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Original Article

Phytochemical Analysis and *in vitro* Cell Viability Effects of Ethanolic Extract of *Ormocarpum cochinchinense* on Mouse Embryonic Fibroblasts

Gayathri Somashekar[1,*], Uma Sudhakar[2], Seshadri Srividya[3], Snophia Suresh[2]

[1]Department of Periodontics, Dr. M.G.R Educational and Research Institute, Chennai, Tamil Nadu, INDIA.
[2]Department of Periodontics, Thai Moogambigai Dental College and Hospital, Chennai, Tamil Nadu, INDIA.
[3]Department of Biochemistry, Sathyabama Dental College and Hospital, Chennai, Tamil Nadu, INDIA.

ABSTRACT

Background: *Ormocarpum cochinchinense* is a medicinal herb known to be used by traditional bone setters for the healing of bone fractures. This herb has many active biomolecules, which are responsible for biological activities like antioxidant, anti-inflammatory, hepatoprotective and anticancer. This study aimed to perform phytochemical screening to identify the phytoconstituents and determine the cell viability effects of ethanolic extract of *Ormocarpum cochinchinense* through MTT assay. **Materials and Methods:** Ethanolic extract of leaves of *Ormocarpum cochinchinense* were subjected to qualitative phytochemical screening through Gas chromatography-Mass spectroscopy analysis. The *in-vitro* cell viability effects on 3T3 cell lines with varying concentrations of ethanolic plant extract were measured through (3- [4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) MTT assay. **Results:** Phytochemical screening identified 24 bioactive phytocompounds with various biologic activities. The herb's *in vitro* cell viability effects of ethanolic extract revealed an IC_{50} value of 58.05µg/ml on 3T3 cell lines. **Conclusion:** It can be suggested that ethanolic extract of leaves of *Ormocarpum cochinchinense* are a potential source of bioactive phytoconstituents and are safe on 3T3 cell lines at lower concentrations. The results of this study provide scientific knowledge about the herb that may be applied in future biomedical research investigations.

Keywords: *Ormocarpum cochinchinense*, MTT assay, GC-MS, Phytocompounds.

Correspondence
Dr. Gayathri Somashekar
Research Scholar, Department of Periodontics, Dr M.G.R Educational and Research Institute, Chennai- 600095, Tamil Nadu, INDIA.
Email id: gayathri.agrahar@gmail.com
ORCID ID 0000-0002-4220-2116

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INTRODUCTION

Ormocarpum cochinchinense (OC) is a small herb belonging to the Fabaceae family. OC herb is commonly called Bone knit or Elumbotti (elumbu- Bone, Otti- to join) and Kattumurungai in Tamil.[1] This herb is available in forest regions of Tamil Nadu. The herb has been used in folk medicine to treat bone fracture healing.[2] The phytocompounds parts of medicinal plants are Alkaloids, Phenols, Terpenoids, Flavonoids, Tannins, Saponins, Phytol, Fatty acids and Coumarins.[1,3] *Ormocarpum cochinchinense* is known to have various biological properties like anti-inflammatory, antioxidant, antimicrobial, and antidiabetic. The bone fracture healing study done by M.D Kumar *et al.* reported a faster bone fracture healing on topical application of the plant extract and radiographic evidence of bone formation. The plant had a rich source of calcium ions and magnesium ions.[4] MTT assay (3- [4, 5- dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) was used to measure cell viability or cytotoxicity. MTT is a tetrazolium salt that dissolves in water. Succinate dehydrogenase enzyme present in metabolically active cells can reduce MTT to water-insoluble formazan product. MTT is a commonly employed method to determine cell cytotoxicity, proliferation, or activation.[5] The goal of this work was to investigate the bioactive phytocompounds in ethanolic extract of *Ormocarpum cochinchinense* leaves using Gas chromatography-mass spectroscopy (GC-MS) and the MTT test was used to examine the *in vitro* cell viability of 3T3 cell lines with various doses of ethanolic extracts of OC plant extract effects.

