In-vivo and in-vitro anti-inflammatory and anti-arthritic activity of Synthetic cis-9 and cis-10 cetyl myristoleate (CMO) isomers in mice

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RESEARCH ARTICLE

IN-VIVO AND *IN-VITRO* ANTI-INFLAMMATORY AND ANTI-ARTHRITIC ACTIVITY OF SYNTHETIC *CIS*-9 AND *CIS*-10 CETYL MYRISTOLEATE (CMO) ISOMERS IN MICE.

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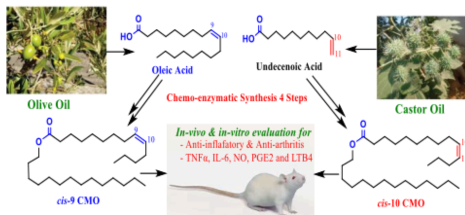
Abstract

Nutraceuticals comprising *cis*-9-cetylmyristoleate (CMO) was reported to block inflammation and prevent adjuvant-induced arthritis in rats. Latter it was also used to reduce knee pain in patients suffering with osteoarthritis. In our earlier study, we have reported the synthesis of pure *cis*-9 and *cis*-10 CMO isomers form oleic acid and undecenoic acid by chemo-enzymatic transformations and their anti-arthritic effect *in-vivo* using Freund's complete adjuvant (FCA; chronic) and carrageen induced paw edema (acute) in male Wistar rats. In this context, we tested the effects of these synthetic *cis*-9 and *cis*-10 CMO isomers for *in-vitro* anti-inflammatory activity using stimulated RAW264.7 mouse macrophage cell line, and destabilization of medial meniscus (DMM) surgery-induced osteoarthritis in C57WT mice. Further, we have treated mouse macrophage cells with CMO isomers to investigate the expression profile of secreted inflammatory cytokines. Both CMOs exhibited dose-dependent reduction of secretion of TNFα, IL-6, nitric oxide, prostaglandin E_2 and leukotriene B_4, in response to lipopolysaccharide (LPS) in mouse macrophage cells. These vegetable oil origin synthetic *cis*-9 and *cis*-10 CMO isomers can be potential sustainable non-steroidal anti-inflammatory drugs (NSAIDs) for the treatment of osteoarthritis and inflammatory diseases.



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Introduction:-

Arthritis is a complex multi factorial disease that is generally known to affect elderly people or a consequence of physical injury that involves inflammation of one or more joints of the body[1]. Recent reports indicate that, there are

approximately 100 causes for arthritis, among them osteoarthritis and rheumatoid arthritis are the two most common types of arthritis[2]. Globally, more than 100 million of people are suffering from arthritis related diseases and it is generally accepted to be the leading cause of movement limitation and disability including swelling, pain, and redness of the area and sometimes dysfunction[3]. Despite the availability of several anti-inflammatory drugs such as steroidal, non-steroidal and immune-suppressive drugs which are effective and are pharmacologically powerful, and they pose several problems due to serious gastrointestinal and cardiovascular side effects, especially upon long-term use [4,5]. In recent years, use of dietary supplements such as glucosamine sulfate (derived from oyster and crab shells), and chondroitin sulfate (derived from shark and cow cartilage) are being widely used as treatment for osteoarthritis (OA) symptoms [5,6]. Dietary supplements generally do not need prescription and that is the reason, glucosamine has been on the market for more than two decades and has succeeded annual global sales of over two billion dollars[6]. Other alternate natural health products including collagen derivatives, ginger, and herbal medicines have been studied and are in use, along with beef tallow extract including *cis*-9-cetylmyristoleate (CMO)[7–9].

Nowadays, CMO is gaining popularity as a dietary supplement for inflammatory response and joint comfort and being sold in the market with different names. The global market size for cetyl myristoleate was valued at approximately USD 1.1 billion in 2023 and is projected to grow to USD 2.3 billion by 2032, reflecting a compound annual growth rate (CAGR) of 8.3%. CMO is fatty acid ester of *cis*-9 myristoleic acid ($C_{14:1}$, MA) and cetyl alcohol (C_{16}-OH) and is naturally occurring fatty acid ester in some animal fat and plant oil sources. CMO was first isolated from Swiss Albino Mice that were naturally resistant to developing joint health issues by Harry W. Diehl in 1972[10]. This finding attracted the researchers to utilize this natural CMO for human health and inflammation, especially related to OA. There was some confusion around the mechanisms of CMO and other fatty acids, and how it impacts joint comfort. The misconception is that they will help to lubricate the joints. In reality, fatty acids support the inflammation pathways that contribute to swelling in the joints, not lubrication. The cyclooxygenase pathway and lipooxygenase pathways are well-known cascade reactions that contribute to inflammation in the body. The rational for the use of CMO is theorized to be a 5-LOX pathway inhibitor and decrease the expression of prostaglandins and leukotrienes that trigger systemic inflammation[11,12].

CMO is an ester of *cis*-9 myristoleic acid ($C_{14:1}$, MA) and cetyl alcohol (C_{16}-OH). It was isolated from Swiss Albino Mice for the first time in 1972 by Harry W. Diehl, at the National Institutes of Health (NIH)[13]. In addition, nutraceuticals comprising CMO have been widely used in the treatment of inflammation to reduce the pain and inflammation in patients suffering with osteoarthritis and fibromyalgia[13-16]. Celadrin is a patented cetylated fatty acids ester complex including cetyl myristoleate, extracted from vegetable oils[17]. Several clinical studies have shown that, Celadrin is supportive to the lubrication of affected joints[18], reduces the production of IL-6, MCP-1 and controls the expression of TNF-α responsible for inflammation[19]. Hunter *et al*., synthesized *cis*-9 CMO from *cis*-9 myristoleic acid (MA, purchased from commercial sources) by PTSA catalysed esterification with cetyl alcohol and confirmed its therapeutic efficacy in a murine model of collagen-induced arthritis in mice[20]. Studies also suggested that, CMO (extracted from beef tallow extracted or vegetable oils) was a safe and effective for patients with osteoarthritis [13-20]. Moreover, there are no proven studies concerning the side effects of the CMO so far[21].

