

Evaluation of cytotoxic, DNA protecting and LPS induced MMP-9 down regulation activities of *Plectranthus amboinicus* (Lour) Spreng. essential oil.

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ABSTRACT

Introduction: *Plectranthus amboinicus*(Lour) Spreng is a known medicinal plant used in Siddha and Ayurveda medicines in India. It has enormous medicinal potential to treat various diseases. **Methods:** The present study focused on the use of essential oil obtained from the leaves of *Plectranthus amboinicus* to test cytotoxicity against breast (MCF-7) and colorectal (HT-29) cancer cell lines, to protect DNA from H₂O₂ induced genotoxicity through comet assay and to treat inflammation in lipopolysaccharide (LPS) induced over expression of matrix metalloproteinase-9 (MMP-9) in human peripheral blood mononuclear cells (PBMCs) by gelatin zymogram and inhibition at transcriptional level confirmed using RT-PCR (reverse transcriptase polymerase chain reaction). **Results:** Cytotoxicity of essential oil against MCF-7 and HT-29 cancer cell lines revealed the IC₅₀ values of 53 ± 0.01 and 87 ± 0.01 µg/mL respectively. At 200 µg/mL essential oil protected against 75% of DNA damage in 3T3-L1 fibroblast cells. Essential oil showed significant reduction in production of MMP-9 in a concentration dependent manner. **Conclusion:** Overall, the results showed that essential oil of *P. amboinicus* is a potent bioactive substance and it could be used in herbal medicine preparations.

Keywords: Essential oil , Cytotoxicity , Antigenotoxicity, Zymogram, RT- PCR.

INTRODUCTION

Matrix metalloproteinases (MMPs) are a family of endopeptidases which digests proteins of the extracellular matrix. They are important for regulating normal tissue development in morphogenesis, angiogenesis and wound

healing. However, unregulated activities of MMPs can play a role in many disease states including cancer expansion, invasion and metastasis, arthritis and atherosclerosis.¹ Numerous factors, namely tissue inhibitors of matrix metalloproteinases (TIMP), plasminogen activator inhibitors and general protease inhibitors such as 2 macroglobulins and 1 antitrypsin may limit or block the enzymatic activity of the activated MMPs.² The use of synthetic inhibitors of MMPs and antibiotics were effectively found to reduce the vascular expression of MMPs, in particular MMP-2 (72kDa) and MMP-9 (92kDa) and delay the progression of atherosclerotic plaque.³ Long term use of synthetic drugs may cause side effects that limit their clinical use. Natural products are an enormous reservoir of structurally diverse secondary metabolites that potentially inhibit the inflammatory process by affecting different molecular targets.⁴ *Plectranthus amboinicus* is cultivated in home gardens throughout India

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for use in traditional medicine, being used to treat cough, chronic asthma, hiccup, bronchitis etc. The leaves have been reported to have anti-inflammatory and antitumor activities.⁵ Antifungal, antibacterial and larvicidal activities of essential oil were reported in the literature.⁶⁻⁹ Based on the medicinal uses of *P. amboinicus*, the present study was focused on cytotoxic, antigenotoxic and MMP-9 down regulation activities of essential oil under *in vitro*.

MATERIALS AND METHODS

Chemicals and reagents

Acrylamide, bisacrylamide, gelatin, sodium sulphate anhydrous, trisHCl, sodium dodecyl sulphate (SDS), bromophenol blue, glycine, methanol, coomassie brilliant blue R250, trypan blue, ethidium bromide and tetramethylethylenediamine (TEMED), glycerol, EDTA, isopropyl alcohol, RPMI-1640, dulbecco's modified eagle medium (DMEM) medium with sodium bicarbonate without L-glutamine and phenol red, phosphate buffered saline (PBS), fetal calf serum (FCS), high melting point agarose (HMP), low melting point agarose (LMP) and triton-X100 were purchased from Hi Media Pvt. Ltd, India. Dimethyl sulfoxide (DMSO) and diethyl pyro carbonate (DEPC) treated water was purchased from Ranbaxy Fine Chemicals, Pvt. Ltd, India. Histopaque-1077, lipopolysaccharide (*E. coli* 0111:B4) and ammonium persulfate were purchased from Sigma Aldrich, USA and trizol reagent from GeneiPvt. Ltd, India.