MATERIALS AND METHODS

The herbal leaves were collected from forests near Hosur, Tamil Nadu, in February 2020. Plant botanist Dr. P Jayaraman of the Plant anatomy research center (PARC), West Tambaram, Chennai, confirmed the herb's species. Accession number Parc/2021/4408 was assigned to the voucher specimen. The herb belonged to species called *Ormocarpum cochinchinense* (OC), Family Fabaceae.

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Chemicals

3T3 cell line (Mouse embryonic fibroblast cell lines) was procured from the Pune National center for cell science. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Penicillin, Streptomycin, Modified Eagle medium by Dulbecco (DMEM), 10% Foetal bovine serum (FBS), Pen strip, Trypsin, Dimethyl sulfoxide (DMSO), Ethanol was procured from Sigma Aldrich.

Preparation of Ethanolic Extract of OC

The leaves were washed and shade dried. The leaves were ground into a fine powder in an electric blender and stored in polyethylene bags. 100g of herbal powder was soaked in 1000ml of 95% ethanol with intermittent shaking for 5 days. Whatman No. 1 filter paper was used for filtration. The ethanolic extract was placed in Petri dishes and dried in an evaporator before being stored at 4°C. The dried extract was dissolved in ethanol before being used for further testing.

Gas Chromatography-Mass Spectroscopy (GC-MS)

The *Ormocarpum cochinchinense* (OC) ethanolic extract was subjected to qualitative analysis to identify phytocompounds using JEOL GCMATE II GCMS equipped with high resolution and secondary electron multiplier. The carrier gas was helium at a 5:4 ratio. The HP 5 column was fused to silica 50 m×0.25 mm. 1µl of the sample was evaporated in an injector with a run speed of 22 min.[6] The mass spectrum was analyzed using the database from the National Institute of Standards and Technology (NIST). The phytocompounds found in the ethanolic extract of OC were compared to those in the NIST library, which contains over 62000 patterns in the published literature.[7] Retention time (RT), Nature of the compound, Molecular formula (MF), Molecular weight (MW), Peak area percentage (PA%) and biological activities were used to describe phytocompounds extracted from OC ethanolic extract.

MTT Assay — *In-vitro* Cell Viability Assay

The cellular ability to cleave the tetrazolium salt, MTT, was used to measure cell viability, as described by Mosman.[5,8] 3T3 cell lines grown at 37°C in a humidified atmosphere of 5% CO_2 in a medium supplemented with 10% inactivated Foetal Bovine Serum (FBS), penicillin (100 IU/ml), and streptomycin (100 g/l). Media containing 10% FBS, the trypsinized monolayer of cell culture and the cell count were adjusted to 1.0 × 105 cells/ml. 100 µl (microlitres) of diluted cell suspension (50,000 cells/well) was added to the 96 well microtiter plates. The supernatant was discarded after 24 hr when a partial monolayer had developed and washed with a medium and 100 µl of various concentrations of DMSO was added to dissolve formazan. Absorbance (abs) was measured at a wavelength of 570 nm (nanometres).

$$\text{Percentage (\%) of Viability} = \text{Sample absorbance} / \text{Control absorbance} \times 100$$

