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Identification of bio-active compounds and invitro anti-inflammatory evaluation of *Couroupita guianensis* Aubl. fruits

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Abstract

Couroupita guianensis, commonly known as Cannon ball tree, is a well-known medicinal and ornamental plant. The tree is known for its broad spectrum of pharmacological activities for treating various human illnesses. The research study was based on identification of bio-actives and evaluation of the invitro anti-inflammatory activity of cannon ball fruits. The methanolic extract of dried fruits was sequentially extracted with Ethyl acetate and Butanol. The marker compounds viz., Tryptanthrin, Indirubin, Methyl perillate and Azealic acid are identified and validated by LCMS and LCMS/MS. Further, Azelaic acid and Methyl perillate are reported for the first time in *Coroupita guianensis* fruit extract. Also, the Ethyl acetate sub extract and its column fraction recorded promising invitro anti-inflammatory activity of 64.50% and 81.93% at 200ug/ml against LPS induced IL-6 Cytokine in THP1 cell line respectively. These research findings establish the preliminary invitro data on effectiveness and pharmacological rationale for use of *C. guianensis* fruit extract as an anti-inflammatory agent.

Key words: Cannon ball fruits, LCMS/MS, Tryptanthrin, Anti-inflammatory

Introduction

Couroupita guianensis Aubl. of Lecythidaceae family is a well-known medicinal and ornamental plant, widely distributed in India, Sri Lanka, South America, and Malaysia. It is commonly known as Cannon ball tree, Nagalinga pushpam, Ayahuma, Kailaspathi and Mallikarjuna (1). Cannon ball tree has been known for its traditional and cultural importance since ancient times and is considered as a sacred tree by Hindus. The shape of the flower resembling the sacred snake, protecting Lord Shiva (2). The tree is known for its broad spectrum of pharmacological properties such as antibacterial, antirheumatic, antithrombotic, antioxidant, anti-inflammatory etc., for treating broad range of human illness such as hemorrhage, scabies, dysentery, scorpion sting, hypertension, tumors, malaria, toothache, and skin diseases (1 & 3). It is reported that the native Brazilians and Amazonian people consumed infusions or tea prepared from leaves, flowers, and barks for overall health benefits. Fruits are edible but their unpleasant smell discourages most people from consuming. However, the Negroes of Guiana prepare the medicinal drink with fruits for treatment of cold, headache, stomach related issues and to disinfect wounds (4). Availability of the literature/research papers on the phytocompounds in cannon ball fruits is minimum. The present research was thus, aimed to completely evaluate the underexplored cannon fruits and to conjoin the scientific rationale to their traditional applications.

Materials and Method

Collection of plant material

Coroupita guianensis dried fruit was collected and authenticated by the pharmacognosy department of Himalaya wellness company with Authentication number of NPD/047/2024.

Preparation of extracts

The dried cannon ball fruits were powdered in a laboratory blender. About 100g of the powdered sample was independently extracted using solvents viz., Hexane, Ethyl acetate Acetone, Methanol, 70% Methanol and Water in a Soxhlet apparatus. The solvent was heated to reflux, with the temperature maintained at 70º C for Solvents and 95 ºC for water extraction. The condenser cools the solvent vapor, which further drips back down into the chamber housing the sample. Sample with respective solvent was extracted for 3times, wherein, each extraction was carried out for 3hours. After the complete extraction, the samples were individually filtered and evaporated to dryness in rotary vacuum evaporator. The extract yield (%) is recorded and stored in an airtight container until further analysis.

High performance liquid chromatography

The preliminary screening of the extracts (Hexane, Ethyl acetate Acetone, Methanol, 70% Methanol and Water) prior to LC-MS is carried out in HPLC, equipped with C18 column (250mm*0.25mm, 0.25µm) using 0.1% Orthophosphoric acid and Acetonitrile in a gradient mode.

