



COCONUT SHELL DERIVED BIOACTIVE COMPOUND OXYRESVERATROL MEDIATES REGULATION OF MATRIX METALLOPROTEINASE 9

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ABSTRACT

Cocos nucifera L., a member of the family Arecaceae is an important fruit crop in tropical countries and is known to possess several pharmacological properties. The oil extracted from the coconut shell is used in Unani medicine to treat severe skin diseases. The present study involves bioactivity guided fractionation and characterization of a potent Matrix Metalloprotease 9(MMP-9) inhibitor from coconut shell extract (CSE). Coconut shell was extracted using soxhlet extractor and was subsequently purified using Sephadex LH 20 column. The effect of the bioactive fraction, F1 was studied on human melanoma cell line, A375. F1 significantly down regulated the activity of MMP-9 which was further corroborated by qRT-PCR studies. A corresponding decrease in the expression of MMP-2, MMP-9 as well as other biomarkers of cancer progression like COX-2, VEGF and EGFR was also seen. Further studies confirmed that F1 inhibited migration of A375 cells and caused cell cycle arrest at G2/M phase. Based on the results obtained, structural characterization of F1 was carried out by NMR and LC/MS which resulted in the identification of F1 as oxyresveratrol, a stilbenoid. Further, molecular docking studies demonstrated that oxyresveratrol forms hydrogen bonds with critical amino acid residues present in the catalytic site of both MMP-9 and MMP-2.

KEYWORDS: *Cocos nucifera* L.; coconut shell; Melanoma; Oxyresveratrol; Matrix metalloproteinase; Epidermal Growth Factor Receptor



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INTRODUCTION

Human skin is usually susceptible to various physical, chemical and environmental agents. Cutaneous overexposure to ultraviolet (UV) radiation has multiple effects on human health, including the progression of melanoma and non-melanoma. Among various other skin diseases, melanoma remains one of the predominant reasons for cancer cell death due to its ability to metastasize. The statistical analysis from the American Cancer Society indicated that more than 70,000 new cases of melanoma were diagnosed in US in 2015¹ which would exceed 112,000 cases by the year 2030². Unlike many other cancers, skin cancer is curable if treated at early stages but are prone to metastasize, and is less submissive for treatment at later stages³. Essential step in different stages of cancer progression like migration, invasion, and metastasis is the degradation of basement membrane and extracellular matrix by matrix metalloproteinases (MMPs)⁴. MMPs and epidermal growth factor receptors (EGFR) are up-regulated in virtually all human and animal tumors⁵⁻⁹. In addition to MMPs and EGFR, cyclooxygenase-2 (COX-2) also plays a significant role in promoting various types of cancer. Exposure of skin to UV radiation, the primary cause of skin cancer¹⁰, induces an increase in the expression of COX-2 which is observed in the majority of human malignancies¹¹. A positive correlation between Vascular endothelial growth factor (VEGF) and COX-2 has also been observed in several cancers¹²⁻¹⁴. VEGF is a potent stimulator of angiogenesis that induces cell proliferation and survival. Angiogenic factors such as MMPs and VEGFs are considered important regulators of tumor growth, both at the primary tumor site and other distant sites of metastasis¹⁵⁻¹⁸. Earlier studies from our laboratory has shown the role of anacardic isolated from the waste obtained from cashew industry in inhibiting MMPs¹⁹. The present work utilizes the waste product obtained from coconut industry i.e. the coconut shell to identify potent modulators of MMP-2 and MMP-9. *Cocos nucifera*, commonly known as coconut tree is well known to possess several pharmacological properties²⁰⁻²². The endocarp of the coconut shell has been used in traditional medicinal practices to cure several ailments including skin infection²³. The present study therefore employs A375, a melanoma cell line, known to exhibit metastatic features to assess the chemotherapeutic effect of CSE on several biomarkers of cancer with special emphasis to gelatinases.

MATERIALS AND METHODS

Extraction of Coconut shell

Mature coconut shell was procured from local sources which was crushed, powdered (500g) and subjected to Soxhlet extraction using petroleum ether, chloroform and ethyl methyl ketone (EMK). The solvents were removed by rotary flash evaporator at a temperature of 40°C. The EMK extract (1.0gm) was fractionated using silica gel column chromatography (60-120 mesh, 50gm). The column elution was initiated using petroleum ether (PE), followed by a mixture of PE containing increasing proportions of chloroform (CHCl₃). The fractions were monitored by thin layer chromatography (toluene: ethyl

acetate: formic acid; 5:5:2). Three major fractions: PI (180mg), PII (250 mg) and PIII (400 mg) were obtained; PI and PII were taken for further purification based on their bioactivity. Sephadex LH 20 column was used for further purification of PI and PII which yielded fractions F1 (80mg) and F2 (30mg) respectively. Characterization of the major bioactive compound, F1, was carried out using UV, IR, HPLC, LCMS, melting point and NMR analysis. All other chemicals used in the study were purchased from Sigma Aldrich, USA.