RESULTS

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

Twenty-four bioactive phytoconstituents such as phytol, terpenoids, flavonoids, alkaloids, fatty acids, esters and amides were identified from ethanolic leaves extract of *Ormocarpum cochinchinense* through GC-MS analysis. Table 1, Table 2, and Graph 1 show the compounds classified based on retention time (RT), molecular formula (MF), molecular weight (MW), peak area percentage (PA%), and biological activity. Different peaks were obtained where each peak corresponds to an important phytocompound. The significant peaks in the order of the retention time, peak area percentage and biological activities have been reported. Out of 24 phytoconstituents, 11 compounds with significant peaks are phytol- a diterpene (RT: 61.194, PA%: 19.63) with biological properties like antioxidant, anti-inflammatory, anticancer, immunostimulant, reducing levels of inflammatory mediators and anti-microbial. Phthalic acid, di(2-propylpentyl) ester-phthalic acid (RT:68.418, PA%:15.32)- antimicrobial and anticancer, 2-hydroxy-1-hydroxymethylethyl ester of hexadecanoic acid - terpenoid (RT: 67.789, PA%:10.93)- antioxidant, hemolytic, reduces levels of inflammatory mediators and hypocholesterolemic. 2,2,5a-trimethyl-1a-[3-oxo-1-butenyl]-1-benzazirene-1-carboxylic acid methyl ester (RT: 78.842, PA%: 6.82)- no activity reported. n- Hexadecanoic acid, - palmitic acid (RT: 58.352, PA%: 6.63)- antioxidant, anti-inflammatory, antimicrobial and hypocholesterolemic. Tetradeconoic acid - fatty acid (RT: 54.291, PA%: 4.09)- antioxidant, antimicrobial and anticancer. N-methyl-s-Triazolo[4,3-a] pyrazine, 3-ethyl-5,8-dimethyl-amide (RT: 68.090, PA%: 3.94)- scaffold for drug delivery, antibacterial and antidiabetic. 1,1,1,3,5,5,5-Heptamethyltrisiloxane (RT:78.085, PA%: 3.59)- antimicrobial and analgesic. 9,12,15 Octadecatrienoic acid -linolenic acid (RT: 61.643, PA%: 3.74)- anti-inflammatory, anticancer, antiarthritic and hepatoprotective. Hexadecanoic acid methyl ester (RT:57.659, PA%:2.30) - antioxidant, anti-inflammatory, antimicrobial and hypocholesterolemic. Octadecanoic acid -fatty acid (RT:62.060, PA%: 1.42)-antioxidant and anti-inflammatory. As far as literature search, GC-MS analysis of leaves of *Ormocarpum cochinchinense,* this is the only study in which 24 phytocompounds were identified.

Effect of Plant Extract on 3T3 Cell Lines- MTT Assay

The IC_{50} value of ethanolic leaves extract of *Ormocarpum cochinchinense* on cell viability on 3T3 cell lines (mouse embryonic fibroblasts) for 24 hr was 58.05 µg/ml (Table 3, Graph 2). Cell viability reduced as concentration increased. Cell viability was 98.41% at a concentration of 3.12 µg/ml OC plant extract.

Table 1: Phytocompounds in the ethanolic leaves extract of *Ormocarpum cochinchinense* (GC-MS).

