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Down Regulation of Inflammatory Cytokines by Protocatachuic Acid Isolated from *Blepharis maderaspatensis* (L.) B.Heyne ex Roth Leaves.

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ABSTRACT

Blepharis maderaspatensis whole plant was collected from near agricultural field in Chengalpattu district, Tamil Nadu. The dried leaf powders extract with 90% aqueous alcohol and re-fractionated with different organic solvents like petroleum ether, chloroform, ethyl acetate and acetone. Totally five extracts were obtained and screened for phytochemical constituents. Altogether, methanolic fraction shows good antioxidant activity using $ABTS^{+}$, DPPH method. And it was shows higher content of total flavanoid, total alkaloid, total saponin, total tannin, total carotenoids, total polyphenolics while compared with other fractions. Finally this was taken for screening of cytotoxicity activity and antiinflamatory activity on normal Vero cell line and macrophage RAW 264.7 cells. A purified fraction from methanolic extract and analyzed using TLC, UVVIS spectrophotometer, FTIR, ESIMS, ^{1}HNMR, ^{13}CNMR for structural elucidation and identified as Protocatachuic acid. This purified compound used for antiinflamtory activity and cells was harvested from drug treated concentrations and analyzed for cytokines mRNA expression studies like iNOS, COX2, TNF α, IL1, IL6 Cytokines using RTPCR. The purified compound shows significantly lowering the cytokine expression at 20µg/ml compared with LPS and $p \leq 0.001$ value consider as significance. Altogether the isolated compound has potential antiinflammatory activity on LPS induced inflammation.

Keywords: *Blepharis maderaspatensis,* RTPCR, Cytokines, Phytochemical, mRNA.

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INTRODUCTION

The term inflammation comes from the Latin word *"inflammo"* and meaning *"I set alight, I ignite"*. Inflammation is part of the complex innate immune response of body tissues to harmful stimuli, such as pathogens, damaged cells or irritants and it may categorized into acute and chronic inflammation. Inflammation is a defensive response that involves immune cells, blood vessels and molecular entities. The role of inflammation is to eliminate the initial cause of cell damage, clear out necrotic cells, tissues damaged from the original rudeness, inflammatory process and to initiate tissue repair. Inflammated macrophages discharge a broad range of mediators such as several proinflammatory cytokines like TNF-α, IL-1, IL-6, IL-8, IL-12, reactive oxygen species, nitric oxide and prostaglandins. And they are involved in the inflammatory response that is a part of the innate immune defense. Due to acute or chronic infection or inflammation can cause release or overproduction of these biomolecules, which are harmful to host tissues and lead to tissue damage, chronic inflammatory diseases such as rheumatoid arthritis [1]. Many clinically used anti-inflammatory agents target and inhibit proinflamatory cytokine functions [2-5]. They inhabit many side effects on human health. For that, many traditionally used medicinal plants are now a day’s taken for scientific experiments. Lipopolysaccharide (LPS) is a well-known gram-negative bacteria outer membrane component, which triggers the inflammatory response and production of pro-inflammatory mediators such as cyclooxygenase-2 (COX-2), cytokines (interleukin-1 beta; IL-1b and IL-6), tumor necrosis factor-alpha (TNF-a) and reactive oxygen species (ROS) These inflammatory mediators are closely associated with the pathogenesis of various inflammatory diseases [6- 9]. Also, generated ROS alter the structure and function of cells and contributes to cell death [10-12].

A vast number of plant species consisting numerous bioactive compounds exhibiting beneficial activities; like antioxidative, antiinflammatory, anticancer, hepatoprotective, anti-febrifuge, antihypertensive, antiallergic and antimicrobial effects etc. Numerous natural compounds have been isolated and tested on various in vitro and in vivo models for the development of new antiinflammatory therapeutics and other health problems [13-16]. *Blepharis maderaspatensis* is belongs to the family Acanthaceae, called as sathai otti, elumbu otti in Tamil and it was collected from agricultural field in Chengalpattu district, Tamil Nadu. *B. maderaspatensis* used in Indian medicine as a medicine when the heart beats unevenly. Root extract used for circulatory system disorders, arrhythmia, Infections/Infestations, syphilis, Leaf juice is used for bronchial complaints and especially leaf paste used for external wound healing and plant-ash used for dropsy, swellings, oedema, gout. In this study aimed to investigate potential anti-inflammatory activity of protocatachuic acid from the *B. maderaspatensis* on LPS induced production of inflammatory mediators in activated macrophages.