Despite its importance for anti-arthritic and anti-inflammatory properties, the availability of *cis*-9 myristoleic acid (MA), raw material for *cis*-9 CMO is scarce and was found in selective natural sources such as, fish oils, whale oils, beef tallow and oilseed of *Pycnanthus kombo* (Kombo butter) and *Serenoa repens* in the range of 8-30% in total fatty acid (FA) mixture [22-27]. As the separation of pure *cis*-9 MA from mixture of FAs is high-priced; thus the cost of the *cis*-9 CMO based on *cis*-9 MA will also be expensive. Because, the commercial source of *cis*-9 MA is mainly beef tallow and has to be purified from FA mixture by employing purification methods such as molecular/or fractional distillation or urea complexation to obtain high percentage of MA[24]. Recent studies also established the importance of *cis*-9 MA in the inhibition of 5-lipoxygenase which is a potent mediator of inflammation[25], anti-fungal activity [26] as well as prevents osteoclast formation and bone resorption[27]. Nevertheless, the literature reports reveals that there is no reports available so far in this direction either for the chemical synthesis of *cis*-9 MA or any

other alternate raw material for the preparation of *cis*-9 CMO, and thus there is a need to develop a synthetic route for this compound. In this context, we reported in our previous study [28], a novel synthetic route for the synthesis of *cis*-9 and *cis*-10 CMO isomers (**12** & **13**). In continuation to this, the present study aimed to study the effect of these synthetic CMO isomers for *in-vitro* anti-inflammatory activity and DMM surgery-induced osteoarthritis in mice. Further, we also treated stimulated mouse macrophage cells with these CMO isomers to investigate the expression profile of the inflammatory cytokines and eicosanoids.

Materials and Methods:-

Commercial immobilized 1,3- specific lipase, *Lipozyme* TLIM was a gift sample from M/s Novozymes South Asia Pvt. Ltd., Bangalore, India. 10-Undecenoic acid (UDA), oleic acid (OA) (purity, 99%) and n-butyl lithium were purchased from M/s Sigma Aldrich, USA. Triphenyl phosphine (TPP), n-butyl bromide, n-bromo pentane, dimethyl sulphide (DMS), cetyl alcohol and anhydrous sodium sulphate were purchased from s.d fine-CHEM Ltd, Mumbai. Pre-coated silica gel-60 F254 (0.5 mm) glass plates were purchased from M/s Merck, Germany. Dichloromethane (DCM) and tetrahydrofuran (THF) were purchased from Ranchem, RFCL Ltd, New Delhi. Hexane, ethyl acetate, dichloromethane (DCM), acetone, methanol, toluene and anhydrous sodium sulphate (Na_2SO_4) were purchased from local firms. Silica gel was purchased from M/s Acme Synthetic Chemicals, Mumbai. All the other chemicals used were of reagent grade. Carrageenan, indomethacin, dexamethasone, TNFα ELISA kit, IL-6 ELISA kit, NO assay kit and Freund's Complete Adjuvant (FCA) were purchased from M/s Sigma Chemicals, USA. The PGE_2 and LTB_4 ELISA kits were procured from Enzo Life Sciences, India. Acacea was purchased from Loba Chemie Pvt. Ltd, India.

Analytical Methods

Spectral and chromatographic analysis: ^{1}H-NMR spectra were recorded on a Varian Gemini-200 MHz or on Avance-300 MHz instrument using TMS as internal standard. The standard abbreviations s, d, t, q, m, br s refer to singlet, doublet, triplet, quartet, multiplet and broad singlet respectively. The chemical shifts were expressed in δ values quoted (ppm) downfield from internal TMS standard. FT-IR spectra were recorded on a 1600 FT-IR Perkin Elmer spectrum BX spectrophotometer in a range of 400-4400 cm^{-1}. Liquid samples were recorded by dissolving the sample in dichloromethane and for solid samples KBr pellets were prepared by mixing 1-2 mg sample with 150 mg of KBr.

GC Analysis: The GC analysis was performed using Agilent 6850 Series Gas Chromatograph equipped with FID detector. The chromatographic conditions for GC were followed according to the class of the compounds. GC was carried out using HP-1 (30 m x 0.25 mm i.d. x 0.25 μm film thickness) column. The column temperature was initially maintained at 150 °C for 2 min, increased to 300 °C at 6 °C/min, and maintained for 15 min at 300 °C. The carrier gas was nitrogen at a flow rate of 1 ml/min. The injector and detector temperatures were maintained at 300 °C and 320 °C respectively.

GC-MS Analysis: The GC-MS analysis was performed using Agilent 6890N Gas Chromatograph connected to Agilent 5973 Mass Spectrometer at 70 eV (m/z 50-600; source at 230 °C and quadruple at 150 °C) in the EI mode with a HP-5 ms capillary column (30 m x 0.25 mm; 0.25 μm). The column temperature was initially maintained at 100 °C for 2 min, increased to 300 °C at 6 °C/min, and maintained for 25 min at 300 °C. The carrier gas was helium at a flow rate of 1.0 ml/min. the inlet temperature was maintained at 300 °C and split ratio was 50:1. Structural assignments were based on interpretation of mass spectrometric fragmentation and confirmed by comparison of retention times as well as fragmentation pattern of authentic samples are given in mass units (*m/z*). The spectral data obtained from the Wiley and NIST libraries.

Animals: Male Albino Wistar rats of weight ranging from 150 to 180 g were procured from National Institute of Nutrition (NIN), Hyderabad, India with commercial rat diet and water ad libitum, which is registered with Committee for the Purpose of Control and Supervision of Experimental Animals (97/1999/CPCSEA, dated 28.04.1999, CPCSEA), Govt., of India. Rats were then kept under standard laboratory conditions such as light and

dark (i.e., 12:12 h light/dark cycle) in polypropylene cages at 24 °C. Animals were quarantined for a period of 7 days prior to start of the experiment. Rats were observed for general health and suitability for testing during this quarantine period. The C57WT mice which were used in this study were bred at Animal House Facility at CSIR-IICT, Hyderabad, India.