Sl. No	RT	Phytochemicals Solvent: Ethanol	Nature of the compounds	MF	MW/ PA%
1	43.449	O-(Carboxymethyl) hydroxylamine, N- dimethylaminomethylene-, methyl ester 3-Hexanone, 2,5-dimethyl-4-nitro-2,3'-Bifuran, octahydro-	Amides	$C_4H_{11}ClN_2O_6$	109.30/ 0.59
2	47.350	1,4 Benzodioxan-6-amine	Amide	C_8H_9NO	151.16/2.33
3	48.748	Benzoic acid, 4-ethoxy-, ethyl ester	Ester	$C_{11}H_{14}O_3$	194.2/3.25
4	54.291	Tetradecanoic acid (mystric acid)	Fatty acid	$C_{14}H_{28}O_2$	228/4.09
5	55.908	Neophytadiene 3,7,11,15-Tetramethylhexadec-2-en- 1-yl acetate	Diterpenoid	$C_{22}H_{42}O_2$	338.5/0.90
6	57.210	Lidocaine	Amide	$C_{14}H_{22}N2_O$	234.34/1.51
7	57.544	7,9-Di-tert-butyl-1-oxaspiro (4,5) deca-6,9-diene-2,8-dione	Flavonoid	$C_{17}H_{24}O_3$	276.4/1.23
8	57.659	Hexadecanoic acid, methyl ester	Fatty acid	$C_{17}H_{34}O_2$	270.5/2.30
9	57.884	Benzothiazole, 2-(2-hydroxyethylthio)- 2-Mercaptobenzothiazole	Amine	$C_9H_9NOS_2$	211.3/0.59
10	58.352	n-Hexadecanoic acid	Fatty acid	$C_{16}H_{32}O_2$	256/6.63
11	58.987	Hexadecanoic acid, ethyl ester Undecanoic acid	Palmitic acid ester	$C_{18}H_{36}O_2$	284.4/0.98
12	60.969	9,12,15-Octadecatrienoic acid, methyl ester	Fatty acid ester	$C_{19}H_{32}O_2$	292.4/2.21
13	61.194	Phytol	Diterpene	$C_{20}H_{40}O$	296.5/19.63
14	61.444	Methyl stearate	Ester	$C_{19}H_{38}O_2$	298.5/0.89
15	61.643	9,12,15-Octadecatrien-1-ol, (Z, Z, Z)	Fatty acid	$C_{13}H_{32}O$	264.4/3.74
16	62.060	Octadecanoic acid	Fatty acid	$C_{18}H_{32}O_2$	284.5/1.42
17	62.182	Methyl (Z)-5,11,14,17-Eicosatetraenoate	Fatty acid	$C_{21}H_{34}O_2$	318.5/1.43
18	67.789	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester Glycerol 1-palmitate	Palmitic acid	$C_{19}H_{34}O_4$	330.5/10.93
19	68.090	N-methyl- s-Triazolo[4,3-a] pyrazine, 3-ethyl-5,8-dimethyl	Amide	$C_5H_4N_4$	120.1/3.94
20	68.418	Phthalic acid, di(2-propylpentyl) ester Phthalic acid, 6-ethyloct-3-yl 2-ethylhexyl ester Bis(2-ethylhexyl) phthalate	Fatty acid ester	$C_{26}H_{42}O_4$	418.6/15.32
21	70.592	Butyl 9,12,15-octadecatrienoate 9,12,15-Octadecatrienoic acid, ethyl ester	Linolenic acid	$C_{20}H_{34}O_2$	306.5/1.89
22	70.881	Octadecanoic acid, 2,3-dihydroxypropyl ester Octadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester	Fatty acid	$C_{21}H_{42}O_4$	358.5/3.09
23	78.085	1,1,1,3,5,5,5-Heptamethyltrisiloxane	Polymer	$C_7H_{21}O_2Si_3$	221.50/3.59
24	78.842	1-Benzazirene-1-carboxylic acid, 2,2,5a-trimethyl-1a-[3-oxo-1-butenyl] perhydro-, methyl ester	Fatty acid	$C_{15}H_{23}NO_3$	265.3/6.82