General analytical methods

Thin layer chromatography (TLC) was performed using silica gel plates (Kieselgel, F254, Merck). HPLC analysis was carried out on Shimadzu-SPD-M20AD, equipped with DAD (Diode Array Detector) and Phenomenex Luna 5 μ C18 (2) 100 Å, size 250 X 4.60 nm. All the compounds were detected at 254 nm at room temperature with an eluent flow rate of 1.2ml/min and an injection volume of 10 μ L. The mobile phase consisted of acetonitrile and water containing formic acid (0.03%). ¹H NMR spectra were recorded on Bruker AM 400 spectrometer (400 MHz, for ¹H NMR) using tetramethylsilane (TMS) as a standard. UV-Vis spectra in methanol was recorded on a Shimadzu UV spectrophotometer (Model UV-1800). IR spectrum in KBr was recorded on Shimadzu IR Affinity-1. LC-MS was recorded using Agilent Technologies 1290 Series HPLC and 6340 Ion Trap LC-MS.

Cell Culture

Human melanoma cell line, A375 was obtained from National Center for Cell Sciences, Pune, Maharashtra, India. The cells were grown in DMEM (Sigma Aldrich, USA), supplemented with 10 % FBS (GIBCO, USA), penstrep (100 mg/ml) and amphotericin B (0.5 μ g/mL) (Sigma Aldrich, USA) and were maintained at 37°C in 5% CO₂.

Zymography

A375 cells were pre-treated with PMA (phorbol 12-myristate 13-acetate) in serum free media for 2h in a 6-well plate prior to other treatments. Gelatinase activity in the conditioned media collected after 24h of treatment was analyzed by gelatin zymography as per the standard protocol²⁴. Electrophoresis was performed using 10% SDS polyacrylamide gel incorporated with 0.05 % (w/v) gelatin at 100 V for 3-4 h. Gels were washed in 2.5 % Triton X-100 (v/v) to remove SDS and then incubated overnight in the collagenase developing buffer (50 mM Tris-HCl (pH 7.6), 200 mM NaCl, 5 mM CaCl₂, 0.2 % (v/v) Brij-35, concentration of ZnCl₂). The gels were stained for 4 h in 0.25 % (w/v) Coomassie brilliant blue G-250 and then destained in water.

Cell viability Assay

The viability of the cells after treatment with the compounds were measured using MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl- tetrazolium bromide) assay as described previously²⁵. The cells were treated with varying doses of the compounds for 24h in a 96 well plate and the absorbance at A570/A690 was determined using a plate reader (BioTek Instruments Inc., USA). Percentage cell viability was calculated as the ratio of absorbance of the treated cells to the untreated cells.

RNA Extraction and RT-PCR

Cells (1×10^5) were seeded in a 6 -well plate and 24h post treatment, the total RNA was extracted using RNeasy Mini Kit (Qiagen, Germany), as per the manufacturer's instructions. 100ng of RNA was

converted to cDNA using the MultiScribe™ reverse transcription kit (ABI, USA). Quantitative real-time PCR analysis was performed using SYBR green fluorescent dye (Step One Plus, ABI, USA).

The primers used for PCR were

Primers	Forward(5'-3')	Reverse(5'-3')
GAPDH	AATCCCATCACCATCTTCCAG	AAATGAGCCCCAGCCTTC
MMP-9	CGAACTTTGACAGCGACAAG	CACTGAGGAATGATCTAAGCCC
MMP-2	ACCCATTACACCTACACCAAG	GTTTGCAGCAGATCTCAGGAGTG
COX1	GCCATGAGCCGGAGTCTCTT	CTGGTGCTGGCATGGATAGT
COX-2	TCCCTTGGGTGTCAAAGGTAAAAGC	CCTGGGGATCAGGGATGAACCTTC
EGFR	TCTGCCGCAAATTCGAGACGAA	TGGCACCAAAGCTGTATTGGCC
VEGF	ATAGAGCAAGACAAGAAATCCCTG	CATTACACGTCTGCGGATCT

The amplification conditions used were: 95°C (10 min) followed by 40 cycles of 95°C (15s), 60°C (30s) and 72°C (30s). $\Delta\Delta$ CT method was used to quantify the relative mRNA levels that were normalized to endogenous housekeeping gene, GAPDH.