Experimental Section:-

Synthesis of *cis*-9 and *cis*-10 CMO Isomers

Preparation of methyl oleate (MOO, 3) / methyl undecenoate (MUDO, 4): Esterification of OA (**1**, 10.0 g, 35.5 mmol) was carried out by treating with methanol (100 mL) in presence of SO_3H-carbon catalyst (2 g, 20 wt.% of OA) at reflux temperature for 5 h. Reaction was monitored by TLC and after completion of the reaction, catalyst was separated by filtration. The filtrate methanol having the esterified product was dried over anhydrous Na_2SO_4 and methanol was removed under reduced pressure on rotary evaporator. The dried crude product was purified by silica gel column chromatography to obtain pure MOO (**3,** 10.8 g) in 98% yield. Similar methodology was employed for the preparation of MUDO (**4**) in 99% yield from UDA (**2**) by esterification with methanol. Purity of the MOO (**3**) and MUDO (**4**) was analysed by GC-FID.

Preparation of 1-al-methyl nonanoate (5)/ 1-al-methyldecanoate (6): Ozonolysis of MOO (**3**, 10.0 g, 32.25 mmol) was carried out in DCM (100 ml) at -78 °C by bubbling ozone gas till the reaction mixture turn into dark blue color due to ozonoid formation. After 1 h, the reaction was quenched by adding DMS (2 ml) and was stirred at ambient temperature for 6 h. The solvents were removed under vacuum and the residue thus obtained was purified by silica gel column chromatography to get pure 1-al-methylnonanoate (**5**, 4.32 g) in 72% yield with 95% purity. The structure and purity of the compound **5** was confirmed by ^{1}H-NMR, FT-IR, GC and GC-MS. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 9.72 (t, 1H, -CH_2-C<u>H</u>O), 3.67 (s, 3H, -OC<u>H</u>$_3$), 2.40 (q, 2H, -C<u>H</u>$_2$-CHO), 2.25 (t, 2H, -CO-C<u>H</u>$_2$-), 1.68 (m, 2H, -C<u>H</u>$_2$-CH_2-CHO), 1.62 (m, 2H, -C<u>H</u>$_2$-CH_2- $OCOCH_3$), 1.29 (m, 6H, -CH_2-C<u>H</u>$_2$-CH_2-); IR (neat, cm^{-1}): 2930, 2855, 1740 and 1260 (C-O-C stretching) cm^{-1}; EI-MS: (M^+): 186.

Similarly, MUDO ((**4**, 10.0 g, 50.5 mmol) on ozonolysis resulted the 1-al-methyldecanoate (**6**, 8.1 g) in 80% yield with 95% purity by GC and was directly used for the Wittig reaction. ^{1}H-NMR: (300 MHz, $CDCl_3$): δ 9.72 (t, 1H, -CH_2-C<u>H</u>O), 3.67 (s, 3H, -OC<u>H</u>$_3$), 2.40 (q, 2H, -C<u>H</u>$_2$-CHO), 2.25 (t, 2H, -OC-C<u>H</u>$_2$-), 1.68 (m, 2H, -C<u>H</u>$_2$-CH_2-CHO), 1.62 (m, 2H, -C<u>H</u>$_2$-CH_2-OC-OCH_3), 1.29 (m, 8H, -CH_2-C<u>H</u>$_2$-CH_2-); IR (neat, cm^{-1})**:** 2930 and 2855 (C-H stretching), 1740 (C=O stretching) and 1260 (C-O-C stretching); EI-MS: (M^+): 200.

Preparation of pentyl-/butyl-triphenylphosphonium bromide salts (7 & 8): n-Pentyl bromide (4.25 g, 31.25 mmol) was added to a solution of triphenylphosphine (TPP, 8.26 g, 31.25 mmol) in toluene (50 ml) at reflux temperature for 20 h under stirring. The reaction mixture was concentrated in vacuum and the white solid precipitate obtained was washed with ether (50 ml x 3 times) and dried under vacuum to obtain pentyl-triphenylphosphonium bromide salt (**7,** 15.4 g). Similarly, butyl-triphenylphosphonium bromide salt (**8,** 13.5 g) was also prepared from n-butyl bromide (8.5 g, 6.25 mmol) and TPP (16.5 g, 62.5 mmol).

Synthesis of *cis*-9 and *cis*-10 methyl myristoleate (MMO, 9 & 10): The pentyl-TPP- bromide salt (**7,** 14.0 g, 34.0 mmol) was suspended in dry THF (50 ml) under nitrogen and cooled to 0 °C. To this slurry, n-butyl lithium (8.5 ml, 1.6 M in hexane) was added, stirred the reaction mixture for 30 min to obtain an orange solution. 1-Al-methylnonanoate (**5**, 4.0 g, 21.6 mmol) in dry THF (20 ml) was added slowly and allowed the reaction mixture to reach to 25 °C and then heated at reflux temperature for 4 h. The THF was evaporated and to the residue distilled water was added and extracted with ether (25 ml x 3 times). The combined ether layer was evaporated, dried under vacuum to get the residue and purified by column chromatography using hexane and ethyl acetate (98:2) as eluant to obtain *cis*-9 MMO (**9**, 3.4 g) in 65% yield with 95% purity by GC. ^{1}H-NMR: (300 MHz, $CDCl_3$): δ 5.32 (m, 2H, -C<u>H</u>=C<u>H</u>-), 3.65 (s, 3H, C<u>H</u>$_3$-O-CO-CH_2-), 2.3 (t, 2H, -OC-C<u>H</u>$_2$-), 1.92 (m, 4H, -C<u>H</u>$_2$-CH=CH-C<u>H</u>$_2$-), 1.60 (m, 2H, -

OC-O-CH$_2$-C$\underline{H}_2$-CH$_2$-), 1.30-1.20 (br, m, 12H –CH$_2$-C$\underline{H}_2$-CH$_2$-), 0.90 (t, 3H, -CH$_2$-C$\underline{H}_3$); IR (neat, cm^{-1}): 2928, 2856, 1742, 1170 cm^{-1}; EI-MS: (M$^+$): 240.

