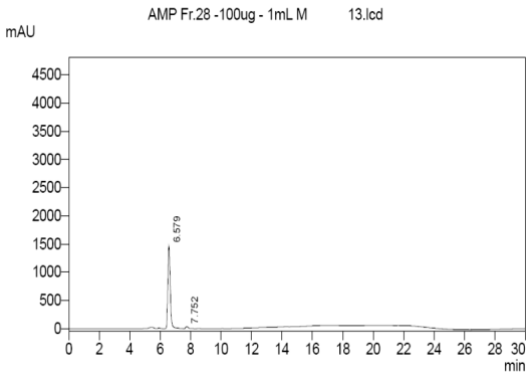
AMP – Hexane Extract



AMP – Chloroform Extract



AMP – Ethanolic Extract



Isolated compound(Heraclenin)

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Fig: 7- HPLC chromatogram for AMFP Hexane, Chloroform and Ethanol extracts compared with the standard Heraclenin.

Table 2: Percentage **(% w/w)** Heraclenin isolated from AMFP extracts

Extract	**Percentage (% w/w) of isolated compound**
Hexane	1.078
Chloroform	4.837
Ethanol	0.9482

3.10 Biotransformation of Heraclenin with the growing cell systems of the fungus

The biotransformation of heraclenin was carried out in an attempt to obtain derivatives of the molecule with enhanced activity. However, the three microorganisms tested for this process were unable to biotransform heraclenin.

3. CONCLUSION

AMFP has been in use as a traditional medicine to treat ailments in human subjects. This has made it an ideal target for several researchers, intended on identifying AMFP phytoconsituents, as well as deconvoluting its role as a therapeutic drug. The current study proceeded with the target of identifying the most suitable polar solvent for generating antioxidant rich extracts of AMFP. In this regard, the AMFP chloroform extract showed the most potent ability to reduce intracellular ROS in THP-I cells compared to both hexane and ethanol extracts. The AMFP choloroform extract also yielded a furanocoumarin, Heraclenin. The isolated compound showed significant intracellular antioxidant reducing ability in THP-1 cells as well as significant radical scavenging effect in the DPPH studies. HPLC analysis showed chloroform to be the most suitable solvent for generating a heraclenin rich AMFP extract. In conclusion, the study demonstrates that, chloroform to be the most suitable solvent for generating heraclenin rich AFMP extract. Also, AFMP extract, due to its low cytotoxicity and high anti oxidant activity, is a potential drug for anti inflammatory disorders involving disease like atherosclerosis.

CONFLICT OF INTEREST

Authors declare no conflict of interest regarding this research paper

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