Extraction and isolation

The coarse dried fruit powder (5Kg) was completely extracted with Methanol and then evaporated to dryness under reduced pressure to obtain the dark green residue of crude methanol extract. The methanolic extract (200 g) was distributed in Ethyl acetate and Butanol respectively and the solvents were evaporated under pressure to obtain the sub extracts viz.; Ethyl acetate (Red colored, 28 g), Butanol (Brown colored, 18 g) and the remainder was insoluble in distributed solvents is considered as Water (126 g). Further, the Ethyl acetate subfraction was subjected to Normal phase column chromatography with Hexane: Ethyl acetate (Gradient, 0-100% Ethyl acetate) to give 5 fractions. These fractions were spotted on the TLC with Hexane: Ethyl acetate, (8:2) (v/v) solvent system. The compounds were detected by illuminating under UV light at λ=254/366 nm and spraying with 10% Vanillin Sulphuric acid reagent and then heating at 105^{0}C for 1-2 min in Hot air oven.

Optimization of LC-MS conditions

A Shimadzu LC-40D X3 series pump and DUG-405 series Shimadzu degasser in binary gradient mode was used to deliver a mobile phase consisting of Pump A: 0.1% formic acid Buffer and Pump B: Acetonitrile. Solvent in pump B was linearly ramped from 5 to 20% in 10 minutes and then from 20 to 45% in 10min, followed by 45% to 60% in 5min and then increased to 90% in the next 5 minutes followed by decreased from 90% to 5% in 10 minutes and re-equilibration at 5% for an additional 5 minutes. The mobile phase was delivered at a flow rate of 1.0 mL/min. The separation was achieved through Phenomenex Luna C18 (250 X 4.6 mm, 5µm) column. The injection volume 5µL was injected through SIL-40C X3 Shimadzu auto sampler and an ambient temperature was achieved through CTO-40 C column oven at 40°C. Total run time was 45 minutes (5).

Validation by LC/MS/MS

LC-MS/MS Analysis was carried out using SCIEX 6500+ QTRAP tandem mass spectrometer with an Electro Spray Ionization (ESI) source, coupled to a Shimadzu HPLC system. Compound identification was performed initially with IDA enabling Enhanced Product Ion (EPI) scan mode and fragmented targeted molecular ion in negative ionization mode. Further, in multiple reaction monitoring (MRM) Scan mode peak elution region was reconfirmed. Ion spray voltage (IS) was set to 5500 V, the source tem-

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perature was maintained at 550 °C, Curtain (CUR) gas 40 psi, source gas 1 (GS 1) and source gas 2 (GS 2) respectively. Batch acquisition was controlled by Analyst 1.7 version software and data processing, and interpretation were performed in SCIEX OS software.

Evaluation of the invitro anti-inflammatory activity

The cannon fruit extract and column fractions were evaluated for its invitro anti-inflammatory activity by estimating the inhibition of LPS induced production of IL-6 cytokine in THP1 cell lines. In this assay, the Human Monocyte cell line, THP-1 was cultured in RPMI1640 media with 10% heat inactivated FBS. The cytotoxicity of the samples was tested by MTT cytotoxicity assay method and non-cytotoxic concentrations were used for assay. The cells were treated in duplicates with different concentrations of test samples along with the stimulant 1µg/ml LPS (to induce inflammatory cytokines) in RPMI1640 media with 2% heat inactivated FBS and incubated at 37°C with 5% CO_2 for 24 hours. After incubation, the cell supernatant was separated by centrifugation. IL-6 levels were estimated by ELISA performed according to the assay procedure provided by the manufacturer (Elabscience ELISA kit). Dexamethasone (100µM) was used as a reference standard for the assay. The percentage Inhibition of cytokine production by samples was calculated based on the LPS treated cell control.