Plant material

The leaves of *Plectranthus amboinicus* (Lour) Spreng. was collected from healthy green plants growing in the medicinal plant garden, Kongunadu Arts and Science College, Coimbatore, Tamil Nadu, India.

Hydrodistillation of leaves

Freshly collected leaves of *P. amboinicus* (1kg) were hydrodistilled for 3 hours using Clevenger apparatus for essential oil extraction. Extracted essential oil was treated with sodium sulphate anhydrous to remove water. The purified oil was then filled in small vials, tightly sealed and stored in a refrigerator (4°C) for further analysis.

Cell lines and culture

The human breast (MCF-7) and colorectal cancer (HT-29) cell lines were cultured in a T25 cm² cell culture flask containing DMEM supplemented with 10% FBS, penicillin

(100U/mL) and streptomycin (100µg/mL). The cell lines were incubated in humidified incubator at 37° C with 5% CO₂.

MTT assay

The cell line culture in the T25cm² flask was harvested using trypsin and the cell number was counted using a hemocytometer. 1x 10⁴ cells/100mL medium was added in each well of 96 well plate and incubated for 24h. Then the cells were treated with various concentrations of essential oil dissolved in medium and further incubated for 48h. A 20 µl of MTT (5mg/mL) in phosphate buffered saline was added to each well and the plate was incubated at 37° C for 4 h. The medium was removed and 100µL of DMSO was added to each well. After 10 minutes of incubation at 37° C the plate was read at 570 nm using a microplate reader.¹⁰ The percentage of cell viability was calculated as 100 (absorbance of test/absorption of the control).

Comet assay

Cell culture and treatment

The 3T3-L1 fibroblasts (4-5x10⁶) were cultured in DMEM supplemented with 10% fetal bovine serum in 25 cm² tissue culture flasks. The cells in culture were treated with various concentrations of oil (25, 50, 100, 150 and 200 mg/mL) for 60 min in a CO₂ incubator. After pretreatment, the cells were exposed to 100 mM of H₂O₂ for 30 min on ice. The cells were harvested, centrifuged for 5 min at 1500 rpm and resuspended in phosphate buffered saline (PBS).

Single cell gel electrophoresis (Comet assay)

A 25 mL of cell suspension was mixed with 75 mL of 0.6% low melting agarose. The suspension was spread on a frosted microscopic slide precoated with 0.8% of normal melting agarose. The cell suspension was covered with a cover slip and kept on ice for 10 min. The cover slips were removed and the slides were incubated overnight in lysis solution containing 1% SDS, 2.5 M NaCl, 100 mM Na₂EDTA, 1% Triton X-100 and 10% DMSO at 4°C. The slides were arranged in an electrophoresis tank filled with prechilled electrophoretic buffer (1mM Na₂EDTA and 300 mM NaOH) and incubated for 20 min. Electrophoresis was carried out at 25 V (300 mA) for 20 min using a power supply (CBS Scientific company, USA). After electrophoresis, the slides were washed with 0.4 M Tris (pH 7.5) and stained with ethidium bromide (20 mg/mL). The slides were viewed using an Olympus BX50 fluorescence microscope. The comet tail length was measured using an

eyepiece micrometer and the DNA damage was calculated as follows:

Comet tail length (lm) = (maximum total length) - (head diameter).¹¹

MMP-9 down regulation

Isolation of mononuclear cells

Human lymphocytes were isolated from fresh whole blood of healthy volunteers by adding RPMI1640 with 10% of fetal calf serum on ice for 30 min, then underlaid with 2.5mL of Ficoll Histopaque 1077 followed by centrifuge at 200g for 3 min at 4°C. Isolated lymphocytes were stained with 0.4% trypan blue and viable cells were counted using Haemocytometer under microscope. Cell viability of more than 95% was taken in to further studies.