MATERIALS AND METHODS

Plant extract

The dried leaf powders extract with 90% aqueous alcohol and re-fractionated with different organic solvents like petroleum ether, chloroform, ethyl acetate. Totally five extracts were obtained and screened for phytochemical constituents. Altogether, methanolic fraction shows good antioxidant activity using $ABTS^+$, DPPH method. And it was shows higher content of total flavanoid, total alkaloid, total saponin, total tannin, total carotenoids, total polyphenolics while compared with other fractions.

Bioautography - DPPH method

1,1-diphenyl-2-picrylhydrazyl free radical scavenging assay was carried out by TLC bioautography method and scavenging activity of extracts for DPPH free radical was measured on the principle that antioxidants reduce the DPPH radical to a yellow-coloured compound (diphenylpicrylhydrazin) and the extent of the reaction will depend on the hydrogen donating ability of the antioxidant. Plant extracts were spotted on silica gel sheets (Silica gel 60 F254 TLC plates [Merck TLC Silica gel 60G F_{254} plates) and developed in Petroleum ether:Hexane:Chloroform:Ethyl Acetate:Acetone:Methanol (7:2:2:1:0.5:0.5) 2% methanolic DPPH radical solutions were sprayed on TLC plate. After 30 minutes antioxidant compound appeared as yellow colour spot on violet background [17].

Total flavanoid of content

Aliquots of 1.5 ml of extracts were mixed with equal volumes of 2% methanolic solution and vigorously shaken the mixture. At 430 nm the absorbance was read after 10 min of incubation. Quercetin was used as a standard for the calibration curve. The total flavonoid content was expressed as mg Quercetin equivalent/g dry weight. The dry weight indicated was the sample dry weight [18].

Total phenolic content

To the 0.1 ml extract, 0.9 ml distilled water, 0.5ml Folin-Ciocalteau reagent and 2.5 ml of sodium carbonate solution were added sequentially and the final solution was mixed thoroughly in vortex shaker. The reaction was kept for 40 min at 30ºC, after which the absorbance was read at 725 nm. TPC was calculated from standard calibration curve based on tannic acid [19].

DPPH free radical scavenging activity

The antioxidant activity using the DPPH (1, 1-diphenyl-2- 8 picrylhydrazyl) assay was assessed by the method of Blois. The reaction mixture contained 100 µM DPPH in different extracts of *B.maderaspatensis*. Incubated in room temperature for 30 min and absorbance were determined at 517 nm and the scavenging activity were calculated as a percentage of the radical reduction. Each experiment was performed in triplicate. Quercetin was used as a reference compound [20].

$$\% \text{ of radical reduction} = (A0 - A1/A0) \times 100)$$

ABTS$^{\cdot+}$ free radical scavenging activity

ABTS was dissolved in GDH_2O (7 µM) radical cation (ABTS$^{\cdot+}$) was mixed with 2.45 µM potassium persulfate (final concentration) and kept in the dark at room temperature for 12h. The reaction mixture was diluted with ethanol or DH_2O adjusted to an absorbance of 0.700 (±0.02) at 734 nm and equilibrated at 30°C. Reagent blank reading was taken (A_0) 1.0 mL of ABTS$^{\cdot+}$ cation mixture added with 5 µL of *Blepharis maderaspatensis* extracts and the absorbance reading was taken at 30°C exactly 5 min after initial mixing (A_t) Appropriate solvent blanks were run in each assay. Replicates were performed for all the extracts. β-carotene standard solution (0 -15 µM) in 80% ethanol was prepared and assayed at the same conditions. The absorbance of the resulting oxidized solution was compared to that of the calibrated β-carotene standard. Results were expressed in terms of β-carotene equivalent antioxidant capacity (µM β-carotene equivalents per g dry weight of plant) [21].

Nitric oxide radical scavenging

Sodium nitroprusside 5 mM was prepared in phosphate buffer pH 7.4. To 1 ml of test compound, sodium nitroprusside 0.3 ml was added. The test tubes were incubated at 25 ºC for 5 hr after which, 0.5 ml of Griess reagent was added. The absorbance of the chromophore was read at 546 nm. The experiment was performed in triplicate [22].

TLC finger printing

10 µl of crude extract were spotted on Merck precoated TLC plate and developed using different proportion of polar and non polar solvents. The developed plates were visualized under normal illumination, Short wave length UV illumination, Long wave length UV illumination and Iodine vapour. The good separated bands on TLC were photographed for the evidence [23].