Under similar reaction conditions, Witting reaction of the butyl-TPP-bromide salt (**8**, 12.55 g, 31.0 mmol) with 10-al-methyldecanoate (**6**, 5.0 g, 25.0 mmol) resulted *cis*-10 MMO (**10**, 4.44 g) in 74% yield with 95% purity by GC. ^{1}H-NMR: (300 MHz, CDCl$_3$) δ: 5.32 (m, 2H, -C$\underline{H}$=C$\underline{H}$-), 3.65 (s, 3H, C$\underline{H}_3$-O-CO-CH$_2$-), 2.3 (t, 2H, -CO-C$\underline{H}_2$-), 1.92 (m, 4H, -C$\underline{H}_2$-CH=CH-C$\underline{H}_2$-), 1.60 (m, 2H, -OC-O-CH$_2$-C$\underline{H}_2$-), 1.30-1.20 (br, m, 12H, –CH$_2$-C$\underline{H}_2$-CH$_2$-), 0.90 (t, 3H, -CH$_2$-C$\underline{H}_3$); FT-IR (neat, cm^{-1}): 2928 and 2856 (C-H stretching), 1742 (C=O stretching) and 1170 (C-O-C stretching); EI-MS: (M$^+$): 240.

Synthesis of *cis*-9 CMO (12) and *cis*-10 CMO (13): The *cis*-9 MMO (**9**, 3.4 g, 14.17 mmol) was subjected for enzymatic transesterification with cetyl alcohol (**11**, 4.1 g, 16.91 mmol) employing *Lipozyme* TLIM (1.7 g, 5 wt.% of **9**) at 65-70 ºC for 8 h. The reaction was monitored by TLC and after completion of the reaction, hexane (20 ml) was added and the enzyme was separated by filtration and the solvent was evaporated to get the crude product and was further purified by column chromatography to obtain *cis*-9 CMO (**12**, 5.86 g) in 92% yield with 98% purity by GC. The structure of *cis*-9 CMO (**12**) was confirmed by ^{1}H-NMR, FT-IR and GC-MS. ^{1}H-NMR: (300 MHz, CDCl$_3$): δ 5.30 (m, 2H, *J* = 5.6 Hz, -C$\underline{H}$=C$\underline{H}$-), 4.01 (t, 2H, -C$\underline{H}_2$-O-CO-CH$_2$-), 2.25 (t, 2H, -CO-C$\underline{H}_2$-), 1.99 (m, 4H, -C$\underline{H}_2$-CH=CH-C$\underline{H}_2$-), 1.60 (m, 2H, -OC-O-CH$_2$-C$\underline{H}_2$-), 1.30-1.20 (br, m, 40H –CH$_2$-C$\underline{H}_2$-CH$_2$-), 0.90 (t, 6H, -CH$_2$-C$\underline{H}_3$); IR (neat, cm^{-1}): 2926 and 2855 (C-H stretching), 1738 (C=O stretching), 1242 (C-O-C stretching); EI-MS: *m/z* calculated for $C_{30}H_{58}O_2$ (M+): 450.78, found (M$^+$): 450.

Similarly, *Lipozyme* TLIM catalysed transesterification of *cis*-10 MMO (**10**, 4.0 g, 16.7 mmol) with cetyl alcohol (**11,** 4.82 g, 19.9 mmol) resulted *cis*-10 CMO (**13**, 7.14 g) in 95% yield with 98% purity by GC. The structure of *cis*-10 CMO (**13**) was confirmed by ^{1}H-NMR, FT-IR and GC-MS. ^{1}H-NMR: (300 MHz, CDCl$_3$): δ 5.31-5.27 (m, 2H, *J* = 5.6 Hz, -C$\underline{H}$=C$\underline{H}$-), 4.01-3.96 (t, 2H, -C$\underline{H}_2$-O-CO-CH$_2$-), 2.20-2.23 (t, 2H, -CO-C$\underline{H}_2$-), 1.95-1.90 (m, 4H, -C$\underline{H}_2$-CH=CH-C$\underline{H}_2$-), 1.56-1.51 (m, 2H, -OC-O-CH$_2$-C$\underline{H}_2$-), 1.30-1.20 (br, m, 40H –CH$_2$-C$\underline{H}_2$-CH$_2$-), 0.85-0.80 (q, 6H, -CH$_2$-C$\underline{H}_3$); IR (neat, cm^{-1}): 2926 and 2855 (C-H stretching), 1738 (C=O stretching), 1242 (C-O-C stretching); EI-MS: *m/z* calculated for $C_{30}H_{58}O_2$ (M+): 450.78, found (M$^+$): 450.

In-vivo and *in-vitro* Biological Evaluations of CMO Isomers

Evaluation for anti-inflammatory potential: All experiments involving laboratory animals were conducted after taking the approval from Institutional Animal Ethics Committee of Indian Institute of Chemical Technology, Hyderabad. The anti-inflammatory activity potential of the CMO isomers were evaluated initially in carrageenan induced rat paw edemas model employing the method of Winter *et al* [36]. The CMO isomers were administered intraperitonially to assess the anti-inflammatory potential against carrageenan induced paw edema model. Male Wistar rats of weighing between 150 to 180 g were divided into 4 groups each containing 6 animals. The CMO isomers were administered intraperitoneally to rats in two different test groups at a dose of 400 mg/kg. The test compounds were administered intraperitoneally by suspending in 1% gum acacia. The standard group received Indomethacin at a dose of 10 mg/kg, which served as standard. The control group received vehicle alone. A zero hour reading of the paw volumes was measured for all the animals using Plethysmometer (Ugo Basile, Italy). One hour after the administration of test compounds carrageenan (1%, 0.1 mL) was injected into the sub plantar region of animals in all the groups. Paw volumes were again measured 3 h after the carrageenan administration. The anti-inflammatory potential of the CMO isomers was assessed by measuring the inhibition of the paw volumes with reference to the mean paw volume of control group.