Cell cycle analysis

A375 cells (0.5×10^5) were seeded in a 6 -well plate and treated with different concentrations of F1 fraction for 24h post PMA induction. After treatment, the cells were trypsinized and suspended in DMEM, centrifuged at 2000 rpm for 10 minutes, re-suspended in 1 mL of cell cycle buffer (0.03% NP-40, 40 μ g/mL RNase A, 40 μ g/mL propidium iodide, 0.1% sodium citrate) and incubated in the dark for 5-10 minutes at room temperature. Cell cycle analysis was performed using BD FACS Calibur flow cytometer. Data acquisition and analysis was performed using Cell Quest Pro and Flow Jo software respectively.

Cell migration assay

The upper chamber of transwell inserts (BD bioscience) contained A375 cells (1.5×10^4 cells/100ul in serum- free medium) treated with 'F1'. 200ul of DMEM containing 10% FBS was added to the lower chamber. The chambers were assembled and kept in incubator for 24h at 37°C. After incubation, cells from the upper surface of the membrane were removed using a cotton swab and the cells on the lower surface were fixed using 5% glutaraldehyde followed by staining with crystal violet. The membranes were microscopically examined and cell migration was determined with respect to the untreated control.

Docking Studies

In order to identify the binding mode of oxyresveratrol, AutoDockTool (<http://mgltools.scripps.edu>) was used. The crystal structure of MMP-2 (PDB code-1QIB) and MMP-9 (PDB code-2OVX) catalytic domain was obtained from RCSB PDB data bank. For docking studies, a grid box of 44X46X60 (MMP-2 and MMP-9)

points on 1-Å grid were used to generate affinity maps. The best docking pose of oxyresveratrol was selected and analyzed.

Statistical Analysis

Statistical analysis was conducted using Prism (GraphPad Software). Student's t-test or one-way analysis of variance were performed for statistical comparisons followed by Dunnett's test. All values are expressed as the mean +S.E.M. from three independent experiments.

RESULTS**Regulation of MMP-9 and MMP-2 activity by different fractions obtained from coconut shell**

In order to study the regulation of MMP-2 and MMP-9 by the major constituents of CSE, A375 cells were treated with EMK-CSE extracts, F1 and F2, for 24h post induction with PMA (50ng/ml). Treatment with PMA gave a significant induction of MMP-9 activity (9 fold). The other extracts, i.e. water, petroleum ether, and chloroform were not subjected for further analysis either due to lack of significant gelatinase inhibitory property or enhanced cytotoxicity. Gelatin zymography studies performed with the conditioned media obtained after treatment (2.5 μ g/ml to 10 μ g/ml) revealed that among the two major fractions treated, F1 was more potent when compared to F2 (Supplementary Fig.1). Further studies performed with lower concentrations (0.5 μ g/ml to 2 μ g/ml) of both the fractions revealed that F1 fraction exhibited significant inhibition of both MMP-9 and MMP-2 activity (87% and 40% respectively), at concentrations as low as 1 μ g/ml and was therefore subjected to further characterization (Fig.1).

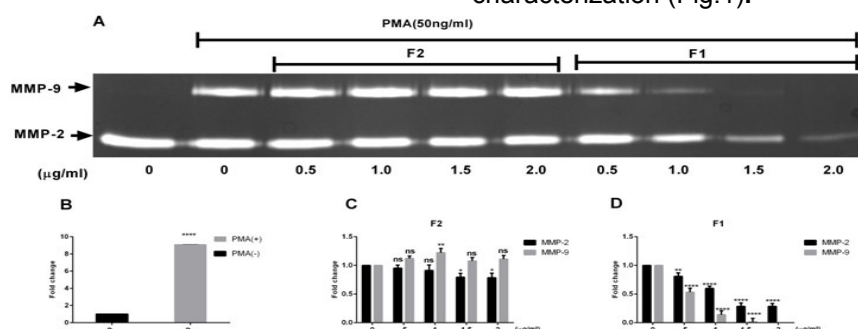


Figure 1

Regulation of MMP-2 and MMP-9 (gelatinases) activity by CSE

Figure 1 A) Zymogram showing gelatinase activity of conditioned media from A375 cells treated with 0.1% DMSO (lane:1), PMA(50ng/ml) (lane:2), 0.5, 1.0, 1.5, 2.0 μ g/ml of F2 (lane:3-6) and 0.5, 1.0, 1.5, 2.0 μ g/ml of F1 (lanes:7-10). (B) A representative plot of the zymogram showing induction of MMP-9 activity in the presence of PMA. (C) A representative plot of the zymogram showing fold change in gelatinase activity on treatment with various concentrations of F2 fraction. (D) A representative plot of the zymogram showing fold change in gelatinase activity on treatment with various concentrations of F1 fraction. Each bar represents the Mean \pm SE of triplicate determinations from three independent experiments. ***P <0.001 (one-way analysis of variance with Dunnett's multiple-comparison)

Effect of F1 fraction on cell viability

In order to ensure that the reduction in gelatinase activity was not due to cytotoxicity, MTT assay was performed. As observed in Fig.2A, MTT analysis showed no significant cytotoxicity in cells that were treated with F1 fraction at the concentrations tested. Additionally, there was no visible morphological alterations in the F1 treated cells when compared to control cells (Fig.2B).