Effect of protocatachuic acid on cytokinin expression levels

Determination of mRNA expression of cytokines, TNF-α, IL1, IL6, iNOS and COX-2 were studied. Macrophages RAW 264.7 Cells were seeded at a density of 1×106/well. After 24 hour incubation they were treated with different concentrations of protocatachuic acid (10, 20, 30 µg/ml) Twenty four later the drug treatment, the medium was discarded, followed by the addition of LPS (1µg/ml) containing medium. After 4

hours of LPS treatment the medium was aspirated and cells were washed twice with ice cold PBS (pH 7.4) Then the cells were harvested using 0.25% Trypsin-EDTA centrifuged and the pellet formed was stored in refrigerator for further experimental uses.

RNA isolation and real-time RT-PCR

Total RNA was extracted from treated RAW 264.7 cells using RNA easy mini kit (Qiagen, Hilden, Germany; Cat. No. 75161) according to the manufacturer's instructions and quantified spectrophotometrically. Reverse transcription was carried out as follows: A mixture of 2 µg total RNA and 2 µl Oligo (dT) was heated at 70 °C for 5 min, rapidly cooled down on ice and mixed with reaction mixture containing 5 µl M-MLV RT 5X Buffer (Cat. No M531A, Promega, USA), 0.5 µl deoxyribonucleotide triphosphate (dNTP, 25 µM, Cat No. U1240, Promega, USA), 0.7 µl RNAse inhibitor (2500U, RNasin® Cat. No N211A, Promega, USA) and 1 µl M-MLV reverse transcriptase (10000u; Cat. No M170A, Promega, USA) The reaction was made up to 25 µl with RNA-free water and then incubated at 42 °C for 1 h and at 70 °C for 10 min. cDNA was synthesized and stored at -80°C until its use. RT-PCR was carried out on a ABI PRISM® 7000 Sequence Detection System instrument using QuantiTect SYBR Green PCR Kit (Cat. No. 204141, QIAGEN, USA) according to the manufacturer's instructions. For RT-PCR, reaction mixture containing 5 µl QuantiTect SYBR Green PCR Master Mix, 0.5 µl of 10 µM forward primer, 0.5 µl of 10 µM reverse primer, cDNA template and RNAse-free water was prepared and transferred into capillary glass tubes. PCR was performed under the following conditions: denaturation at 95°C for 15 min, followed by 45 cycles of denaturation at 94°C for 15s, annealing at 61°C for 22s, and extension at 72°C for 30s. Expression of target genes was measured in triplicate and was normalized to β- actin, an internal control. The RT-PCR products were subjected to 2% agarose gel electrophoresis, stained with 1mg/ml ethidium bromide, and photographed using ChemiGenius2 image analyzer (SYNGENE, Cambridge, UK) Forward and reverse primer sequences for each gene and their corresponding amplicon sizes are provided in Table 1. The predicted size and single band confirmed the amplicons and that the respective primers were specific. Fold change of target genes' expression was calculated according to the formula: fold change = 2-((Ct (control) gene X-Ct (control) actin)-(Ct (activated) gene X-Ct (activated) actin). cDNA amplification by polymerase chain reaction [PCR] was performed using the following sense and antisense primers (5'-3') [24, 25].

Table 1: Primers used for RT-PCR

Name	Sense(5'-3')	Antisense(5'-3')	*Size (bp)*
TNFα	CGTCAGCCGATTTGCTATC	CGGACTCCGCAAAGTCTAAG	205
IL-1	GCCCATCCTCTGTGACTCAT	AGGCCACAGGTATTTTGTCG	229
IL-6	AACGATGATGCACTTGCAGA	GGAAATTGGGGTAGGAAGGA	276
iNOS	GCTTGTCTCTGGGTCCTCTG	CTCACTGGGACAGCACAGAA	217
Cox2	CCCCCACAGTCAAAGACACT	CTCATCACCCCACTCAGGAT	191
β-actin	TCACCCACACTGTGCCCATCTACGA	GGATGCCACAGGATTCCATACCCA	314

Column chromatography

60-120 mesh size silica gel was dissolved in the low polarity solvent hexane and tightly packed in 50 X 150 mm glass column up to 100 mm height without air bubbles. Then the experimental extracts were loaded individual glass column and fractionated with solvents hexane, petroleum ether, chloroform, ethyl acetate, acetone and methanol at various proportion of solvent mixture. 15 ml of fractions were collected using each solvent and the collected fractions were screened for purity using thin layer chromatography (Merck TLC Readymade sheets 20 X 20 cm) with appropriate solvent systems and the fractions are spotted on TLC for purity. After, clear separation the TLC plate visualized using day light, UV light short and long wave length and Iodine vapour. The isolated compounds were scanned with Shimatzu UV spectrophotometer and Thermoscientific spectrophotometer.