Effect on the arthritic rats: The studies on anti-arthritic activity of the CMO isomers were undertaken employing the method of Pearson CM *et al* [37]. The CMO isomers were subjected to evaluation for anti-arthritic potential in Freund's complete adjuvant induced arthritis model in rats. Male Wistar rats were divided into 4 groups each containing 6 rats. Arthritis was induced in rats in all the groups by administering Freund's Complete Adjuvant (FCA) at the dose of 1 mg per rat by injecting at sub plantar region of the right hind paw. Paw volumes were measured for

both paws on every alternate day till 14 days and till full arthritis were developed. The CMO isomers (100 mg/kg/day), and indomethacin (2.5 mg/Kg/day) were administered from 14th day till 22nd day to the 3 test groups. Control group received the vehicle solution without drug. Paw volumes were again measured daily from 15th day to 22nd day for all rats in all groups. The percent inhibition of arthritis in the treated groups was calculated with reference to control group

***In-vitro* anti-inflammatory effects on macrophages:** The RAW264.7 macrophages were incubated with CMO isomers or dexamethasone (0.5 µM) as indicated in the Fig. 4 and Fig. 5 for 3 h. Later, the cells were treated with bacterial lipopolysaccharide (LPS, 2 µg/ml) for 24 h and the cell medium was collected. The cytokines and lipid mediators including TNFα, IL-6, NO, PGE_2 and LTB_4 respectively were analyzed in the medium by ELISA or NO assay kit. Data are shown as mean ± SD of 3 independent experiments.

Induction of osteoarthritis by DMM-surgery in mice knee tissues: The DMM surgery was performed in the right hind knee of 10-12 week-old C57WT mice following administration of general anesthesia (inhalation of isoflurane in oxygen) by transection of the anterior attachment of the medial meniscotibial ligament in the knee [38,39]. Later, the buprenorphine (1.0 mg/kg) was given subcutaneously to alleviate pain after surgery. Only the skin of the right knee joint was resected in the sham-operated group. After recovery from anesthesia, all mice were weight bearing and returned to the cages. The *cis*-9 and *cis*-10 CMO isomers (30 mg/kg/day) were administered weekly thrice intraarticularly for 8-weeks post DMM-surgery. The mice were sacrificed 8 weeks after DMM-surgery, and the knee tissues were harvested, fixed in buffered formalin for 72 h, and transferred to 70% alcohol until they used. The joint tissues were decalcified by immersing in decalcifier (stat-lab) for 48 h and then embedded in paraffin for sectioning. The tissues were sectioned (6-µm thick) and selected sections were stained with safranin O-fast green staining. The cartilage damage was measured using the Osteoarthritis Research Society International (OARSI) scoring system [40,41]. In brief, grade 0= healthy cartilage; 0.5= loss of Safranin O staining no structural changes; 1= small fibrillations with no loss of cartilage; 2= vertical clefts down to the layer below the superficial layer; 3-6= vertical clefts and erosion of calcified cartilage in the articular surface with <25%, 25%–50%, 50%–75% and >75%, respectively. The OARSI scoring of cartilage damage was performed by 2 blinded observers. The mean values of scores were used for analysis.

Statistical Analysis: Results were represented as mean ± standard error measurement (SEM), and analysed by ANOVA to reveal differences and interactions between treatments or using the Tukey HSD test when parametricity of data did not prevail. Sigma Plot software for Windows (Version 11.0, Systat Software, Inc.), ddifferences at $p < 0.05$ was considered to be statistically different. All measurements were carried out in triplicate.

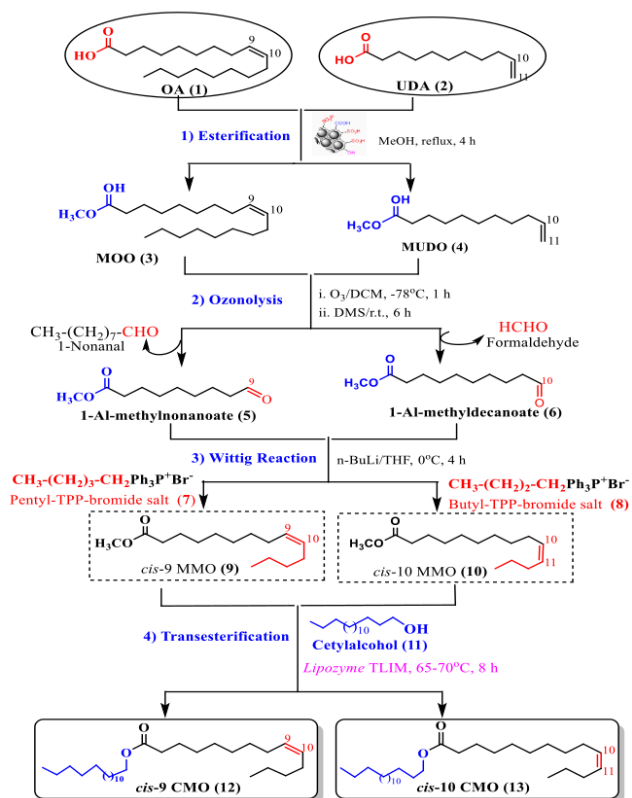
Results and Discussion:-

Synthesis of cis-9 and cis-10 CMO isomers (12 &13)

Synthesis of the *cis*-9 CMO (**12**) and *cis*-10 CMO (**13**) isomers from *cis*-9 and *cis*-10 MMOs (**9** & **10**) which in turn were derived from commercially available natural plant oil sources such as OA (**1**) and UDA (**2**) respectively, employing chemo-enzymatic methods as reported in our patent[28]. Accordingly, the synthesis of the main precursors i.e., *cis*-9 & *cis*-10 MMOs (**9** and **10**) for the preparation of the targeted CMO isomers **12** and **13** from OA (**1**) and UDA (**2**) involves three steps namely, esterification, ozonolysis and Wittig reaction and then followed by enzymatic transesterification with cetyl alcohol as shown in **Scheme 1.** The methyl oleate (MOO, **3**) and methyl undecenoate (MUDO, **4**) prepared by SO_3H-carbon acid catalysed esterification with methanol[29] were subjected for ozonolysis reaction to obtain 1-al-methylnonoate (**5**) and 1-al-methyldecanoate (**6**) by the oxidative cleavage of double bond at C9-10 and C10-11 carbons respectively.