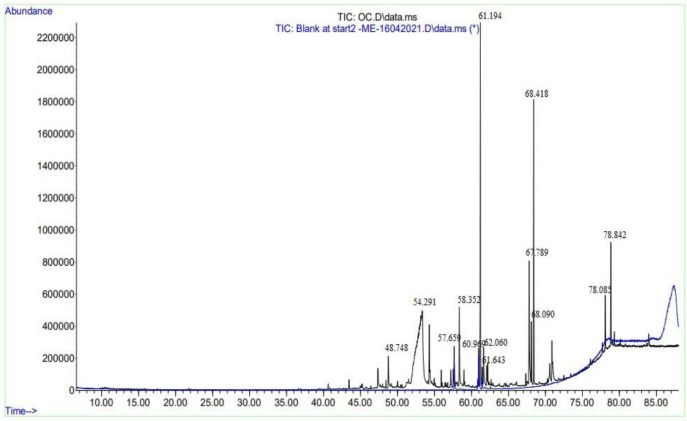
DISCUSSION

The existence of 24 bioactive phytocompounds was discovered in ethanolic extract of *Ormocarpum cochinchinense* leaves in the current study. Out of these, there were 11 phytocompounds with significant peaks with important biological activities. Phytol was found at 19.63 PA% to be antioxidant, anti-inflammatory, antimicrobial, anticancer and reduces the levels of inflammatory mediators[7,9-11] The second highest compound was Phthalic acid, di(2-propylpentyl) ester with antimicrobial and anticancer activity.[12] The third compound in abundance was Hexadecanoic acid which has antioxidant, anti-inflammatory, antimicrobial, and hypocholesterolemic.[13] Most of the phytocompounds have mainly antioxidant, anti-inflammatory and antimicrobial properties. The results of the GC-MS are consistent with the study by Sivakumar *et al.*[14] Phytol, an immunostimulant compound that was identified in other species of *Ormocarpum*.[15] The identification of phytocompounds is considered a part of plant defense mechanisms. These phytoconstituents are called 'phytoprotectants' which are of ecological significance in biomaterial research.[16,17] Studies on extract of *Ormocarpum sennoides,* a species of the *Ormocarpum* herb, have revealed anticancer, antioxidant, antimicrobial, callus induction and presence of calcium and magnesium.[18,19]

In-vitro cell viability assay is done to predict cell viability before *in vivo* studies and the general cytotoxic screening of new materials or chemicals. MTT assay results showed that 3T3 cell line viability was gradually decreased with an increasing concentration of the OC extract. IC_{50} values for ethanolic extract of *Ormocarpum cochinchinense* were 58.05µg/ml Table 3, Graph 2. The concentration of the plant extract below 25µg/ml had more than 85% of cell viability. A study on *Ormocarpum kirkii,* a subspecies of OC plant, reported that isoflavones and bioflavonoids were cytotoxic on cancer cells *in-vitro* and can be used as a potential for cancer therapy.[20]

Table 2: Phytocompounds and their biological activities.

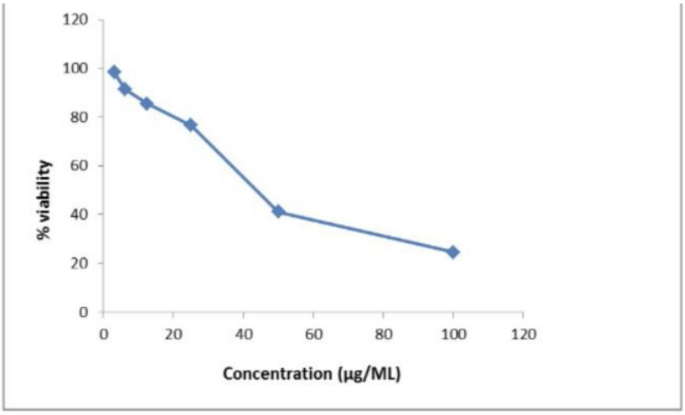
Sl. No	Phytocompounds	Biological activities
1	O-(Carboxymethyl) hydroxylamine, N- dimethylaminomethylene-, methyl ester 3-Hexanone, 2,5-dimethyl-4-nitro-2,3'-Bifuran, octahydro	Anti-inflammatory Anti-allergic
2	1,4 Benzodioxan-6-amine	Anti-inflammatory Anti-hepatotoxic
3	Benzoic acid, 4-ethoxy-, ethyl ester	Anti-microbial
4	Tetradecanoic acid (mystric acid)	Anti-oxidant Anti-microbial Anti-cancer
5	Neophytadiene 3,7,11,15-Tetramethylhexadec-2-en-1-yl acetate	Anti-microbial
6	Lidocaine	Anaesthetic
7	7,9-Di-tert-butyl-1-oxaspiro (4,5) deca-6,9-diene-2,8-dione	Anti-oxidant Anti-microbial Anti-inflammatory Anti-carcinogenic
8	Hexadecanoic acid, methyl ester	Anti-inflammatory Anti-microbial Anti-oxidant Hypocholesterolemic
9	Benzothiazole, 2-(2-hydroxyethylthio)-2-Mercaptobenzothiazole	Anti-microbial Anti-oxidant
10	n-Hexadecanoic acid	Anti-inflammatory Anti-oxidant Hypocholesterolemic
11	Hexadecanoic acid, ethyl ester Undecanoic acid	Anti-inflammatory Anti-oxidant
12	9,12,15-Octadecatrienoic acid, methyl ester	Anti-inflammatory Anti-arthritic Anti-cancer
13	Phytol	Anti-oxidant Anti-inflammatory Immunostimulant Anticancer Anti-microbial Reduces the level of TNF -α, IL-6, and IL-8
14	Methyl stearate	Anti-inflammatory
15	9,12,15-Octadecatrien-1-ol, (Z, Z, Z)	Anti-inflammatory Anti-cancer Hepatoprotective Anti-arthritic
16	Octadecanoic acid	Anti-inflammatory Anti-oxidant Anti-microbial
17	Methyl (Z)-5,11,14,17-Eicosatetraenoate	Anti-bacterial Anti-oxidant Anti-fungal Anti-tumor
18	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester Glycerol 1-palmitate	Anti-oxidant Haemolytic Hypocholesterolemic Reduces levels of TNFα, PGE2, and IL-10 Inhibitor of NF κ B
19	N-methyl- s-Triazolo[4,3-a] pyrazine, 3-ethyl-5,8-dimethyl	Scaffold for drug discovery Anti-bacterial Anti-diabetic
20	Phthalic acid, di(2-propylpentyl) ester Phthalic acid, 6-ethyloct-3-yl 2-ethylhexyl ester Bis(2-ethylhexyl) phthalate	Anti-microbial Anti-cancer
21	Butyl 9,12,15-octadecatrienoate 9,12,15-Octadecatrienoic acid, ethyl ester	Anti-oxidant Anti-carcinogenic Hypocholesterolemic
22	Octadecanoic acid, 2,3-dihydroxy propyl ester Octadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester	Anti-oxidant Anti-inflammatory
23	1,1,1,3,5,5,5-Heptamethyltrisiloxane	Anti-microbial Analgesic Surfactant
24	1-Benzazirene-1-carboxylic acid, 2,2,5a-trimethyl-1a-[3-oxo-1-butenyl] perhydro-, methyl ester	None