Spectroscopic characterization

The purified compound was characterized using FTIR (Agilent Resolutions Pro), ESI-MS (Shimadzu Lab Solutions), Nuclear magnetic resonance spectroscopy (Bruker 500 Mhz) in SRM university. The structural elucidation were performed and compared with data base.

Statistical analysis

The recoded data were expressed as mean ± standard error and one-way ANOVA followed by Turkey's post hoc test was used for comparison between control and treatment groups using statistic program SPSS version 10.0. The P-value ≤0.05was considered statistically significant difference.

RESULTS

Plant extract: 22.5g of crude extract was obtained from 5kg of leaf powder. Re-fractionated with petroleum ether (3.6g), Chloroform (4.74g), ethyl acetate (3.92g), acetone (1.73g) methanol (7.48g).

Total flavanoid of content: The total flavanoid content was accessed and recorded in Table 2. And it was higher content in methanol fraction than all the fractions and least was recorded in petroleum ether fraction.

Table 2: Total flavanoid of content of experimental plant leaf extracts (mg/g of dried leaf powder)

S. No.	Solvents fractions	*Blepharis maderaspatensis*
1	Petroleum ether	12.2
2	Chloroform	19.3
3	Ethyl acetate	15.5
4	Acetone	13.0
5	Methanol	30.9

Table 3: Total phenolic content of content of experimental plant leaf extracts (mg/g of dried leaf powder)

S. No.	Solvents fractions	*Blepharis maderaspatensis*
1	Petroleum ether	27.6
2	Chloroform	59.1
3	Ethyl acetate	68.1
4	Acetone	42.7
5	Methanol	186.4

Total phenolic content: Higher phenolic content was recorded in methanolic fraction as 186.4 mg/g of dried leaf powder and followed by ethyl acetate, chloroform, acetone, and petroleum ether fractions.

Total antioxidant content

Total antioxidant content was analyzed using three different free radical scavenging methods and recorded data's presented in the Table 4. Altogether, methanolic fraction showed higher content of antioxidant in all the three methods and followed by ethyl acetate fraction. The rest of fractions showed considerable amount of antioxidant content. The Table 4 shows higher total antioxidant content as 89.53, 81.75 and 87.45 mg/g dry weight of leaf powder in $ABTS^{\cdot+}$, DPPH and Nitric oxide free radical scavenging methods respectively.

Table 4: Total antioxidant of content of experimental plant leaf extracts leaf extracts (mg/g of dried weight)

S. No	Plant extract	Free radical scavenging activity (%)		
		$ABTS^{+}$	DPPH	Nitric oxide
1	Petroleum Ether	63.65	45.72	47.25
2	Chloroform	69.92	80.93	60.32
3	Ethyl acetate	80.23	72.33	72.28
4	Acetone	75.43	67.82	65.93
5	Methanol	89.53	81.75	87.45

TLC finger printing

TLC finger printing was performed for listing of approximate number compound present in methanolic extract. Fig.**A** shows more than eighteen clear spots on TLC plate and it is indicating many compounds; till many compound**s** not separated and remain together on bottom.

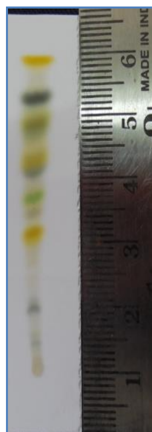


Fig A. *Blepharis maderaspatensis*- Methanolic fraction TLC finger printing

Bioautography - DPPH method

In this method the free-radical scavengers appear as yellow spots against a purple background behalf of the conversion of purple coloured DPPH in to colourless by antioxidant compound and appeared yellow colour spot on TLC plate. Fig **B** shows the *B. maderaspatensis* methanolic fraction showed good low molecular weight antioxidant on top and higher molecular weight compound at bottom of the plate as yellow colour.

Fig B. *Blepharis maderaspatensis*- Methanolic fraction antioxidant bioautograpy

Cytotoxic activity of protocatachuic acid

Table 5. shows *In vitro* cytotoxic effect of protocatachuic acid isolated form *Blepharis maderaspatensis* leaf against Vero cells at the various concentration of 100 - 1.5625 µg/ml by double dilution method. This test was performed at different time interval 12 to 72 hrs. There is no cytotoxicity was recorded up to 25 µg concentration. The cytotoxicity was observed at 50 and 100 µg concentration on 72hr only. The rest of time interval does not show any toxicity in these concentrations.