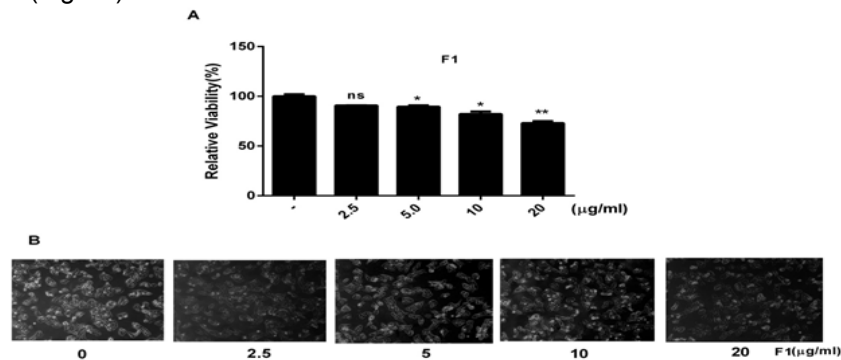


Figure 2

Effect of F1 fraction on A375 cells viability

Figure 2. (A) Cell viability using a MTT cytotoxicity assay was performed for the cells treated with F1 for 24h (at a concentration range of 2.5 to 20 μ g/ml) in serum-free DMEM. Each bar represents the mean \pm SE of triplicate determinations from three independent experiments. (B) Morphological examination of A375 cells treated with different concentrations of F1 for 24h. Each bar represents the Mean \pm SE of triplicate determinations from three independent experiments. ***P <0.001 (one-way analysis of variance with Dunnett's multiple-comparison)

F1 modulates the expression of MMP-2 and MMP-9 in A375 cells

qRT-PCR studies with the 'F1' fraction indicated that both MMP-2 and MMP-9 mRNA levels were down regulated in a dose dependent manner, in PMA induced A375 cells. At a concentration of 5 μ g/ml, F1 inhibited both MMP-2 and MMP-9 mRNA expression by 67% and 84% respectively (Fig.3).

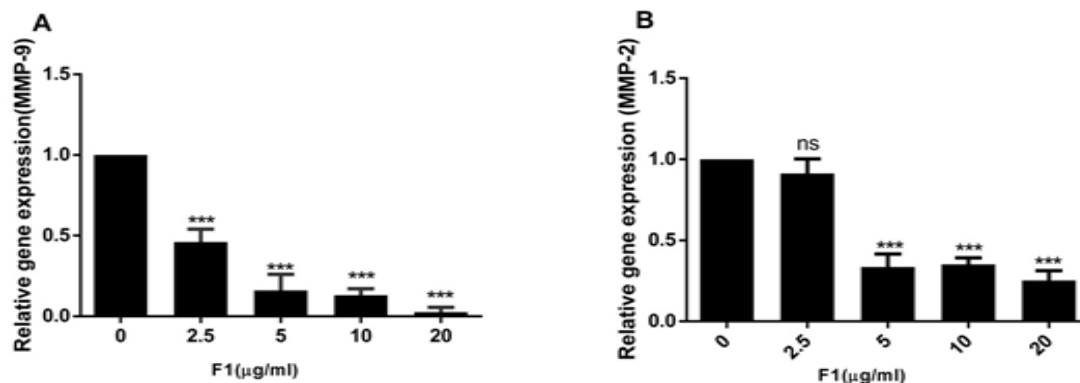


Figure 3

Regulation of F1 fraction on MMP-2 and MMP-9 expression

Figure 3. Dose dependent regulation of (A) MMP-9 and (B) MMP-2 expression on PMA induced A375 cells by F1. Total RNA was extracted after 24h of treatment and qPCR was performed to determine the gene expression. Each bar represents the Mean \pm SE of triplicate determinations from three independent experiments. ***P <0.001 (one-way analysis of variance with Dunnett's multiple-comparison)

F1 regulates the expression of EGFR, VEGF and COX-2 in A375 cells.

The effect of F1 on the regulation of COX-2, EGFR and VEGF, known to play prominent roles in cell proliferation, angiogenesis and metastasis in a variety of cancers was studied using qRT-PCR analysis.

Treatment with F1 for 24h showed significant dose dependent inhibition of VEGF, EGFR and COX-2. F1 showed a substantial decrease in VEGF (70%), EGFR (60%) and COX-2 expression (90%) at a concentration of 5 μ g/ml (Fig.4).