Results and Discussion

The *Coroupita guianensis* (Cannon ball) fruits were extracted in a Soxhlet apparatus with solvents of polarity ranging from non-polar to polar respectively. The extract yield (%) (w/w) is tabulated in Table 1. The results reported that the aqueous solvent recorded the highest extract yield of 18%, followed by 70% Methanol (14%), Methanol (12%), Acetone (4.54%), Ethyl acetate (3.95%) and the lowest yield was recorded in Hexane (1.32%).

Table 1: Cannon ball fruit extract yield (%) with the respective solvent systems

Sl.no	Solvent	Extract Yield (%)
1	Water	18.00
2	70% Methanol	14.00
3	Methanol	12.00
4	Acetone	4.54
5	Ethyl acetate	3.95
6	Hexane	1.32

HPLC analysis

The preliminary data on the compounds present in different extracts were evaluated through HPLC analysis. The pattern was established by eluting the respective extracts in 0.1% O-Phosphoric acid and Acetonitrile in a gradient manner at three different wavelengths (205 nm, 254 nm and 330 nm) (Fig 1, 2 and 3).

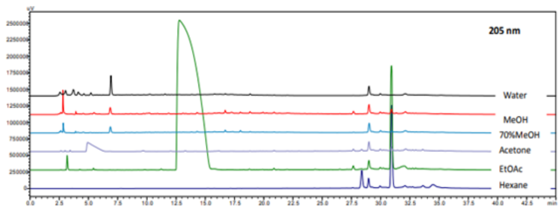

Fig 1: HPLC overlay of the Cannon ball fruit extracts at 205 nm

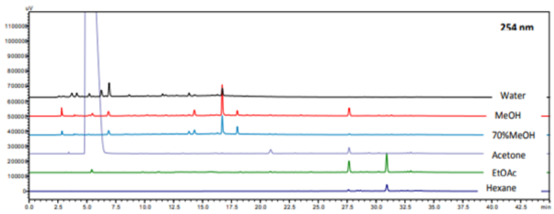

Fig 2: HPLC overlay of the Cannon ball fruit extracts at 254 nm

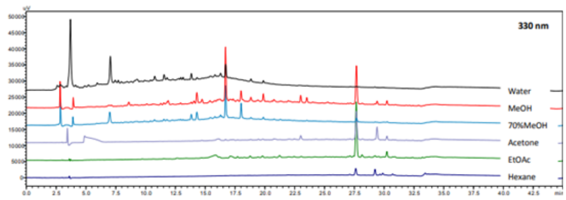

Fig 3: HPLC overlay of the Cannon ball fruit extracts at 330 nm

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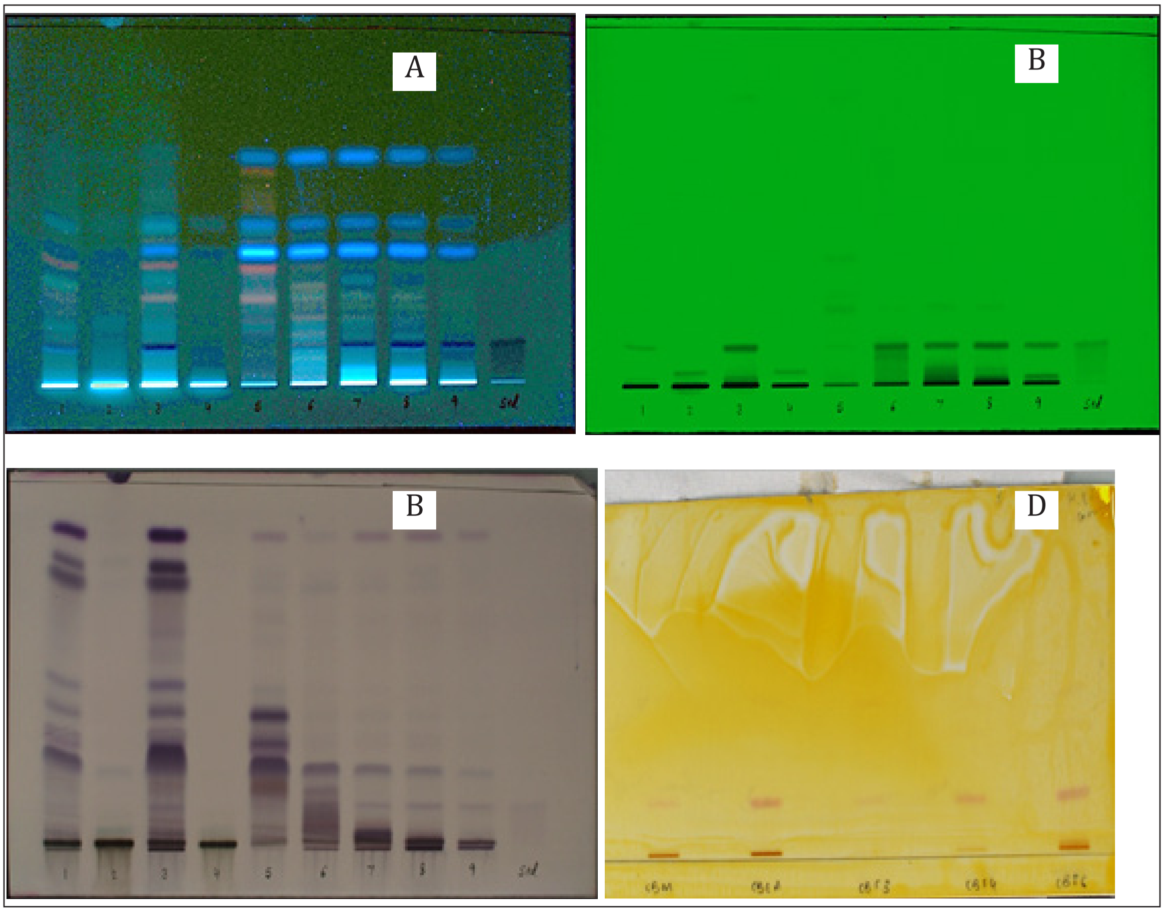
Isolation by column chromatography

The Ethyl acetate sub extract slurry was eluted with Hexane and Ethyl acetate (v/v) solvent system. The yield of 5 different fractions collected are reported in Table 2. These fractions were spotted by illuminating under UV light at λ=254/366 nm and spraying with 10% Vanillin Sulphuric acid reagent and Dragendorff reagent and then heating at 105^{0}C for 1-2 min on a hot plate (Fig 4). The results infer that the compounds separated are a class of alkaloids and phenolic acids.