Stimulation of inflammation and sample treatment

Cells (1×10^5 cells/well) cultured in 96 well plates were induced inflammation using 1 µg/mL LPS.¹² After 24h incubation, *P. amboinicus* essential oil in different concentration (50, 100, 150, 200 and 250 µg/mL) were added to each well and incubated for 24 h. At the end of incubation, the cell free media was collected and assayed for MMP-9 inhibition by gelatin zymography and reverse transcriptase polymerase chain reaction (RT-PCR) method.

Gelatinzymography

SDS-PAGE was carried out according to the method of Laemmli¹³ using 7.5% gel. Zymogram gel consisted of 7.5% polyacrylamide gel copolymerized with gelatin (1 mg/mL). Following electrophoresis, the gel was washed successively with 50 mL of 2.5% (v/v) Triton X-100 in distilled water (for an hour) to remove SDS. The gel was then incubated with developing solution (CaCl₂ 10mM, Triton X-100 1% and Tris buffer, 50mM pH 7.4) at 32°C for 18 h. Further, the gel was stained with coomassie brilliant blue R250 for 2 h and destained overnight to reveal the bands.

RNA isolation and RT-PCR analysis

The cells were harvested after LPS and essential oil treatment and transferred to fresh 1.5 mL centrifuge tubes and centrifuged at 1,400rpm for 10min at 4°C. The supernatant was discarded and the pellet was suspended in 1mL of trizol to lyse the cells. About 200 µL of chloroform was added and mixed by pipetting for 30sec and centrifuged at 14,000rpm for 15min at 4°C. Aqueous phase was carefully transferred to a fresh microfuge tube and 500 µL of isopropyl alcohol

was added and incubated at room temperature for 10min. The tubes were then centrifuged at 1,400rpm for 10min at 4°C and the supernatant was discarded. RNA pellet was washed with 75% ethanol by spinning at 1,400 rpm for 5min at 4°C. The supernatant was discarded, the pellet was air dried and suspended in 9µL of deionised autoclaved diethyl pyro carbonate (DEPC) treated water.

Reverse transcription was carried out as follows. To the above sample, 2µL of dNTP, 5µL of cDNA synthesis buffer, 1µL of oligo d (T), 1µL of reverse transcriptase enzyme mix (Thermo Fischer Scientific, India) and 9µL of nuclease free water were added and reverse transcription was carried out in a thermocycler (Eppendorf) to synthesis cDNA. The synthesized cDNA was further used for PCR. Primers for human MMP-9 and β-actin (Helini, India) were as follows forward primer: 5'-AAG ATG CTG CTG TTC AGC GGG-3' and reverse primer 5'-GTCCTCAGGGCACTGCAGGAT-3' for MMP-9¹² and forward primer 5'-CGGATGTCCACGTACACTT-3' and reverse primer: 5'-GTTGCTATCCAGGCTGTGCT-3' for β-actin. PCR conditions for MMP-9 and β-actin, initial denaturation (94°C for 5 min), denaturation (94°C for 30 sec), annealing (57°C for 30 sec for MMP-9) and (60°C for 30 sec for β-actin), extension (72°C for 1 min) and final extension (72°C for 10 min).

RESULTS AND DISCUSSION

Essential oil extraction

Essential oils are complex mixtures of biologically active substances used since a long time as flavouring agents and constituents of a number of commercial products. Scientific literatures have revealed the cytotoxicity, anticholinesterase, antimicrobial, antiinflammatory and antioxidant activities of essential oils. Hydrodistillation of *Plectranthus amboinicus* (Lour) Spreng leaves yielded light yellow colour oil with strong aromatic smell. Out of 1.0 kg leaves 1500µL of oil was extracted. The collected oil was treated with sodium sulphate anhydrous and excess water was removed and kept at 4 °C for further use.