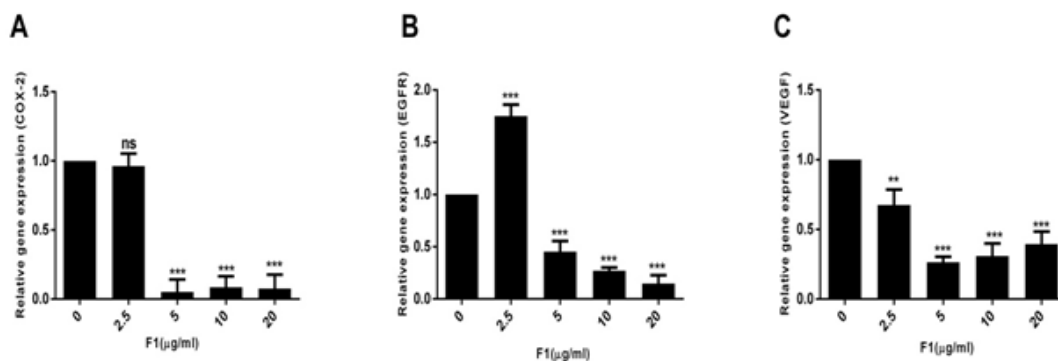


Figure 4

Regulation of COX-2, EGFR and VEGF expression by F1 fraction

Figure 4. Dose dependent regulation of (A) COX-2 (B) EGFR (C) VEGF mRNA on PMA induced A375 cells by F1. Total RNA was extracted after 24h of treatment and qPCR was performed to determine the gene expression. Each bar represents the Mean \pm SE of triplicate determinations from three independent experiments. ***P <0.001 (one-way analysis of variance with Dunnett's multiple-comparison)

Regulation of cell migration and cell cycle progression by F1 in A375 cells.

Since EGFR and MMP-9 are well known to regulate cellular processes like proliferation and migration, the effect of F1 fraction on cell migration and cell cycle progression was studied using transwell migration assay and FACS analysis respectively. A375 cells were treated with various concentrations of F1 for 24hrs post PMA

treatment. As evident from our results, treatment with F1 fraction showed significant accumulation of cells in the G2-M phase (Fig.5A). Furthermore, cell migration studies clearly indicated that treatment of A375 cells with different concentrations (5µg/ml and 10µg/ml) of 'F1' for 24h resulted in a dose-dependent inhibition of migration when compared to the untreated control (Fig. 5B).

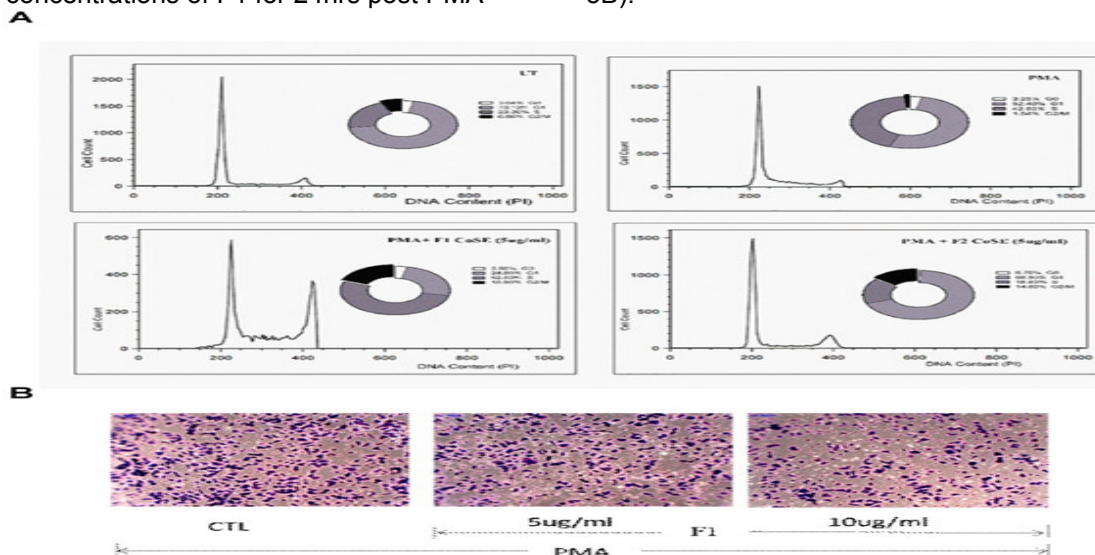


Figure 5

Regulation of cell migration and cell cycle progression by F1 in A375 cells.