Table 2: Yield (%) of the Ethyl acetate sub extract collected fractions

Sl.no	Fractions	Yield (%)
1	Fraction 1	7.25
2	Fraction 2	2.25
3	Fraction 3	2.45
4	Fraction 4	1.87
5	Fraction 5	5.00



1: Methanol Extract; 2: Butanol sub extract; 3: Ethyl acetate sub extract; 4: water sub extract; 5: Fraction 1; 6: Fraction 2; 7: Fraction 3; 8: Fraction 4; 9: Fraction 5; STD: Indirubin standard

Fig 4: TLC fingerprint of Cannon ball fruit extracts and column fraction as compared to the standard observed at A: 366nm; B: 254nm; C: After spraying with vanillin Sulphuric acid reagent; D: After spraying with Dragendorff reagent

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Lc-ms/ms analysis of cannon ball fruit extracts

In this study, QTRAP enabled Liquid Chromatography-tandem mass spectrometry technique is applied to get higher selectivity and sensitivity for analyzing complex herbal matrices. Both positive and negative ionization mode are employed for analysis and identification of the targeted natural compounds in cannon ball extract. LC-MS/MS analysis was carried out for Methanol extracts, Ethyl acetate sub extract, Butanol sub extract and Water sub extract by accurately weighing 0.5 g of extracts and transferring into 50 mL falcon tube separately. The extracts were dissolved and sonicated in 50 ml of methanol for 10 mins at room temperature. The processed extracts were filtered through 0.22 µm PTFE filters and collected 1ml in autosampler vial. The final test solutions were then subjected to LC-MS/MS analysis.