Cytotoxicity

The cytotoxic activity of essential oil against breast (MCF-7) and colorectal (HT-29) cell lines were significant and IC50 value calculated as 53 ± 0.01 and 87 ± 0.01 mg/mL respectively. Plant essential oils and their individual components have anticancer activity when tested on a number of human cancer cell lines including colon, breast, leukemia, lung and gastric cancer. The observed

cytotoxicity of oil may be due to bioactive phenolic compound, carvacrol. Similar result is reported in the literature that carvacrol has cytotoxic effect against various cell lines.¹⁴⁻¹⁶ At the same time other constituents of the oil such as γ - terpinene, p-cymene, β - caryophyllene and α -humulene could also be taken into account for their possible synergistic effects.

DNA protecting activity of oil

DNA protecting effect of oil on H₂O₂ induced toxicity was studied. Cells (3T3-L1) treated with 100mM H₂O₂ produced the maximum tail length of 10.63±0.70mm (Table 1). Cells pretreated with different concentrations of oil showed decrease in tail length. The antigenotoxic activity of oil was observed by the reduction in tail length with increasing concentration and the optimum concentration was observed as 200mg/mL (Figure 1). H₂O₂ is a natural source of oxidative damage in cells causing a spectrum of DNA lesions, including single and double strand breaks. According to Collins¹⁷, DNA damage due to H₂O₂ results from the production of hydroxyl radicals in the presence of transition metal ions such as iron via Fenton reaction. The observed DNA protecting activity of oil may thus be due to hydroxyl radical scavenging activity. In addition, the protective activity of oil may also be attributed to electrons donated for neutralization of free radicals.

MMP-9 inhibition and down regulation

The optimum concentration of 1µg/mL of LPS was used to induce inflammation. After incubation, different concentration of essential oil was added (50, 100, 150, 200 and 250 µg/mL) and gelatinase activity was measured by gelatin zymography. Cells treated with oil showed significant reduction in MMP-9 expression. While increasing the concentration the intensity of the band decreased (Figure 2), 200 µg/mL of oil observed as optimum concentration needed to inhibit MMP-9 expression. The total RNA of the cells was isolated and RT-PCR was performed to test the down regulating activity of oil on MMP-9 production

Concentration of oil (mg/mL)	Tail length (mm) ^a (Mean ± SD)
Control	0.51 ±0.41*
H ₂ O ₂ 100 (mM)	10.63 ±0.70*
25	7.42 ±0.47*
50	6.22 ±0.50*
100	5.9 ±0.48*
150	4.0 ±0.40*
200	3.17 ±0.38*

^aRepresents p < 0.05 vs. control, as tested by the Student's 't'-test

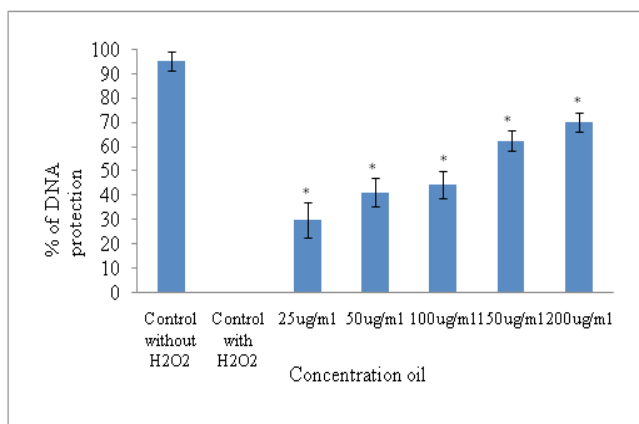


Figure 1 DNA damage induced by H₂O₂ in 3T3-L1 fibroblasts

The cells were incubated with 100 µM H₂O₂ and 25, 50, 100, 150 and 200 µg/mL oil on ice for 4h and then used comet assay. At 100 µM, a maximum tail length of 10.63 ±0.70 µm was observed. Means ± standard deviations are presented