Table 5: *In vitro* cytotoxic effect of protocatachuic acid isolated form *Blepharis maderaspatensis* leaf against Vero cells

Cell line	Extract (µg/ml)	12 hr	24 hr	48 hr	72 hr
Vero cells	100	Nd*	Nd	Nd	16.25 ± 2.01
	50	Nd	Nd	Nd	10.07 ± 0.98
	25	Nd	Nd	Nd	Nd
	12.5	Nd	Nd	Nd	Nd
	6.25	Nd	Nd	Nd	Nd
	3.125	Nd	Nd	Nd	Nd
	1.5625	Nd	Nd	Nd	Nd

* Nd - Not detectable

Table 6: Effect of protocatachuic acid on cytokinin expression levels of iNOS, COX2, TNF-α, IL1 and IL-6 in the macrophage RAW 264.7 cell line

Experimental groups	TNF-α	IL-1	IL-6	iNOS	COX-2
LPS	25.69 ± 19.06	25.69 ± 19.06	1.03 ± 0.68	0.44 ± 0.26	2.83 ± 0.39
PA 10 µg	0.50 ± 0.14*	0.50 ± 0.14*	0.66 ± 0.23*	0.27 ± 0.01*	0.06 ± 0.01*
PA 20 µg	0.13 ± 0.04*	0.13 ± 0.04*	0.55 ± 0.08*	0.28 ± 0.07*	0.40 ± 0.21*
PA 30 µg	0.62 ± 0.01*	0.62 ± 0.01*	0.91 ± 0.27*	0.21 ± 0.024*	0.55 ± 0.23*

LPS-Lipopolysaccharide; iNOS-Inducible nitric oxide synthase; COX-2 - Cyclooxygenase-2; TNF-α-Tumour necrosis factor-α; IL-1-Interleukin-1; IL-6-Interleukin-6
* - $p \leq 0.001$Compared with LPS

Effects of the protocatachuic acid on LPS-induced pro-inflammatory cytokines transcription

Based on the cytotoxicity data, the treatment dose was designed about three different concentrations at 10, 20 and 30 µg/ml on macrophage RAW 264.7 cell line. MTT assay was performed at different time intervals about 12, 24, 48 and 72 hrs. The IC_{50} value was inferred and cells were used for cytokine expression using real time PCR method. The results presented follwoingly.

In acute and chronic inflammatory condition, the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α were secreted at elevated level. Hence forth, the present investigation was involved to find out the effects of the protocatachuic acid on the mRNA levels of IL-1β, IL-6 and TNF-α on LPS induced macrophages inflammatory cells. And also we investigate pro-inflammatory mediators COX2 and iNOS mRNA expression levels.

Expression of cytokines and pro-inflammatory mediators at the mRNA levels were significantly increased in LPS induced state and it was significantly reduced while treated with different concentrations of protocatachuic acid at $p \leq 0.001$. Among three different concentrations treatment, 20 µg/ml shows significantly decreased than the lower concentration 10 µg/ml; higher concentration 30 µg/ml at $p \leq 0.001$. This experimental results finding, infer the protocatachuic acid is able to suppress or disrupting signal transduction pathways and consequently preventing the transcription of pro-inflammatory factors.

Spectroscopic characterization

Based on spectral recorded data's the isolated and identified compound is protocatachuic acid. The recorded spectral data are followingly.

TLC plate the isolated compound apparently appeared s pale brown spot and the R_f value was 0.62 (Fig. C) and λmax observed at 270 and 300 nm (Fig. D); Fig. F depict FTIR datas of protocatachuic acid - 3497, 3425, 3269, 2982, 1684, 1610, 1518, 1477, 1448, 1368, 1335, 1293, 1234, 1186, 1128, 1024, 955, 912, 874, 824, 764, 722, 668, 589, 541. Fig. F shows molecular weight of isolated compound protocatachuic acid as 154 g/mol in ESI-MS. The fig G and H are ^{1}H and ^{13}C spectrum obtained 500 Mhz using DMSO. δ 2.04, 3.09, 2.07,

1.03, 1.01 and 1.00 are the ^{1}H NMR and δ 14.26, 39.01, 39.18, 39.34, 39.51, 39.68, 39.85, 40.01, 60.01 ,115.30, 116.26, 120.82, 121.74, 145.05, 150.35, 165.68 are 13C-NMR. The recorded spectral results revealed the compound protocatachuic acid with reference of spectral database literatures and existing literature (Fig. I).