The initial fragmentation pattern reported Tryptanthrin, Indirubin in positive ion mode, while Azelaic acid and Methyl perillate in negative ion mode in all the extracts except for Butanol sub extract and water sub extract. Further validation of Tryptanthrin and Indirubin was carried out through MRM mode (Table 3, Fig 5).

Table 3: Compounds identified through LCMS/MS

Sl.no	Name	Ion mode	Molecular weight	Molecular formula
1	Tryptanthrin	Positive	248.24 g/mol	$C_{15}H_8N_2O_2$
2	Indirubin	Positive	262.26 g/mol	$C_{16}H_{10}N_2O_2$
3	Azelaic acid	Negative	188.22 g/mol	$C_9H_{16}O_4$
4	Methyl perillate	Negative	180.24 g/mol	$C_{11}H_{16}O_2$

The ions in the MRM mode of the mass ion chromatogram for Tryptanthrin and indirubin were at m/z 249 and 263 respectively, and the corresponding retention times were 26.55 and 29.27 min.

For Tryptanthrin, the protonated molecular ion at *m/z* 249.1 $[M+H]^+$ was observed at 26.55 mins retention time and product ion scan yielded m/z 221.0, 130.0, 102.0 which shown great intensity as well as characteristic fragments in the MS2 scan possess the quinazoline ring which confirms the compound reported as Tryptanthrin. Further, Multiple Reaction Monitoring (MRM) mode was enabled with optimized Collision energy (v) and Declustering potential (v) (6). The final transition ion (m/z) for Tryptanthrin are given in Table 4 and Fig 6. Tryptanthrin, plant alkaloid, is one of the medicinally important phytocompound, known for anti-inflammatory, anticancer, antimicrobial; suppress angiogenesis, and is also considered as a remedy against Athletes foot (7). It is also reported to block the leukotriene production in neutrophils and in whole blood assays, inhibit P-glycoprotein and sensitizes resistant cancer cell lines to killing by cytotoxic agents (8).

Similarly, another protonated molecular ion *m/z* 263.1 $[M+H]^+$ obtained at 29.27 mins and shown the fragmented ion *m/z* 219.0, 190.0, 167.1 established the presence of indole alkaloid, which further confirms the presence of Indirubin, a natural pigment compound. Further, Multiple Reaction Monitoring (MRM) mode was enabled with optimized Collision energy (v) and Declustering potential (v) (5). The final transition ion (m/z) for Indirubin are given in Table 4, Fig 7Aand B. Indirubin is the red isomer of indigotin, also known as the “red” of indigo. Indirubin occurs only in natural indigo and not in a synthetically produced pigment. Indirubin is reported to ameliorate ulcerative colitis, due to its anti-inflammation, immune regulation, intestinal microbiota regulation, oxidative stress regulation, and intestinal mucosal repair (9-12).

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Table 4: MRM Transition of Tryptanthrin and Indirubin

Name of the compound	Q1 mass	Q3 Mass	Collision Energy (CE) in Volts	Declustering Potential (DP) in Volts
Tryptanthrin	249.1	221.0	30	20
		130.1	40	
		102.1	50	
Indirubin	263.1	219.0	20	35
		190.0	35	
		167.1	45	

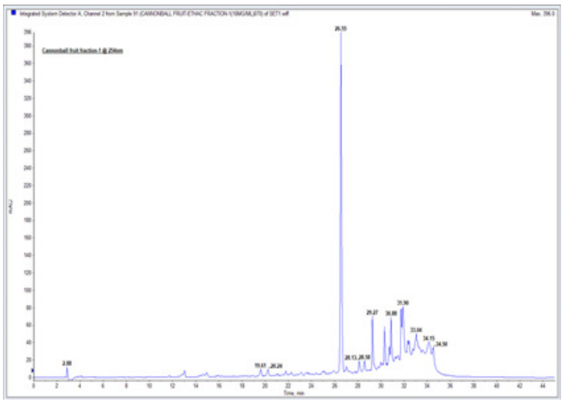
Fig 5: LCMSMS profile of Cannonball fruit extract fraction