Wittig reaction was chosen for synthesis of *cis*-9 MMO (**9**) and is a key step for the formation of -C=C- bond in between C9-10 and C10-11 position. Non-stabilized ylide (Wittig reagent) prepared greatly improved *cis- or* (Z)-

alkene selectivity in the reaction, due to the stability of oxaphosphetane intermediate [30,31]. Accordingly, aldehyde compounds **8** & **10** when subjected for Wittig reaction with TTP salts **7** & **8** resulted the corresponding *cis*-9 MMO (**9**) and *cis*-10 MMO (**10**) in 65% and 95% yields with >95% purity. This is the first report on the synthesis of pure MMO isomers having *cis*-9 as well as *cis*-10 double bond. In addition, the resulted *cis*-10 MMO (**10**) is an omega-4 (ω-4) fatty acid which is quite different from naturally available vegetable/fish oil-based ω-3, ω-6 and ω-9 fatty acids.



Scheme 1:- Synthesis of *cis*-9 and cis-10 CMO isomers.

Enzyme catalyzed reactions in recent years have gained much attention in synthetic organic chemistry, pharmaceuticals and food ingredients compared with conventional chemical synthesis. The main advantages for employing enzymatic catalysis over the chemical process in the final step of transesterification reaction is that, inorganic chemical contamination can be minimized in the end-product and in addition immobilized enzymes can be reused for several times as well as safer and environmentally friendly[32]. Hence, *Lipozyme* TLIM catalysed

transesterification methodology was adapted for the preparation of *cis*-9 and *cis*-10 CMO isomers from cetyl alcohol and the *cis*-9 and *cis*-10 MMO (**9** & **10**) in 92 and 95% yields with 98% purity. Purity of all the products was checked by GC and the structures were confirmed by ^{1}H-NMR, FT-IR and GC-MS. The ^{1}H-NMR spectra (Figure 1) of the CMO isomers **12** and **13**, showed the characteristic olefinic bond protons at 5.27-5.30 ppm, with coupling constant (*J*) <6 *Hz*, thus confirming the -C<u>H</u>=C<u>H</u>- in *cis*/*Z* configuration.

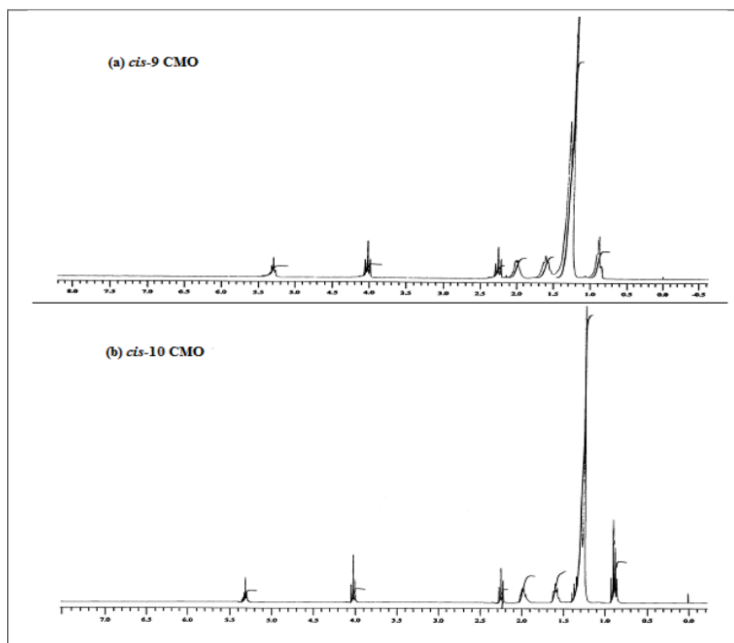


Figure 1:- ^{1}H NMR Spectrum of **(a)** *cis*-9 CMO and (**b**) cis-10 CMO.

In-vivo and in-vitro biological evaluation of cis-9 and cis-10 CMO isomers (12 &13)

Evaluation for anti-inflammatory potential: Administration of the inflammatory agent i.e., carrageenan in the rat hind edema paws is a standard and well-known, and thus widely used to study inflammation and evaluate the anti-inflammatory activities of various drugs[33]. Carrageenan induced inflammation is considered to be biphasic, where the 1st phase involves the release of several inflammatory mediators for examples serotonin and histamine in the early phase (i.e., up to 1 h) and the 2nd phase is mediated mainly by prostaglandins and cyclooxygenase substances (over 1 h) as a result, increase in vascular permeability, accumulation of neutrophils and macrophages[34,35]. However, oxidative stress is a major cause of inflammatory events associated in a large number of diseases, such as cancer, neurodegenerative, and cardio-vascular diseases, or diabetes [35]. The anti-inflammatory activity of the *cis*-9 and *cis*-10 CMO isomers **12** and **13** was evaluated employing carrageenan induced rat paw edemas model[36] by measuring the inhibition of the paw volumes induced by carrageenan with reference to those in control group. Both the CMO isomers were found to reduce the inflammation and edema of the animals equally up to 36% compared to about 58%

for control group (Figure 2). Moreover, the anti-inflamatory potential of the new *cis*-10 CMO (**13**) was found to be comparable to that of known *cis*-9 CMO in the reduction of the inflammation and edema of the animals.