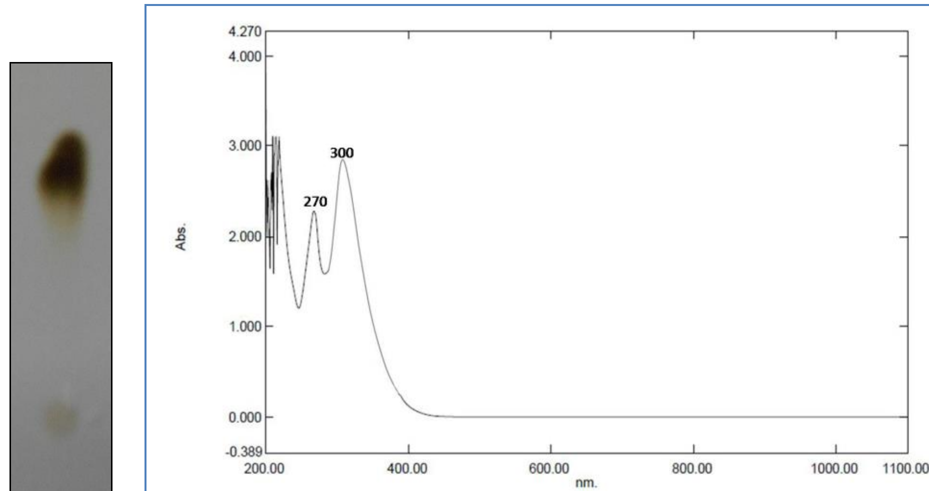
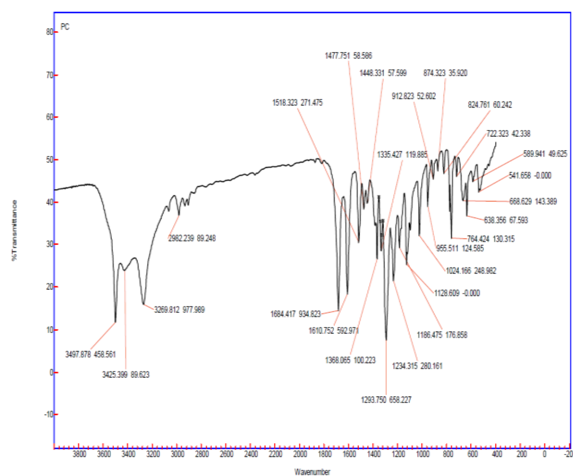


Fig: C

Fig: D



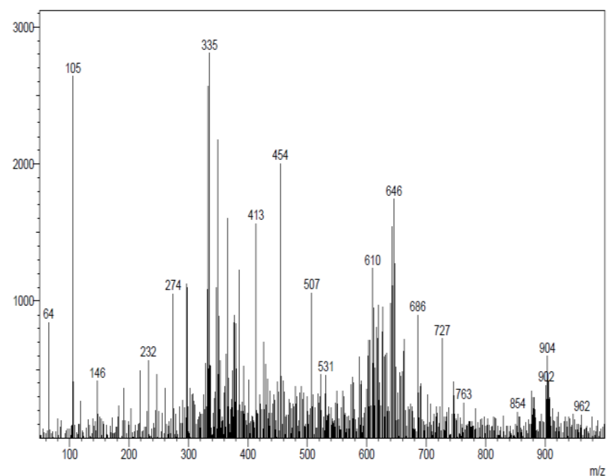
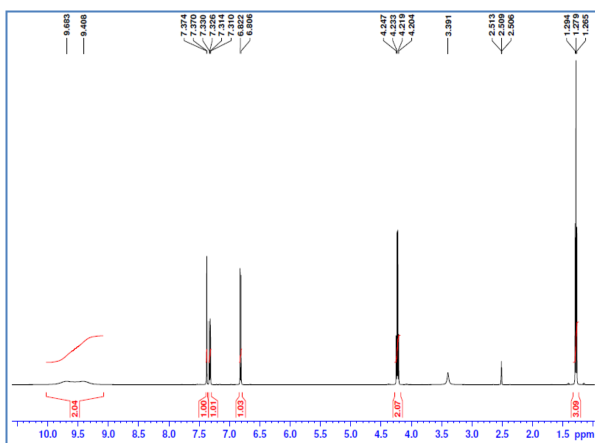