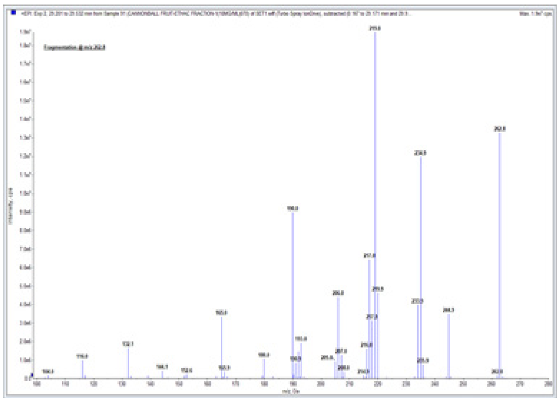
Fig 7A: Indirubin Mass fragmentation at 29.27 mins RT

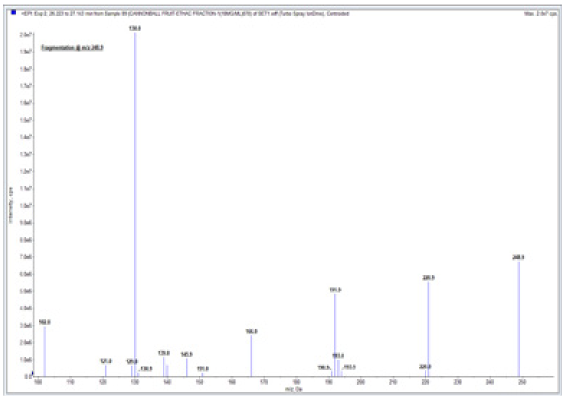
Fig 6: Tryptanthrin Mass fragmentation at 26.55 mins RT

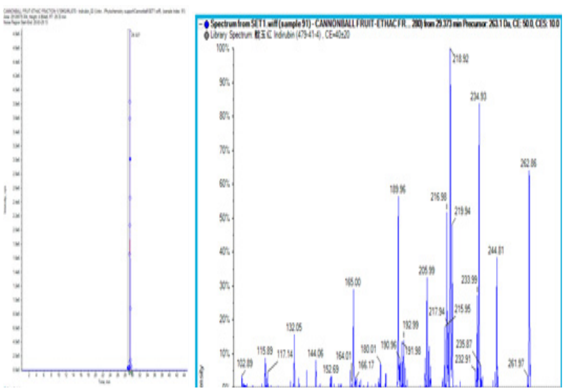
Fig 7B: LCMS profile of Indirubin compound

Azelaic acid was identified in negative ion mode with m/z 187.0968 $[M-H]^-$, $C_9H_{16}O_4$. The fragment ion at m/z 143.1070 $[M-H-CO_2]^-$ was formed due to the loss of a CO_2 molecule. The fragment ion at m/z 125.0967 $[M-H- CO_2 -H_2O]^-$ was derived from the ion at m/z 143.1070 by the loss of an H_2O molecule. The fragment ions at m/z 97.0654 $[M-H-CO_2- H_2OC_2H_4]$ − was derived from the ion at m/z 125.0967 by the loss of fragment ion of C_2H_4 (13) Azelaic acid inhibits mitochondrial respiration, Inhibits *S. epidermidis* and *P. acnes* by interfering with protein syn-

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thesis, reduces DNA and RNA synthesis. Azelaic acid is a competitive inhibitor of mitochondrial oxidoreductases and of 5 alpha-reductase. Possesses bacteriostatic activity to both aerobic and anaerobic bacteria. Also acts as anti-keratinizing agent, with antiproliferative cytostatic effects on keratinocytes (14-15).