Figure 2: Gelatinzymogram of MMP-9

MMP-9 inhibition of different concentrations of *P.amboinicus* essential oil after LPS treatment (C) Control, (1) 50, (2) 100, (3) 150, (4) 200 and (5) 250 µg/mL of essential oil

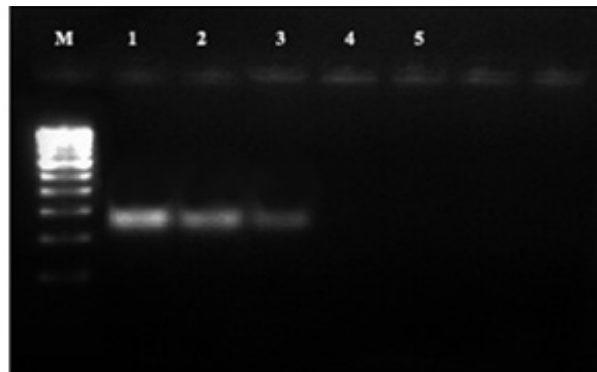


Figure 3 : Effect of *P.amboinicus* essential oil on down regulation of MMP-9

Lane M: marker ;Lane 1: 50 ; Lane 2: 100 ;Lane 3: 150 ;Lane 4: 200 and Lane 5: 250 µg/mL of essential oil

at transcriptional level. The results of RT-PCR clearly indicated that cells treated with LPS alone showed over expression of MMP-9 but cells treated with different concentration of oil showed significant reduction of MMP-9 expression. The down regulation activity of oil was time and concentration dependent (Figure 3). β -actin served as an internal marker (Figure 4). Inhibition of inflammatory cytokine and mediator production or function serves as a key mechanism in the control of inflammation and agents that suppress the expression of these inflammation

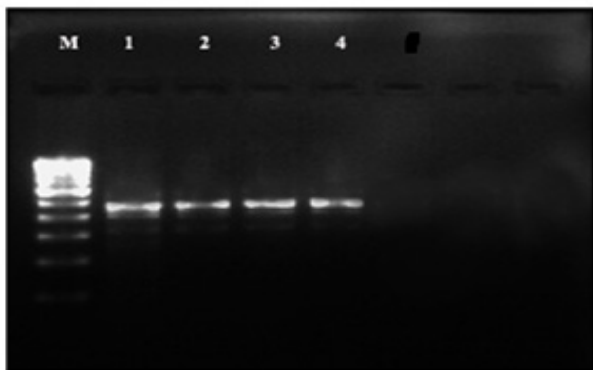


Figure 4 β -actin served as an internal marker

associated genes have therapeutic potential in the treatment of inflammatory diseases.¹⁸ Result obtained in the present study shows, *P.amboinicus* essential oil is a potent MMP-9 inhibitory agent under in vitro. The observed activity may be due to the presence of bioactive mono and sesquiterpenes. However the known anticancer and antiinflammatory agent carvacrol which is the major compound⁸ in oil might be the reason for observed MMP-9 down regulation. In human macrophage like U937 cells, carvacrol suppressed LPS induced COX-2 mRNA expression, suggesting that carvacrol regulates COX-2 expression through its agonistic effect on PPAR γ .¹⁹ We assumed that mechanism of down regulation may be associated with the inhibition of nuclear factor kappa B (NF κ B).¹² using *Aloe vera* where down regulation of MMP-9 either by directly inhibiting the activation of NF κ B and its subsequent binding to MMP-9 or by inhibiting the production of mediators such as prostaglandins which induce MMP-9 expression.

CONCLUSIONS

P.amboinicus essential oil is a good source of bioactive mono and sesquiterpenes. It is clear that oil may be considered as a potential natural product and could be used as a part of daily supplement in food to prevent health related problems.

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CONFLICTS OF INTEREST

All authors have none to declare.

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