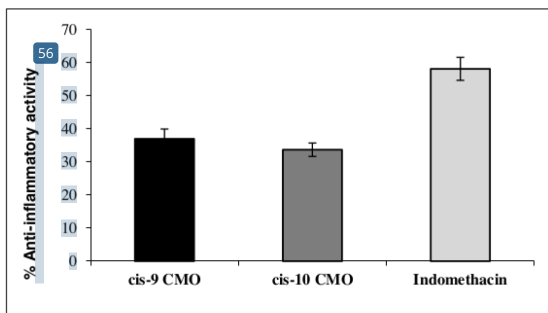


Figure 2:- Anti-inflammatory activity of *cis*-9 and *cis*-10 CMO isomers.

Effect on the arthritic rats: The studies on anti-arthritic activity of the CMO isomers **12** and **13** were undertaken employing the method of Pearson et al [37]. Freund's complete adjuvant (FCA) induced arthritis has been selected in our studies which is commonly used as chronic model for inflammation due to strong correlation between efficacy of therapeutic drugs in this model and rheumatoid arthritis in human[35]. The studies indicated that the anti-arthritic activity of *cis*-10 CMO **13** was found to be more effective compared to that of *cis*-9 CMO **12** and comparable with that of standard indomethacin in inhibiting the inflammation and reduction of adjuvant-induced arthritis in rats. It was also found that both CMO isomers reduced the paw volumes and the symptoms associated with the arthritis equally up to 10 to 18% at the dose of 100 mg/kg (Figure 3). The inhibition was dose dependent when dose was increased from 100 mg/kg to 300 mg/kg, which indicated that the CMO isomers are useful in alleviating the symptoms associated with the established arthritis.

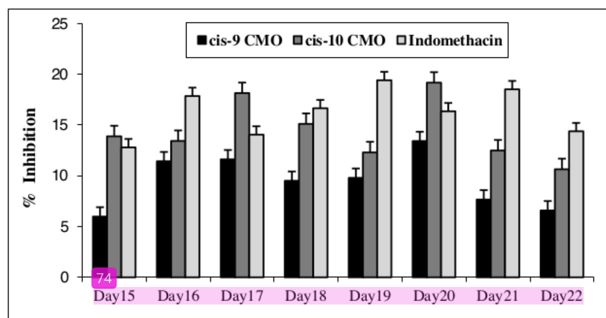


Figure 3:- Effect of *cis*-9 and *cis*-10 CMO isomers on the arthritic rats.

***In-vitro* anti-inflammatory effects on macrophages:** The activation of macrophages and secretion of cytokines (TNFα, IL-6), nitric oxide (NO) and eicosanoids (PGE_2 and LTB_4) propagate edema and inflammation in arthritis.

We have assessed the effect of the *cis*-9 and *cis*-10 CMO isomers **12** and **13** on RAW264.7 mouse macrophage activation and secretion of inflammatory mediators. We found that there was 45-55% reduction in the secretion of TNFα, IL-6, NO, PGE_2 and LTB_4 in the cells incubated with either *cis*-9 CMO (200 μm) or *cis*-10 CMO (200 μm) compared to LPS control. This inhibition of secretion of cytokines by both the CMO isomers is comparable to dexamethasone (Figure 4).

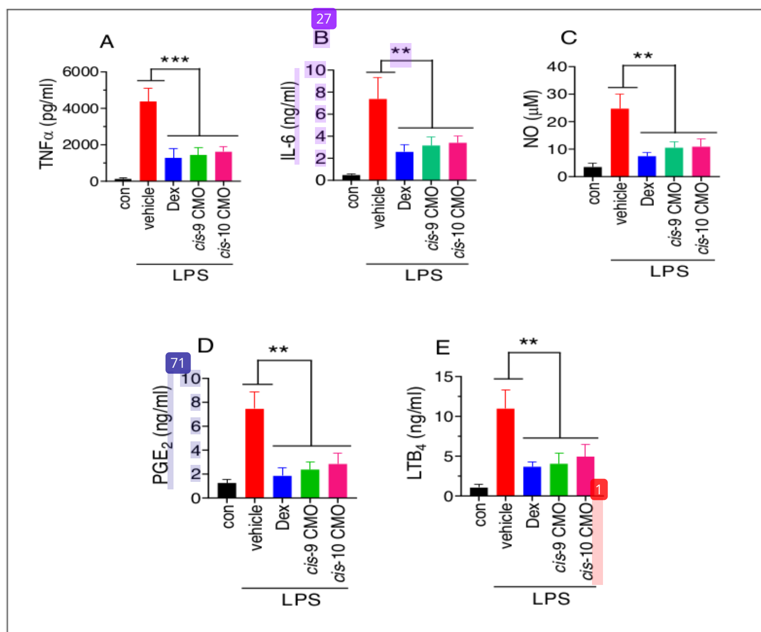


Figure 4:- Effect of *cis*-9 and *cis*-10 CMO isomers on cytokine secretion of LPS-stimulated RAW macrophages.

The cells (1 x 10^6 cells/ mL) were treated with CMO 100 (μM) for 6h, and then stimulated with 5mg/mL LPS for 24h. The TNFα (A), IL-6 (B), PGE_2 (D) and LTB_4(E) wereanalyzed by ELISA. The NO (C) was estimated in the medium using the procedure described in the ''Materials and methods'' section. Data represent the mean±SD of three independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Further, dose dependent studies on mouse macrophage cell lines revealed that there was negligible suppression of cytokines and NO in the cells incubated with 5 μm of CMO isomers. However, there was significant reduction in the secretion of TNFα, IL-6, NO, PGE_2 and LTB_4 at doses 50-200 μm (Figure 5). Hence, the inhibition of paw edema and inflammation in the *cis*-9 CMO **12** and *cis*-10 CMO **13** administered rats could be attributed to suppression of cytokines and eicosanoids.