Figure 5. (A) A375 cells were treated with 5µg/ml of F1 for 24h post PMA treatment and the cells were subjected to cell cycle analysis. Propidium iodide was used to stain DNA. Flow cytometry analysis shows that the F1 extract at a concentration of 5µg/ml arrested the cells in G2/M phase of the cell cycle. (B) Transwell migration assay showing the dose-dependent inhibition in migration of A375 cells after treatment with

Characterization of F1

The potent inhibition of MMP-9, EGFR, COX-2 and VEGF by F1 prompted us to structurally characterize F1. F1 was obtained as a dull white colored powder which gave pale brown color with FeCl₃ and readily dissolves in NaOH (1% aqueous). The structural characteristics of F1 were: MP 204-205°C, UV λ_{max} (MeOH)/nm; 220, 324. IR (vcm⁻¹); 1650, 960, 800, 652. ¹H NMR (400MHz, DMSO-d₆, in ppm); 6.12, t, 1H (H-4), 6.37, d, 2H (H-2 and 6), 6.33, d, 1H (H-3'), 6.70, d, 1H (H-8), 6.73, d, 1H (H-7), 6.83, dd, 1H (H-5'), 6.96, d, 1H (H-6'). ¹³C NMR (100MHz, DMSO-d₆, in ppm); 101, 104, 113, 115, 118, 125, 128.2, 128.6, 139, 145.3, 145.4 and 158. LC-MS; Rt-15.3, Purity-95%, [M+1]⁺ (m/e) = 245,

MS/MS= 227, 199, 135, 107. From the data obtained, F1 was characterized as oxyresveratrol (2,3',4,5'-Tetrahydroxy-trans-stilbene)[PubChem CID:5281717], a naturally occurring polyphenol (Fig.6) that possesses anti-tumor and anti-oxidant activities in addition to its ability to lower blood fat and sugar levels²⁶. Though earlier studies suggested the occurrence of ferric acid as major component in coconut endocarp²⁷, none of the fractions obtained in our study showed the presence of ferric acid (except UV which appears similar to F1). Since EMK extract and F2 did not show significant inhibition of MMP-9 and other modulators of cancer, the data on their structural characterization has not been incorporated here (Supplementary Fig. 2).

Characterization of F1

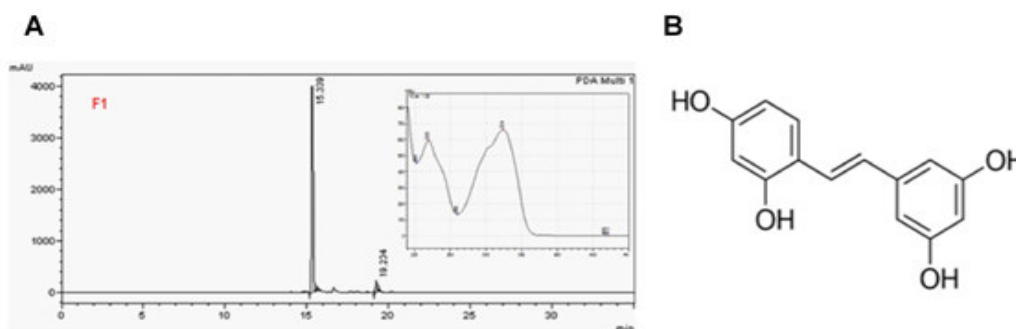


Figure 6
(A) HPLC and UV profile of F1 (inset). (B) Structure of Oxyresveratrol.

Molecular docking studies

To understand the possible molecular interaction of oxyresveratrol with MMPs, docking study was performed using Auto-Dock tools. The docking parameters were fixed based on our previously published results¹⁹. A similar docking energy of -8.9kcal/mol was observed with both MMP-2 and MMP-9. Oxyresveratrol was found to bind at the active site S1' pocket of both MMP-2 and MMP-9. In case of MMP-2, oxyresveratrol forms five hydrogen bonds with Leu164, Ala165, His201, Glu202 and Ala220, all of which are present in the active site of the enzyme (Fig. 7A). The distance from the Zn²⁺ atom

was found to be 4.4 Å⁰. Whereas, in case of MMP-9, oxyresveratrol forms three hydrogen bonds with Ala417, Tyr420 and Met422 (Fig. 7B). The importance of these residues have been reported in earlier studies²⁸. In both MMP-2 and MMP-9, the ligand did not seem to interact with Zn²⁺ atom. In spite of having similarity in the binding energies, oxyresveratrol showed better inhibition of MMP-9 (87%) when compared to MMP-2(40%) at a concentration of 1µg/ml. This difference in inhibition of MMP-2 and MMP-9 could be due to the fact that the three interacting residues in MMP-9 (Ala417, Tyr420 and Met422) is more significant than those in MMP-2 (Leu164, Ala165, His201, Glu202 and Ala220).