Fig: E

Fig: F



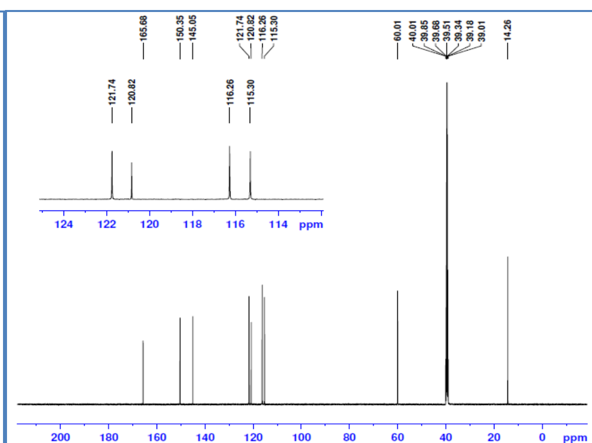


Fig: G

Fig: H

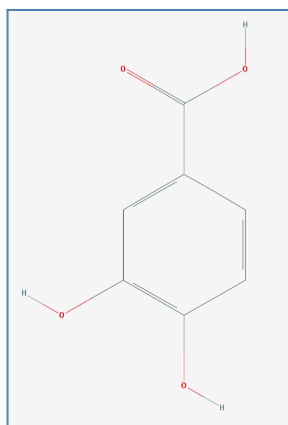


Fig: I

Fig C. TLC; Fig D. UV-VIS; Fig E. FTIR; Fig F. ESIMS; Fig G. ^{1}HNMR; Fig H. ^{13}CNMR; Fig I. Protocatachuic acid structure (Mol. Formula: $C_7H_6O_4$ Molar mass: 154.12 g/mol).

DISCUSSION

Plant kingdom has been found as significant contributors for the pharmaceutical, agriculture and food industries etc. Due to civilization the pharmaceutical industries are manufacturing vast number of synthetic drugs for human and animal diseases. Among thus many are become prominent like severe side effects and resistance of microbes against these drugs. And also they are expensive, too nuisance as pollution to environment by its wastage. In recent decades research on medicinal plants has been increased all over the world. From the natural source many drugs are obtained by single or by combination with few or many plants part and also safe, efficacious and cost effective medicines with incredible benefits [26].

Due to modern civilization of human life and their daily food habits consisting many contaminants like pathogens, allergens, chemicals and many inflammations causing agents. They indirectly cause chronic or acute inflammation by altering immune pathway and a few are generating free radicals or by the pathogenesis of inflammation and successively imbalance of antioxidant homeostasis [27-29]. In general, plants containing many compounds as primary and secondary metabolites. The phytochemicals are accumulate in different parts of the plants, such as in the leaves, flowers, stems, fruits, seeds and roots [30].

A vast number of plant extracts or pure substances have antioxidant activity and they are scavenging the free radicals which was generated by toxic materials, pathogens, chemicals, allergens and etc., Flavonoids, phenols and few known substances are play an important role on disease defense mechanisms whether microbes or through vectors. And they are exhibiting numerous beneficial properties to humans, animals, plants and microbes as food, protector, antimicrobial, cytotoxicity, anti-inflammatory, antiallergic antitumor, cytotoxic, gastroprotective, treatment of neurodegenerative diseases, vasodilatory action, flavour and many medicinal property etc., [31-39].

In *B. madaraspetensis* extracts shows good quantity of total flavanoid and total phenol in methanolic fraction while compared with other fractions. It indicates methanol is efficient extractor of various compounds from plant parts. The high quantities of these phytochemicals are possessing free radical scavenging activity, antiinflamatory activity. Consequently, antioxidant activity was studied in all the extracts and at this juncture also methanolic extract showed high-quality free radical scavenging activity.

The antiinflamatory activities both *in vitro* and *in vivo* models were studied and reported on many medicinal plant in India as well as worldwide. According to researchers and familiar scientist's reports about medicinal plants extracts or purified substances have significant potential antiinflamtory activity and other activities [40].

An extensive variety of *in vitro* methods are available for proving antioxidant activity and among thus ABTS, DPPH, Nitric oxide free radicals are useful to ascertain antioxidant ability of certain substances from

natural and synthetic sources. Free radicals of ABTS, DPPH nitric oxide are highly stable until exposed in air or any oxidative substances. These free radicals are green and purple coloured and substances activity is determined based on its ability to decolorize at its own wave length to light yellow to forming a stable molecule [41, 42].