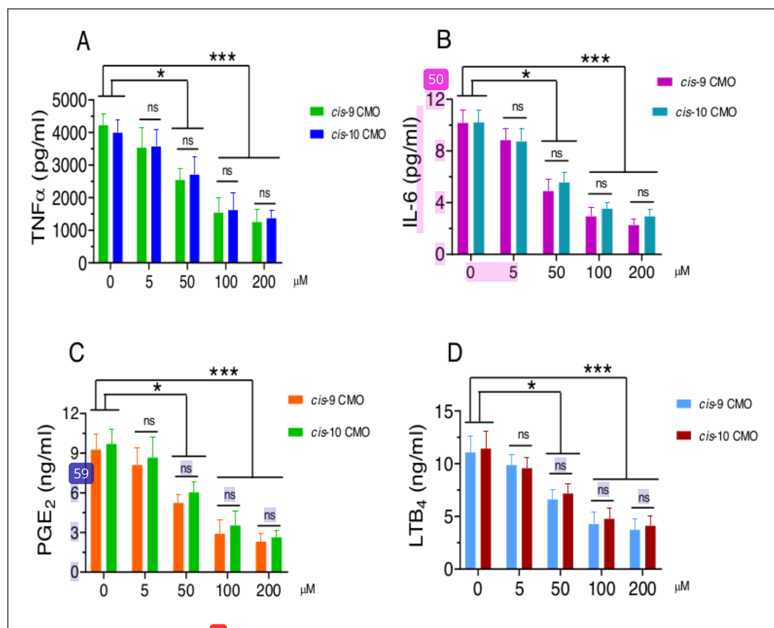


Figure 5:- Dose-dependent anti-inflammatory effects of *cis*-9 and *cis*-10 CMO isomers on macrophages.

The cells (1 x 10^6 cells/ ml) were treated with different doses of CMO 6h, and then stimulated with 5mg/mL LPS for 24h. The TNFα (A), IL-6 (B), PGE_2 (C) and LTB_4 (D) were analyzed by ELISA. Data represent the mean ± SD of three independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Effect on osteoarthritis in mice: The activation of macrophages and inflammation plays a critical role in the degradation of cartilage in osteoarthritis. *In-vitro* studies on macrophages and *in-vivo* paw edema studies suggest that both the CMO isomers **12** and **13** exhibited potent anti-inflammatory effect. Therefore, we have also analyzed the effect of these CMO isomers on osteoarthritis in mice. The destabilization of medial meniscus (DMM) surgery-induced osteoarthritis is a widely used model to study the disease mechanisms and test the effect of therapeutics [38, 39]. In this study, mice undergone DMM-surgery manifest significant loss of articular cartilage compared to sham-group. The mice administered with both *cis*-9 CMO **12** and *cis*-10 CMO **13** significantly protected the DMM-induced cartilage destruction compared to vehicle group. The OARSI score [40,41], which represents the cumulative damage of synovium in mice confirmed the similar result (Figure 6).

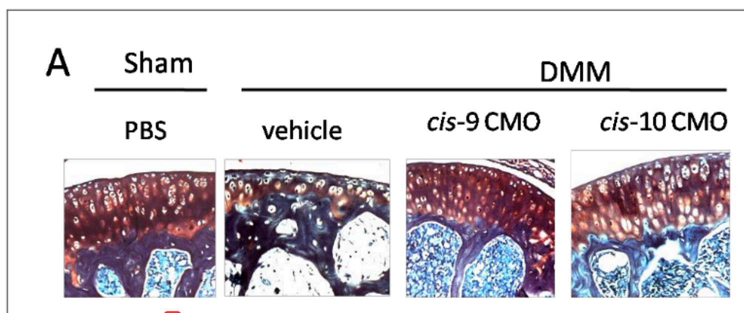


Figure 6:- Effect of *cis*-9 and *cis*-10 CMO isomers on osteoarthritis in mice.

The DMM surgery was performed in the right hind knee of 10-12 week-old C57WT mice. The *cis*-9 and *cis*-10CMO isomers (30 mg/kg/day) were administered weekly thrice intraarticularly for 8-weeks post DMM-surgery. The mice were sacrificed 8 weeks after DMM-surgery, and the knee tissues were harvested, fixed,and the tissues were sectioned (6-μm thick) and stained with safranin O-fast green staining.The images represent safranin stained knee tissues in each group.

Conclusion

In conclusion, synthetically prepared *cis*-9 and *cis*-10 methyl myristoleate (MMO) isomers **(9** & **10)** from methyl oleate and methyl undecenoate were enzymatically transesterified with cetyl alcohol to obtain pure *cis*-9 CMO (**12**) and *cis*-10 CMO (**13**) isomers in excellent (>90%) yields. The **9** & **10** MMO isomers are omega-5 and omega-4 monounsaturated fatty acids respectively, which produced for the first time from vegetable oils, thus can be new source of fatty acids in the interest of pharmaceutical importance. The results of anti-inflammatory and anti-arthritic properties exhibited by the novel *cis*-10 CMO (**13**) was found to be similar to that of *cis*-9 CMO (**12**) in reducing the pain and inflammation in a fully developed arthritis models. In addition, they also exhibited dose-dependent reduction in secretion of TNFα, IL-6, NO, PGE_2 and LTB_4 in response to LPS in stimulated RAW264.7 mouse macrophages. Finally, these vegetable oil based *cis*-9 and *cis*-10 CMO isomers (**12** & **13**) can be economical in the future as potential nonsteroidal anti-inflammatory drugs (NSAIDs) for the treatment of arthritis and inflammatory diseases. In perspective, further *in-vivo* efficacy, safety and toxicity studies on animal models have to be developed to validate the *in-vitro* findings of these synthetic *cis*-9 and *cis*-10 CMO isomers.

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Conflicts of interest: The authors declare no conflicts of interest.

Abbreviations

CMO	cetyl myristoleate
PTSA	*p*-toluene sulfonic acid
OA	osteoarthritis
FCA	Freund's complete adjuvant
LPS	lipopolysaccharide
TNFα	tumor necrosis factor
IL-6	interleukin-6
NO	nitric oxide
PGE_2	prostaglandin E_2
LTB_4	leukotriene B_4
DMM	destabilization of medial meniscus

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In-vivo and in-vitro anti-inflammatory and anti-arthritic activity of Synthetic cis-9 and cis-10 cetyl myristoleate (CMO) isomers in mice

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