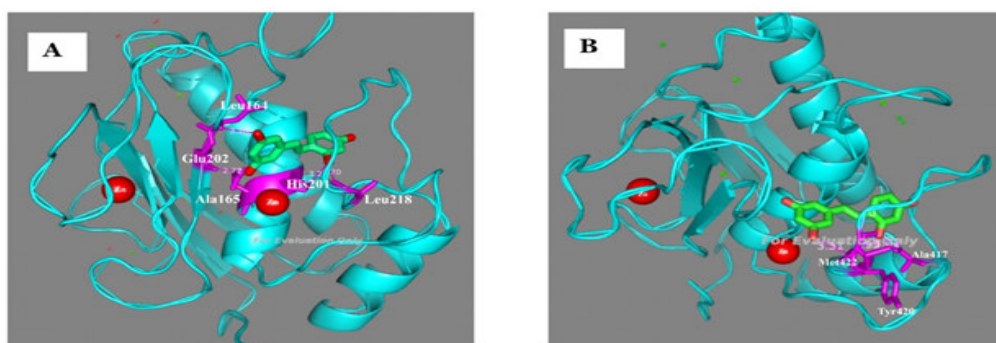


Figure 7
Predicted binding mode of Oxyresveratrol to MMP-2 and MMP-9
Figure 7. (A) Ligand (stick model) interaction with (A) MMP-2 and (B) MMP-9 (both indicated in coil and ribbon structured; red color-zinc metal, hydrogen bonds and amino acid residues are indicated in violet color).

DISCUSSION

Natural products serve as valuable repositories of therapeutic compounds which forms the basis for the development of majority of the well established drugs^{29,30}. Recently, there has been a renewed interest in natural product research as a means to deliver lead compounds in key therapeutic areas such as immunosuppression, anti-infectives, cancer and metabolic diseases^{25,31}. *Cocos nucifera* (CN) is well known for its wide range of therapeutic effects including antiseptic, bactericidal, and diuretic properties^{20,32}. The present study was focused on bioactivity based screening of MMP-9 modulators from coconut shell, the underutilized part of *Cocos nucifera*. The studies were performed on human melanoma cells, A375, since

melanoma is considered as one of the most fatal form of skin cancer due to its ability to metastasize. Therefore the development of novel therapeutic alternatives that can inhibit the metastatic ability of melanoma cells is of considerable importance. Expression of MMP-2, MMP-9, COX-2, EGFR and VEGF are closely linked to various stages of cancer progression like growth, invasion, metastasis and angiogenesis³³. Treatment of cells with F1 (later characterized as oxyresveratrol) inhibited gelatinase activity and also showed reduction in migration of A375 cells. This inhibition in migratory potential could be attributed to the down regulation of MMP-9 and COX-2 gene expression that are known to play a vital role in cancer cell metastasis. These results were supported by our docking analyses which suggested that the inhibition of MMP-2 and MMP-9

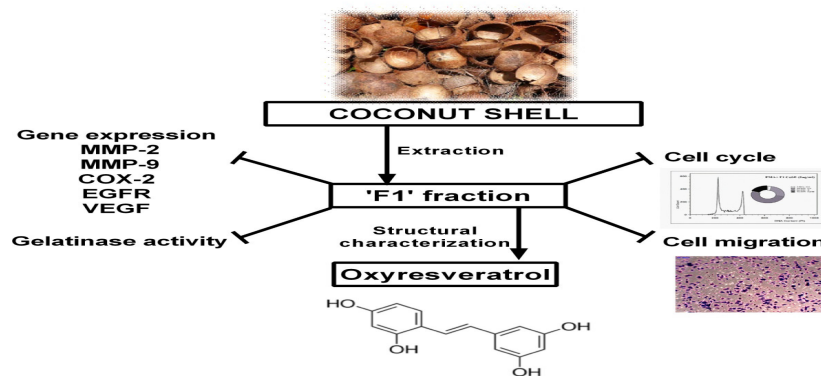
occurs through binding of oxyresveratrol directly to the active site of MMP-2 and MMP-9. In addition to MMPs and COX-2, several growth factor-driven signaling pathways play prominent role in the pathogenesis of human cancers³⁴. Since EGFR is frequently over expressed in melanoma, it is considered an important target in anticancer drug development along with MMPs and COX-2. Earlier studies have shown that combined inhibition of COX-2 and EGFR results in enhanced anti-tumor activity when compared to inhibitors against a single target³⁵. Furthermore, inhibition of these proteins were also found to assist in sensitizing human melanoma cells to many of the currently available pharmaceutical therapies³⁶. A significant down regulation of EGFR gene expression was seen in the presence of Oxyresveratrol. Similarly, VEGF, found to be up regulated in malignant melanoma cells³⁷ plays a dominant role in promoting tumor angiogenesis³⁸. In addition to inhibition of MMPs, COX-2 and EGFR, oxyresveratrol also showed significant inhibition of VEGF gene expression. The present study also highlights the role of Oxyresveratrol in inducing G2/M arrest and inhibiting migration of A375 cells. Therefore

the present work highlights the need for the development of such novel gelatinase and VEGF inhibitors which are likely to be of importance in combating a wide range of pathophysiological conditions such as inflammatory diseases and various forms of cancer.