B. madaraspetensis methanolic fraction TLC finger printing shows many distinguished separated spots and it was indicating the presence of many phytochemical accumulations in methanolic fraction, as well as this extract shows low molecular antioxidant apparently on top of the TLC plate. In TLC bottom shows cumulative antioxidant spot, it is indicate that many compounds still not separate and remain together on TLC bioautography.

Many medicinal plant were explored its phytochemical contents and as well as its antiinflamatory activity using different *in vitro* and *in vivo* model and reviewed many reviewers.[43-49] Some of the phytochemicals against herbivorous insects also end up being harmful to humans nucleic acid, carbohydrate and lipid metabolism, neurochemicals, neuropeptides, hormones and neurotransmitters; agonistic activity on neurotransmitter systems, cholinesterase [50-57]. For the cell based assay was well developed and screened for *in vitro* cyto**to**xicity study using normal cells. In this study also cytotoxicity was determined using various concentrations at different time interval. The result was clearly indicating no toxicity up to between 25 to almost 45 µg/ml of protocatachuic acid.

The inflammatory disease caused by various inflammatory stimuli including viruses, chemicals, and reactive oxygen/nitrogen species, which consequently increases the synthesis and secretion of proinflammatory cytokines. Phytochemicals have been shown to modulate various points in these inflammatory processes. These modulations serve as controlling points where the amplification of the inflammatory processes can be disconnected and thereby reduce subsequent diseases risk [58]. Over the past few decades many modern therapeutic drugs were developed or obtained from natural products [59]. There are variety of substances are acquired from natural products like arachidonic acid metabolites, peptides, cytokines, excitatory amino acids, essential oils, flavanoids, waxes, resins, secondary metabolites as flavanoids, terpenoids, alkaloids and etc and they are act as modulate various inflammatory mediators the production or action of second messengers to cGMP, cAMP, protein kinases and calcium. As well as key pro-inflammatory molecules like inducible NO synthase, cyclooxygenase-2, cytokines - IL-1β, TNF-α, neuropeptides, proteases, proto-oncogenes *c-jun*, *c-fos*, and *c-myc* and expression of transcription factors such as AP-1, NF-κB were modulate or suppression studies revealed from many researches [58 - 61].

A number of mechanisms of anti-inflammatory actions for phytoconstituents were proposed and it consist antioxidative and radical scavenging activities, modulation of mast cells, macrophages, lymphocytes, and neutrophils cellular activities of inflammation-related cells, modulation of proinflammatory enzyme activities such as phospholipase A2, cyclooxygenase, and lipoxygenase; nitric oxide producing enzyme nitric oxide synthase, proinflammatory molecules [62].

In *B. madaraspetensis* methanolic fraction showed high content of total flavanoid, phenol and free radical scavenging activity, hence it was further taken for purification of pure substance. Finally protocatachuic acid was isolated, identified; as well as taken for cytotoxicity study and exploring proinflamatory cytokinin expression levels at different doses.

Upto 25 µg/ml dose of protocatachuic acid does not show any cytotoxycity and at 50 µg/ml showed cytotoxic on 72hrs. These results indicate that first cytotoxicity dose may be between 26 to 50 µg/ml of protocatachuic acid on normal Vero cell line. Hence, different concentrations of protocatachuic acid (10, 20, 30 µg/ml) significantly suppress the LPS induced TNF-α, IL-1, IL-6, iNOS, COX-2 proinflamatory cytokinin expressions. At 20 µg/ml of protocatachuic acid shows preeminent inhibitory activity on macrophage RAW 264.7 cell line while compared with other concentrations. The isolated compound from *B. madaraspetensis* extract methanolic fraction was identified as protocatechuic acid by comparing their physical and spectral data with those reported [63-68] while by comparing with authentic sample (Fig. I).

CONCLUSION

Altogether, in this present investigation the recorded results of *B. madaraspetensis* leaf have significant potential free radical scavenging activity with higher content of total flavanoid and phenolics. The isolated protocatachuic acid may facilitate to inhibitory effects on LPS induced inflammation by blocking NF-B signaling pathway via inhibition of IκB phosphorylation, subunits p65/p50 of NF-B translocation in nuclear and proinflamatory mediators COX-2, iNOS etc. Finally recorded results are confirmed the antiinflamatory activity of LPS induced inflammation on macrophage RAW 264.7 cell line **(Fig. I).**

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