Similarly, Methyl perillate was identified in negative ion mode with m/z 180.24 [M+H], $C_{11}H_{16}O_2$. Methyl perillate is the new compound identified in the collected fractions of ethyl acetate extract of cannon ball fruits. Methyl perillate was reported to be the effective against mosquito (*A.aegypti*) larvae with the LC_{50} of 16 ppm in larval bioassays and can be considered as a potential biodegradable mosquito controlling agent (16). It is a highly functionalized plant compound which is considered as a precursor for terephthalic acid. Another area of application includes the conversion of perillartine to methyl perillate for synthesis of juvabione, a juvenile hormone (17-18).

Invitro anti-inflammatory activity of the C. guainensis fruit extract and its column fraction

Chronic inflammation can become fatal and can lead to heart attacks and even strokes as it destabilizes cholesterol deposits in the coronary arteries. Cytokines mediate and regulate the inflammatory responses through anti-inflammatory interleukins and adhesion molecules. In the present study, the Invitro Anti-inflammatory activity was evaluated for Ethyl acetate sub extract and its column fraction F-1 by estimating the inhibition of LPS induced IL-6 cytokine production in human acute monocytic leukemia (THP-1) cells under inflammatory conditions. The samples in duplicates have reported dose-dependent inhibition at non-cytotoxic concentrations of 200 µg/ml and 100 µg/ml against Dexamethasone as the reference standard (Table 5 and Fig 8). The Ethyl acetate sub extract and its column fraction-1 recorded promising invitro anti-inflammatory activity of 64.50% and 81.93% at 200ug/ml against LPS induced IL-6 Cytokine in THP1 cell line respectively. The activity can be attributed to phytocompounds viz., Tryptanthrin, Indirubin and Azelaic acid. As per the previous literature, the fruit pulp at 50 mg exhibited the anti-inflammatory activity of 55.56 % concentration by in vitro HRBC membrane stabilization method (19-20).

Table 5: % Inhibition of IL-6 production by Ethyl acetate sub extract and its column fraction in THP-1 cells.

Sample (µg/ml)		% IL-6 Inhibition
LPS control		-
Fraction-1	200	81.93± 2.09
	100	40.26± 1.95
Extract	200	64.50± 1.51
	100	30.25± 0.97
Dexamethasone (100µM)		91.62%

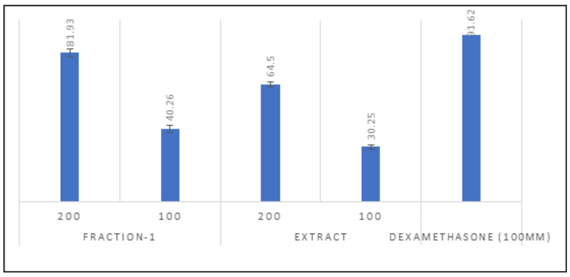

Fig 8: % IL-6 Inhibition of Fraction 1 and Ethyl acetate sub extract on LPS (1µg/ml) induced THP-1 cells. Dexamethasone was taken as reference standard.

Conclusion

Coroupita guianensis (Cannon ball tree) is known for its broad spectrum of pharmacological activities. The present study aims at profiling of *C.guianensis* fruit extract and screening for its anti-inflammatory activity. Marker compounds viz., Tryptanthrin, Indirubin, Azelaic acid and Methyl perillate are identified and validated through LCMS, of which, Azelaic acid and Methyl perillate are reported for the first time in *Coroupita guianensis* fruit extract. The Ethyl acetate column fractions recorded promising an-

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ti-inflammatory activity of 81.93% against LPS induced IL-6 Cytokine in THP1 cell line as compared to Dexamethasone (91.62%). The preliminary invitro study reported that *C.guianensis* fruit extract can be considered as a promising anti-inflammatory ingredient against inflammatory diseases. Additionally, in-vivo validation is required for further understanding of the metabolic pathway and safety assessment.

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