Graph 1: Chromatograph of OC ethanolic extract.

Table 3: *In -vitro* cell viability of OC on 3T3 cell lines.

Concentration in µg/ml	% of viability
3.12	98.41
6.25	91.26
12.50	85.54
25	76.60
50	41.13
100	24.56



Graph 2: *In-vitro* cell viability assay on 3T3 cell lines.

CONCLUSION

The study's findings suggest that ethanolic extract of *Ormocarpum cochinchinense* contain essential phytocompounds with biological activity. On 3T3 cell lines, the cell viability of OC extract reduced as the concentration of the plant extract increased. The initial stage in identifying the components in the herbal extract was GC-MS analysis, which was used to look for active phytoconstituents. More research on the herb's application in bone tissue engineering and *in vivo* action on bone fracture studies is needed.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

MTT: (3- [4, 5- dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide); **IC_{50}:** Half maximal inhibitory concentration; **GC-MS:** Gas Chromatography-Mass Spectroscopy; **OC:** *Ormocarpum Cochinchinense*; **PARC:** Plant Anatomy Research Centre; **DMEM:** Modified Eagle medium by Dulbecco; **FBS:** Foetal Bovine Serum; **DMSO:** Dimethyl Sulfoxide; **NIST:** National Institute of Standards and Technology; **RT:** Retention Time; **MF:** Molecular Formula; **MW:** Molecular weight; **PA%:** Peak Area Percentage; **abs:** Absorbance.

SUMMARY

The leaves of *Ormocarpum cochinchinense* was subjected to ethanolic extraction. The crude extract of leaves was phytochemically analysed by Gas Chromatography-Mass Spectroscopy to explore the bioactive phytoconstituents.

In-vitro cell viability affects plant extract on mouse embryonic fibroblasts (3T3 cell lines) was analysed through an MTT assay.

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