CONCLUSION

The present study highlights the role of an underutilized part of coconut i.e. the coconut shell in promoting anti-cancer effects. The result from our study have identified and characterized oxyresveratrol from coconut shell for the first time. The current work also demonstrates the potential anti cancer properties possessed by oxyresveratrol that includes inhibition of MMP-2, MMP-9, VEGF, EGFR and COX-2 expression, inhibition of cell migration and induction of cell cycle arrest at G2/M phase. These observations enable us to utilize oxyresveratrol as a novel template for developing anticancer therapeutics.

Supplementary figures Schematic summary of Oxyresveratrol mediated regulation of various process involved in cancer progression



Regulation of MMP-2 and MMP-9(gelatinases)activity by CSE

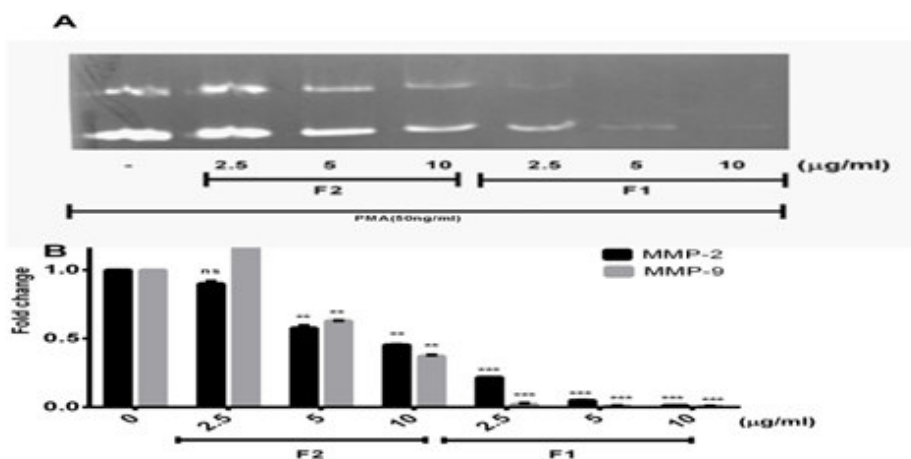


Figure 1

Regulation of MMP-2 and MMP-9(gelatinases)activity by CSE

Supplementary Fig 1.(A) Zymogram showing gelatinase activity of conditioned media from A375 cells treated with PMA(50ng/ml) (lane:1), 10µg/ml 'F2'(lane:2-4) and 2.5, 5, 10µg/ml 'F1'(lane:5-7) (B) A representative plot of the zymogram showing fold change in gelatinase activity on treatment with various concentrations of 'F1' and 'F2' Each bar represents the Mean ± SE of triplicate determinations from three independent experiments. ***P < 0.001 (one-way analysis of variance with Dunnett's multiple-comparison)

Characterization of EMK extract and 'F2' fraction.

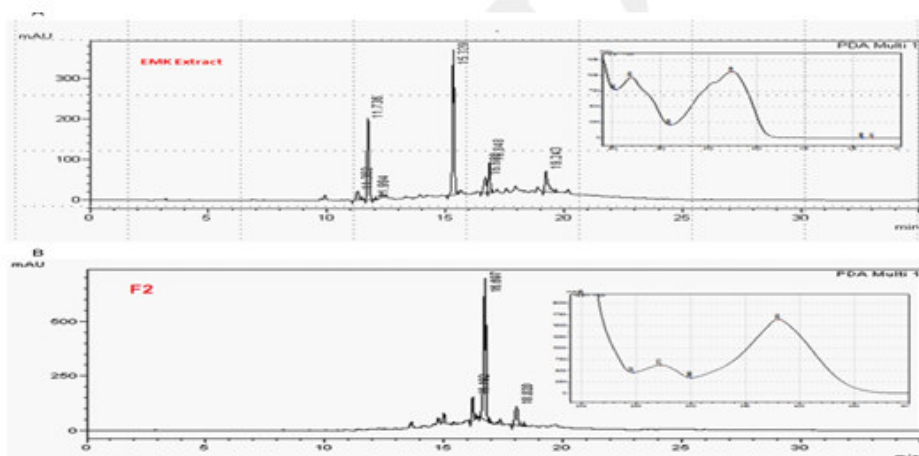


Figure 2

The HPLC and UV profile (inlet) of (A) EMK extract and (B) 'F2' fraction.

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